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Norfloxacin-loaded collagen/chitosan scaffolds for skin reconstruction: Preparation, evaluation and *in-vivo* wound healing assessment

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ABSTRACT

Biomaterial scaffolds are versatile tools as drug carrier for treatment of wounds. A series of norfloxacin-loaded scaffolds were synthesized for treatment of wounds by combining collagen with two different types of chitosan using freeze-drying technique. Subsequently, scaffolds were screened in terms of morphology, water absorption and retention capacity, biodegradation, *ex-vivo* bioadhesive strength, *in-vitro* drug release biological compatibility, X-ray diffractometry, differential scanning calorimetry as well as *in-vivo* evaluation. The results indicate that the scaffold mechanical strength is dependent on the type of used chitosan. The prepared scaffolds contained interconnected porous architecture. The scaffolds had high water uptake and retention capacity with extended biodegradation rate. Scaffolds prepared with chitosan HCl showed superior bioadhesive strength compared to those prepared with low molecular weight chitosan. All scaffolds showed almost 100% drug release within 24 h. As identified by the terahertz pulsed imaging measurements, there is single scaffold area with the same concentration. After 28 days of wound dressing with selected norfoloxacin-loaded or unloaded collagen/chitosan scaffolds in Albino rats, it was found that the tissue regeneration time was fast compared to non-treated wounds. Furthermore, the drug-loaded scaffolds showed normal structure of an intact epidermal layer as well as the underlying dermis as revealed by histopathological studies. The obtained results suggest that the investigated norfloxacin-loaded collagen/chitosan scaffold is a potential candidate for skin regeneration application.

1. Introduction

Treatment of chronic wounds, including diabetic foot ulcers, pressure sores, and other types of wounds, has been a challenging subject for medical teams and researchers. Natural or synthetic bands, cotton and linen gauze and sterilized bandage have been conventionally used for wound dressing for many years. However, nowadays various novel types of wound healing products are available all over the world to prevent and treat chronic wounds (O'Meara et al., 1999). Among these novel products are biomaterial scaffolds. They are versatile tools to create a permissive cellular microenvironment for regenerative medicine. Their tailorable architecture provides a structural niche for the engraftment of transplanted cells and the infiltration of endogenous progenitor cells (Thomas and Shea, 2013).

In tissue engineering, a porous scaffold is required to function as a template and guide in cell adhesion, extension, proliferation, and differentiation. A target tissue would be in growth when the scaffold is biomimetic for the physiological need of the regenerating tissue. Generally, an ideal skin tissue engineering material should have high liquid absorbing capacity, proper gas permeation, biocompatibility and antibacterial properties to protect the skin defect from infections, dehydration and subsequently tissue damaging (Han et al., 2014).

One of the most clinically effective materials that is used for wound healing and skin regeneration is collagen. Collagen is the major protein of the extracellular matrix (ECM) (Kleinman et al., 1987; Rao, 1995). Studies on collagen-based products have processed continuously over decades because of a diversity of manufacturing methods and potential end uses. They have been processed into various shapes such as gel, film, sponge and fiber (Lee et al., 2001). Among these various types of collagen-based products, porous and dense collagen membranes have been favorably applied to healing of skin defects (Kane et al., 1996b; Rao, 1995). Porous collagen membrane was designed as a permanent wound dressing or a scaffold for tissue engineering (Chvapil, 1977; Kane et al., 1996b). Porous membrane has been reported to canalize fibroplast migration from the wound edge because of its porous structure, which leads to the production of newly synthesized extracellular matrix by cells (Lee et al., 2001). Generally, the porous structure of collagen membranes has been attained by the freeze-drying technique. The collagen molecule is specifically degraded by collagenase and its denaturated product is spontaneously denaturated to gelatin at physiological temperature. These gelatinized fragments are then cleaved by several nonspecific proteases. Concomitantly, cells infiltering the membrane should synthesize new extracellular matrix (ECM) components for tissue regeneration. The balance

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between the rates of these two processes is considered to be an important feature of wound healing (Yannas, 1990). Based on the fact that uncross-linked collagen-based materials rapidly degrade into wound fluids, it becomes a necessity to perform membrane cross-linking to control the biodegradation rate (Lee et al., 2001).

Chitosan has been popular in tissue engineering application as a tissue culture scaffold and wound dressing (Florence and Christine, 2013). It has many properties such as biocompatible, biodegradable, nontoxic and antibacterial capabilities (Jayakumar et al., 2011). Chitosan has also been reported to stimulate the activity of growth factors (Ueno et al., 2001) and to contribute to the maintenance of the chondrogenic phenotype, especially in terms of its morphology (Sechriest et al., 2000). Chitosan based scaffolds and sponges have been investigated for wound care in different situations. Chitosan sponges can prevent the wound from infection and dehydration (Anisha et al., 2013; Dutta et al., 2011). These sponges have a typical porous structure and exhibit enhanced liquid absorbing capacity and cell interaction. However, one major limitation for chitosan materials is its brittle behavior. Blends with other synthetic materials are believed to be an effective way to develop a suitable tissue engineering material.

Chitosan was blended with different polymers as alginate and collagen to obtain wound healing scaffolds loaded with drug as drug delivery system for tissue engineering (He et al., 2014; Ti et al., 2015). These scaffolds had enhanced mechanical performance and had controlled drug release to promote wound healing. A study conducted by Ti et al. investigated the use of collagen/chitosan spongy scaffolds loaded with thymosin beta 4 (Tβ4) to accelerate wound healing process in diabetic rats. The investigated scaffolds showed sustained Tβ4 release which resulted in increase in both the migratory and proliferative activities of high glucose-treated human umbilical vein endothelial cells as well as improve angiogenesis (Ti et al., 2015). Kirk et al. fabricated biocompatible collagen scaffolds attached to glycosaminoglycan hyaluronic acid with a covalent bond for chronic wound healing. The tested scaffold supported L929 fibroblast cells attachment and proliferation (Kirk et al., 2013). Scaffold for burn treatment prepared using gelatin microspheres loaded with gentamycin sulfate embedded in silk fibroin was investigated by Lan et al. The fabricated scaffolds were potentially effective against *Pseudomonas aeruginosa* and gave promising results for treatment of full-thickness infected burns as they accelerated both the dermis regeneration and the epithelialization rates (Lan et al., 2014).

In this work, collagen was blended with chitosan to develop an antibiotic medicated-collagen/chitosan sponge scaffold as a skin tissue engineering material. Furthermore, the incorporation of chitosan into a collagen scaffold is known to increase the mechanical strength, as it forms an ionic complex between the positively charged chitosan and the negatively charged collagen (Lee et al., 2004). Norfloxacin was used as a model antibiotic in this study. Norfloxacin is a fluorquinolone with a broad-spectrum activity against gram-positive and gram-negative bacteria, including *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* (Kim et al., 2005) as well as *Salmonella*, *Campylobacter*, *Vibrio cholera* and *Shigella* (Emmerson and Jones, 2003; Van Bambeke et al., 2005). Norfloxacin has effectively been used in wound dressing materials (Dua et al., 2010; Malipeddi et al., 2006). This medicated scaffold can play a dual role in wound healing being considered as an absorption system with excellent water uptake capacity for wound exudates as well as a template and guide in cell adhesion, extension, proliferation and differentiation.

2. Materials and methods

2.1. Materials

Low viscosity chitosan (LMwt CS; 150 kDa), high viscosity chitosan (HMwt CS; 360 kDa) and collagenase were purchased from Sigma-Aldrich Chemie GmbH, Japan. Chitosan hydrochloride was a kind gift from Zhejiang Chemicals Import & Export Cooperation, China. Norfloxacin was supplied by Memphis Co. for Pharmaceutical and Chemical Industries, Cairo, Egypt. Pharmaceutical grade of collagen type 1 obtained from fish with molecular weight of 50,000 Da was purchased from Shaanxi Pioneer Biotech Co., Ltd., China. All other chemicals and solvents were of analytical reagent grade.

2.2. Methods

2.2.1. Fabrication of norfloxacin-loaded scaffolds

Preliminary experiments were done to investigate the proper compositions from collagen and chitosan for scaffold preparation. Different types of chitosan were used in scaffold formulation; chitosan with low molecular weight, chitosan with high molecular weight and chitosan hydrochloride. Five weight ratios of collagen to chitosan were used to formulate the scaffolds; 90:10, 80:20, 70:30, 60:40 and 50:50. The total polymer contents for the scaffold preparation were 4, 8 and 16%. Norfloxacin was added to the scaffold preparation in a concentration of 1%.

Blended collagen and chitosan scaffolds were made by the freeze-drying method described previously (Lee et al., 2001, 2004). First norfloxacin was dissolved in 1% acetic acid solution, after that collagen was dissolved and then chitosan was added in concentrations presented in Table 1. The mixed blends were placed into circular blisters (7.5 mm in diameter), then frozen at −80 °C and then subjected to lyophilization for 24 h using Christ freeze dryer (ALPHA 2-4 LD plus, Germany). The final drug concentration in the lyophilized scaffold was 4 mg.

2.2.2. Characterization of norfloxacin-loaded scaffolds

2.2.2.1. Morphological examination using scanning electron microscopy (SEM). Selected scaffold was subjected to morphological study using SEM (Jeol JSM-6400 Microscope, Tokyo, Japan). The surface and cross-section samples of the scaffold were coated with an ultra-thin layer of gold (~150 Å). The scaffolds pictures were taken at an excitation voltage of 10 kV.

2.2.2.2. Porosity measurements. A digital caliper was used to measure scaffolds dimensions to calculate its volume. Grain volumes of the scaffolds were measured using a helium pycnometer (Ultrapyc 1200e Quantachrome pycnometer, USA). Scaffolds masses were determined using sensitive balance (Sartorius TE 124S, UK). Scaffold density was calculated from scaffold weight and volume. Scaffold porosity was calculated from scaffold grain density and the scaffold true density.

Table 1
Compositions for norfloxacin loaded collagen/chitosan scaffolds.

Formulation code	Collagen	Chitosan	Total polymer content (%)	Type of chitosan
SC-F1	70	30	4	Low Mwt chitosan
SC-F2	60	40	4	Low Mwt chitosan
SC-F3	70	30	4	Chitosan HCl
SC-F4	60	40	4	Chitosan HCl
SC-F5	70	30	8	Chitosan HCl
SC-F6	60	40	8	Chitosan HCl
SC-F7	70	30	16	Chitosan HCl
SC-F8	60	40	16	Chitosan HCl

Norfloxacin was added to scaffold forming solution in a concentration of 1%.