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Collagen Wound Dressings with Anti-Inflammatory Activity

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The aim of this study was to develop modern wound dressings such as controlled drug delivery systems. These systems consist in collagen as release support and niflumic acid as drug. The scaffolds were prepared by lyophilization in order to obtain porous structures and were evaluated by release profile of niflumic acid, water absorption, collagenase degradation and biocompatibility with fibroblast cells. The collagen scaffold with 0.75% niflumic acid solved in laurel oil was optimal in terms of biodegradability, absorbability and fibroblast cells biocompatibility. Thus, the obtained collagen scaffolds could be used as wound dressings with absorbent, antibacterial and anti-inflammatory properties.

Keywords Collagen; fibroblast cells; niflumic acid; scaffolds; wound dressing

Introduction

Wound healing is a specific biological process, which involves cells growth and tissue regeneration [1]. In order to improve healing on the wounds many polymeric wound dressings or skin substitutes have been developed and investigated [2] because they should replace all the structures and functions of native skin [3]. Among the biocompatible wound dressings, the biological ones derived from natural polymers such as collagen, hyaluronic acid, chitosan, alginates and elastin [1] play an active part in the wound healing process and new tissue formation. Biological dressings play an important role in the wound healing process.

The most promising wound dressing is collagen biomaterial due to its excellent biocompatibility and biodegradability, haemostatic properties, well established structure, biologic characteristics and to the way it interacts with the body [4]. As wound dressing, collagen

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stimulates formation of fibroblasts and accelerates the migration of endothelial cells upon contact with damaged tissues [5]. An ideal wound dressing requires adherence to wounds, histocompatibility, control of fluid loss, preventing infection, absence of antigenicity and toxicity and mechanical stability [6,7]. Being a natural protein, collagen is not able to heal the infected tissues by itself, bacteria using it as a substrate [8].

The best solution for skin wound treatment remains the topical antimicrobial therapy, because the local application of drug can provide high concentrations in the area of affected tissues and can avoid systemic effects [9,10]. Infection prolongs the inflammatory process which delays wound healing [11]. The new generations of dressings with therapeutic value incorporate different drugs with active role due to their controlled delivery at local level. In this study we developed new collagen scaffolds for local treatment of inflamed wound. These scaffolds in form of controlled drug delivery systems consist of collagen as released support and niflumic acid as anti-inflammatory drug. However, pathogenic bacteria such as *Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus pyrogeneus* and some *Proteus, Clostridium and Coliform species* can be critical to the healing process [12]. The essential oils are more and more studied in medicine today because of their antimicrobial, antiviral, antifungal, and antioxidant properties [13]. Our studies presents that laurel oils show good antibacterial properties and for this reason we used it as solvent for niflumic acid.

The aim of this study is to develop modern wound dressings such as controlled drug delivery systems. These systems consist in collagen as release support, niflumic acid as drug with anti-inflammatory properties and laurel essential oils with known antibacterial properties. The drug delivery systems are prepared by lyophilization method in order to obtain absorbent wound dressings with porous structures. These scaffolds were investigated by the release of niflumic acid, the water up-take and biodegradability properties as well as their biocompatibility with human fibroblast cells in order to select the proper wound dressing with antimicrobial and anti-inflammatory properties.

Materials and Methods

Materials

Type I fibrillar collagen gel (Coll) having a concentration of 2.11% (w/w) was extracted from calf hide as previously described [4]. Niflumic acid (NA) was purchased from ICN Biomedicals Inc. (USA) and glutaraldehyde (GA) from Merck (Germany). Sodium hydroxide, ethanol (Et) and phosphate buffer solution (PBS), pH, 7.4 were of analytical grade. The laurel oil (LO) was obtained from wild plants from the province of Hatay in the period when they were blooming. The plants were dried at room temperature in dark place at the Laboratory of Medicinal and Aromatic Plants, Department of Field Crops, Faculty of Agriculture, Mustafa Kemal University. Laurel essential oil (2.8%) was obtained by steam distillation in neo-clevenger apparatus from dry leaves.

Collagen Scaffolds Preparation

The concentration of each collagen gel was adjusted at 1.2% and pH at 7.4 with 1 M sodium hydroxide (under stirring). 0.25; 0.50, 0.75 and 1.00% NA reported to the dry substance was solved in ethylic alcohol or laurel oil and then added into collagen gels. The

obtained collagen-NA gels were cross-linked with 0.25% GA and then freeze-dried using the freeze-dryer Delta 2-24 LSC (Martin Christ, Germany), as previously described [14].

Determination of Water Uptake Capacity of Collagen Scaffolds

The water uptake capacity was carried out using phosphate buffer pH 7.4 as immersion medium (n = 3). Pieces of collagen matrices of ~2 cm^2 area were weighed (W_d) and then immersed in phosphate buffer pH 7.4. At established time intervals, the hydrated scaffolds were weighed (W_w) and water uptake was calculated using the following equation [15]:

$$\text{Water uptake} = \frac{W_w - W_d}{W_d} g/g \tag{1}$$

where W_w represents the weight of wet matrices at immersion time t and W_d denotes the weight of dry scaffolds.

Enzymatic Degradation

Enzymatic degradation of collagen scaffolds was investigated by monitoring the weight loss depending on exposure time to collagenase solution. 2 × 2 cm^2 of collagen scaffolds were accurately weighed, placed in PBS solution and collagenase (1 μg/mL) and incubated at 37°C. At regular time intervals, the swollen scaffolds were removed from degradation solution, blotted dry and weighted. The percentage of hydrogel degradation was determined by the following relation:

$$\%\text{weight loss} = \frac{W_i - W_t}{W_i} \cdot 100 \tag{2}$$

where W_i is the initial weight and W_t is the weight after time t.

Niflumic Acid Release

In vitro release of niflumic acid was determined in triplicate at 37 ± 0.5°C using a modified USP paddle method ("sandwich" device). The paddle was rotated with 50 rpm. The phosphate buffer having the pH 7.4 was used as release/dissolution medium (200 mL). Aliquots of 5mL were withdrawn from the reaction medium at different times and replaced with the same volume of fresh pre-heated phosphate buffer. The amount of niflumic acid released was determined spectrophotometrically at 297 nm.

Collagen Scaffolds Colonization

For in vitro colonization we used the human fibroblasts cell line (obtained from human derma) grown in DMEM supplemented with 4,5‰ glucose medium, 10% fetal bovine serum, and sodium selenite 20 μg/l, 30 mg/l ascorbic acid, and antibiotics (100 U/l penicillin, 100 U/l streptomycin, 50 U/l neomycin). Collagen scaffolds were sterilized with 70% ethanol for 24 hours, and then were conditioned in the same culture medium for 24 hours and inoculated with fibroblasts cells at the fifth passages (75 000 cells/ml). Cells were maintained in culture at 37°C in incubators with 5% CO_2 in air (v/v), and relative humidity over 95%. Experiments were done after two days in culture.

Results and Discussion

Collagen has a hydrophilic nature because of its molecular structure characterized by high content of diamino dicarboxylic amino acids and carbohydrate moieties, which provides a surface geometry very suitable for cell adhesion [14]. Niflumic acid is a frequently used anti-inflammatory drug with low aqueous solubility. The niflumic acid has high solubility in organic solvents as ethanol (0.262 M) and ether (0.268 M) while the solubility in water is 0.00 M [16]. In order to avoid phase separation between collagen and drug and to have homogeneous scaffolds the 0.25, 0.50, 0.75 and 1.00% niflumic acid reported to weight of collagen gel was solved in ethanol or laurel essential oil which contains 46.01% eucalyptol (1,8-cineol)—cyclic ether. Also, eucalyptol is a natural organic compound which inhibits inflammatory cytokine, reducing inflammation and pain when applied topically [17]. The compositions and codes of collagen scaffolds are shown in Table 1.

Water uptake studies determined the maximum amount absorbed and retained by scaffold reported to its initial weight during a certain period. Figure 1 showed that swelling is influenced by the following factors:

- the degree of cross-linking: Coll (reference sample) is less absorbable than the cross-linked sample—Coll-R;
- the concentration of niflumic acid from collagen scaffolds: the higher NA content, the higher absorbability ability of scaffolds;
- the solvent of niflumic acid: Coll-N-E samples are less absorbable than Coll-N-L ones;

The lowest water uptake was recorded for scaffold, which has the minimum amount NA solved in ethanol (Coll-N-E-0.25), and the highest for cross-linked reference sample (Coll-R).

The control of degradation rate of the wound dressings is an important feature, as the in vivo resorption influences tissue regeneration ability. Collagenase is the unique enzyme that is able to cleave collagen triple helical region under physiological conditions of pH and temperature. The cross-linking degree can also be indirectly evaluated by biodegradation,

Table 1. Composition and code of collagen scaffolds

Scaffold codes	Coll*, %	NA**, %	LO**, %	Et**, %	GA**, %
Coll	1.2	0	0	0	0
Coll-R	1.2	0	0	0	0.25
Coll-N-E-0.25	1.2	0.25	0	0.5	0.25
Coll-N-E-0.50	1.2	0.50	0	0.5	0.25
Coll-N-E-0.75	1.2	0.75	0	0.5	0.25
Coll-N-E-1.00	1.2	1.00	0	0.5	0.25
Coll-N-L-0.25	1.2	0.25	0.5	0	0.25
Coll-N-L-0.50	1.2	0.50	0.5	0	0.25
Coll-N-L-0.75	1.2	0.75	0.5	0	0.25
Coll-N-L-1.00	1.2	1.00	0.5	0	0.25

* reported to dry substance.
** reported to collagen gel.

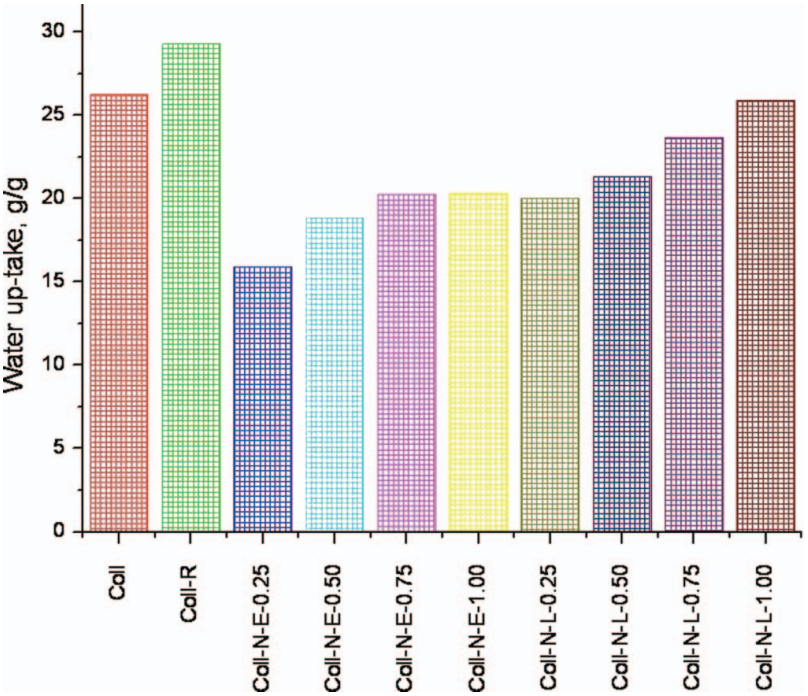

Figure 1. Water uptake for collagen scaffolds.

collagenase being able to cleave only the main chains of collagen and unable to cleave its intermolecular bonds [4].

Our experimental studies shown that reference sample (Coll) was totally digested in one hour; meanwhile the cross-linked sample was digested in 8 hours. Samples that contained 0.25 and 0.50% niflumic acid, both solved in ethanol and laurel oil were totally digested in 24 hours. This fact demonstrated that the niflumic acid forms strong bonds with collagen having also role of cross-linker. Comparing samples with the same concentration of niflumic acid but solved in different solvents, it can be seen in Fig. 2a and b as the dependence of percentage weight loss (%) on the degradation time (4, 8, 24, 48 and 72 hours) that samples Coll-N-E were totally digested after 72 hours, meanwhile the samples Coll-N-L were not totally digested in 96 hours. After this time, the scaffold Coll-N-L-0.75 was digested 97% and Coll-N-L-1.00 was digested 75%. The most resistant to collagenase degradation is the collagen scaffold with 1% NA solved in laurel oil.

The drug release was performed both for collagen scaffolds containing NA solved in ethanol and laurel oil in order to evaluate the amount of NA released. The release profiles of niflumic acid from the collagen scaffolds described in Table 1 are presented in Fig. 3a and 3b.

As it can be seen in Fig. 3a and b, there were significant differences between the release patterns of Coll-N-E and Coll-N-L samples and niflumic acid is released in different amounts: when NA was solved in laurel oil, a smaller amount of NA was released compared with the one solved in ethanol.

The highest amount of NA released was 53.91 mg from Coll-N-E-1.00 (which contain 1% NA) and the least amount was 11.58 mg from Coll-N-E-0.25 (which contain 0.25%

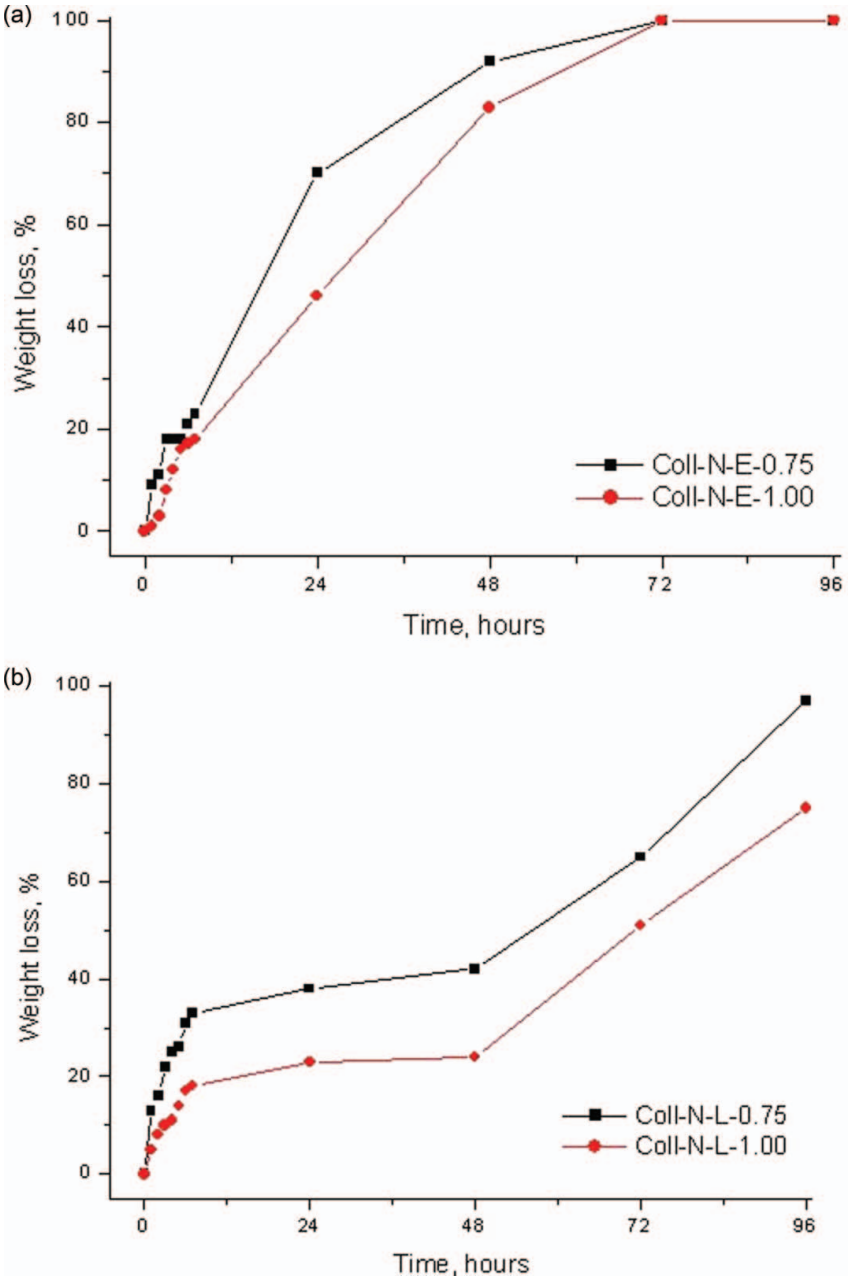

Figure 2. In vitro enzymatic degradation of collagen scaffolds which contain niflumic acid solved in: a) ethanol and b) laurel oil.

NA). Although the amount of NA released from collagen scaffolds increased with NA concentration contained in samples, as Fig. 3a and b showed the percentage of NA released decreased with NA concentration increasing. This means that there is a higher amount of niflumic acid, which is bond by collagen during preparation. Moreover, the bond is stronger when NA is solved in laurel oil, as the enzymatic and water uptake analyses showed.

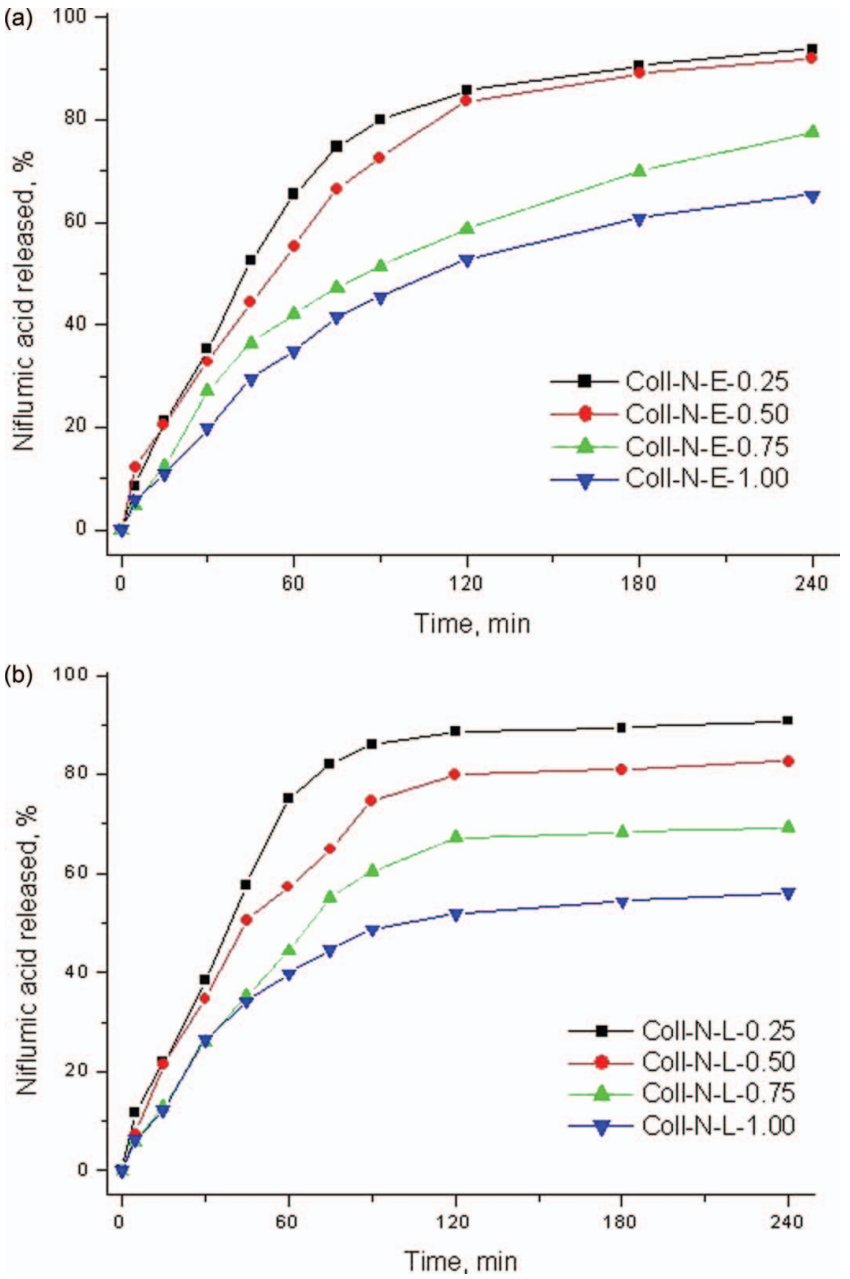

Figure 3. In vitro release of niflumic acid from collagen scaffolds that contain drug solved in: a) ethanol and b) laurel oil.

The biocompatibility of collagen scaffolds with human fibroblast cells was tested only for cross-linked scaffolds, due to the fact the uncross-linked one (Coll) was dissolved in medium culture. The fibroblast cells developed normally around scaffolds (Fig. 4b and i) which indicated that these materials were biocompatible with human fibroblast cells. A

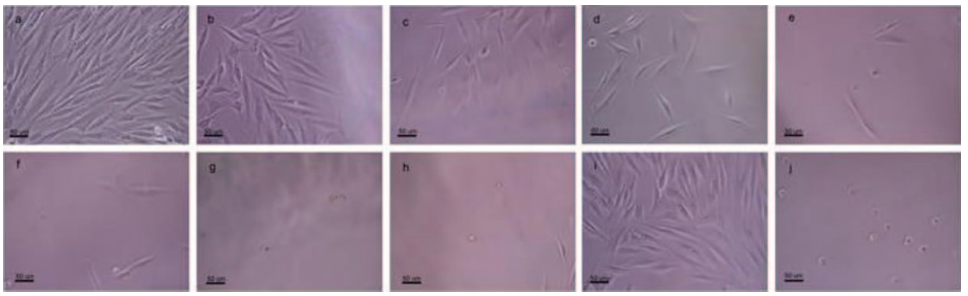

Figure 4. Fibroblasts cells - passage 5 grown three days near collagen scaffolds: a) cells grown on culture plate (control); b) Coll-R; c) Coll-N-E-0.25; d) Coll-N-E-0.50; e) Coll-N-E-0.75; f) Coll-N-E-1.00; g) Coll-N-L-0.25; h) Coll-N-L-0.50; i) Coll-N-L-0.75; j) Coll-N-L-1.00.

few cells were observed near collagen scaffolds with niflumic acid solved in ethanol (Fig. 4c to 4f) and collagen scaffolds with niflumic acid solved in laurel oil had cytotoxic effect (Fig. 4g to 4j) except the sample Coll-N-L-0.75 (Fig. 4i).

These demonstrated that collagen-niflumic acid scaffolds can be used as wound dressing due to their biocompatibility with fibroblast cells.

Conclusions

Various drug delivery systems based on collagen as support and niflumic acid as drug were developed. Niflumic acid, an anti-inflammatory drug insoluble in water was solubilised both in ethanol and laurel essential oil, embedded in collagen gel, cross-linked and then lyophilized in order to obtain spongious form of collagen scaffolds. Water uptake studies showed that the collagen scaffolds have a high hydrophilic ability, which allows them to be used as absorbable wound dressing; the scaffolds which contain niflumic acid solved in laurel oil absorb larger amount of water compared with ones which contain niflumic acid solved in ethanol. The scaffolds which contained niflumic acid solved in laurel essential oil are less biodegradable than the ones with niflumic acid solved in ethanol; the resistance to collagenase biodegradability increases with niflumic acid concentration increasing. The amount of niflumic acid released could be determined from release profiles. The fibroblast cells developed normally around scaffolds which contain less than 0.75% niflumic acid and had cytotoxic effect for the ones with 1.00% niflumic acid. The most promising wound dressing with antimicrobial and anti-inflammatory properties was the one which contains 1.2% collagen and 0.75% niflumic acid solved in laurel essential oil.

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