Antibacterial Agent Loaded Fungal Polymer for Use As A Wound Dressing

Yara Örtü Malzemesi Olarak Kullanılmak Üzere Antibakteriyel Ajan Yüklü Fungal Polimer

Research Article

Eylem Güven[1*], Mesut Şam[2], Ebru Erdal[1,2], Zeynep Karahaliloğlu[1,2], Dilek Candan[3], Necdet Sağlam[1], Emir Baki Denkbaş[1,4]

[1]Hacettepe University, Nanotechnology and Nanomedicine Division, Ankara, Turkey
[2]Aksaray University, Department of Biology, Aksaray, Turkey
[3]The Scientific and Technological Research Council of Turkey, Ankara, Turkey
[4]Hacettepe University, Department of Chemistry, Biochemistry Division, Ankara, Turkey

ABSTRACT

This study is mainly concerned with the preparation and characterization of a novel wound dressing, in the form of three-dimensional microporous structure, from extracellular polysaccharide produced by *Trametes versicolor*, a white-rot basidiomycete. A model antibiotic (i.e., ciprofloxacin) was loaded into these wound dressings in the preparation procedure. The prepared wound dressings were investigated and evaluated in four main sections. In the first part, morphological evaluations were made by using scanning electron microscopy and the obtained images revealed that the wound dressings were having highly porous structure with interconnected pores. Antibacterial agent loaded-wound dressings were also investigated with swelling behavior, *in vitro* drug release and biodegradation studies. Obtained results showed that wound dressings from fungal polymer including antibacterial agent could provide a great potential in the treatment of dermal wounds as a new kind of wound dressing material.

Key Words
Wound healing, wound dressing, *Trametes versicolor,* ciprofloxacin, natural polymer, exopolysaccharide, characterization.

ÖZET

Bu çalışma beyaz çürükçül bir mantar suşu olan *Trametes versicolor*'ın ürettiği hücredışı polisakkaritten üç-boyutlu mikrogözenekli yapıya sahip yeni bir yara örtü malzemesinin hazırlanması ve karakterizasyonunu kapsamaktadır. Hazırlama işlemi esnasında model bir antibiyotik (siprofloksasin) yara örtü malzemelerine yüklenmiştir. Elde edilen yara örtü malzemeleri 4 ana bölüm altında incelenmiştir. İlk bölümde taramalı elektron mikroskobu kullanılarak morfolojik değerlendirmeler yapılmış olup elde edilen görüntüler sonucunda yara örtü malzemelerinin birbirleri ile bağlantılı yüksek gözenekliliğe sahip olduğu gözlenmiştir. Antibakteriyel ajan yüklü yara örtü malzemeleri ayrıca şişme ve in vitro ilaç salımı çalışmaları, biyobozunurluk deneyleri yapılarak karakterize edilmiştir. Elde edilen sonuçlar mantar polimerden elde edilen antibakteriyel ajan yüklü yara örtü malzemelerinin yeni nesil yara örtü malzemesi olarak deri yaralarının tedavisinde iyi bir potansiyel olusturabileceğini göstermektedir.

Anahtar Kelimeler
Yara iyileşmesi, yara örtü malzemesi, *Trametes versicolor*, siprofloksasin, dogal polimer, ekzopolisakkarit, karakterizasyon.

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Correspondence to: Eylem Güven, Hacettepe University, Nanotechnology and Nanomedicine Division, Beytepe, Ankara, Turkey

Tel: +90 312 297 61 95 Fax: +90 312 299 2163 E-Mail: eylemoz@hacettepe.edu.tr

INTRODUCTION

Wounds and injuries can result from a range of potential hazards including fire, natural disasters, transport accidents, diseases, operations, and so on. Most wounds are due to small injuries and heal quickly, with very little attention. However, many people suffer from chronic or complex wounds that can be very difficult to heal. Chronic and complex wounds can lead to complications such as infection, pain, depression and limb amputation [1]. Patients affected by these types of wounds often require additional help performing common daily tasks and face higher mortality rates. Billions of dollars each year are spent to care and treat these wounds worldwide. Therefore the development of new and effective interventions in wound care is an area of intense research.

Wound healing is the tissue response to injury and the process of regeneration. It is a complex biological process involving chemotaxis, cell proliferation, production of extracellular matrix (ECM) proteins, neovascularization, and so on [2-5]. Chronic wounds in certain clinical conditions are challenging problems for both patients and surgeon. In this type of delayed wound healing, insufficient granulation tissue formation, epithelisation, and lack of contraction contribute to the delayed wound healing [6]. Wound dressings have been employed to speed up the healing process in acute and chronic wounds by keeping healing tissues moist and increasing superficial wound epithelialization [7].

Dermal substitution and wound healing are research areas of medicine in which there have been many recent advances. Currently, there are a variety of wound dressings available ranging from passive adherent/nonadherent to interactive and bioactive products that contribute to the healing process. Most of these products are scaffold type which are spongy matrices [8]. The function of a degradable scaffold is to act as a temporary support matrix for transplanted or host cells so as to restore, maintain, or improve tissue and due to these properties they are widey used as wound dressings. Healing of dermal wounds with scaffolds prepared using macromolecular agents such as natural polymers is preferred to skin substitutes owing to many advantages such as biocompatibility, nonirritant and nontoxic properties, and ease and safety of the application on dermis [9]. Polysaccharides, being naturally occurring biomolecules, were an obvious choice for investigation as potential wound management aids. In recent years it was recognised that not only can polysaccharides be produced with the required physical characteristics for a wound management product but that the actual polysaccharide or polysaccharide derivative may itself actively participate in the process of wound healing [10]. Polysaccharides also have functional groups, primary and secondary hydroxyls, amino and carboxylic acid funtionalities, which can be used as sites for chemical derivatisation or attachment of specific ligands. The naturally occurring polysaccharide molecule can therefore be modified to change its physical characteristics and, hence, improve its applicability for a specific application. Additionally some has antibacterial properties which is a desired characteristic for a wound dressing [11,12].

In the last decade interest in exopolysaccharides (EPS) produced by microorganisms has significantly increased. Some of those isolated from fungi do possess interesting physical and pharmacological properties such as anti-tumor activity which are currently in clinical use, and hypoglycemic activity [13]. The EPS from fungi are also used as dietary supplements for enhancing stamina and as a remedy for blood circulatory problems. In addition, microbial EPS are employed in wide range of industries depending on their different properties. Some of the polysaccharides produced by a white-rot fungal strain from Bacidomycetes family,*Trametes versicolor,* is known to have antibacterial, antioxidant, antitumor, antiviral activities and immune enhancer properties [14].

In order to develop an ideal wound dressing, a combination of biopolymer produced by white-rot fungal strain *Trametes versicolor* (ATCC200801) and an antibacterial agent (i.e., ciprofloxacin) was designed in this study. Next, prepared wound dressings were characterized by morphological evaluations, *in vitro* drug release, swelling behavior studies and biodegradation tests.

EXPERIMENTAL

Microorganism and Chemicals

A fungus, Trametes (Coriolus) Versicolor (ATTC 200801) used in this study was obtained from the American Type Culture Collection. Stock cultures of the fungi were stored on malt agar at +4°C and periodically subcultured. All culture media and chemicals were supplied by Sigma-Aldrich and Fluka.

Culture Conditions

The culture medium used for the production of biopolymer (extracellular polysaccharide) from *T.versicolor* was a modified form of Vogel's minimal medium [15,16]. Trace element stock solution was consisted of (g per 100 mililiters of distilled water): citric acid. H_2O: 2.5, $ZnSO_4$: 2.5, $Fe(NH_4)_2(SO_4)_2.6H_2O$: 0.5, $CuSO_4.5H_2O$: 0.125, $MnSO_4$. H_2O: 0.025, H_3BO_3: 0.025, $H_3P(Mo_3O_{10})$. H_2O: 0.025. Vogel's stock solution was consisted of (g per 100 mililiters of distilled water): $Na_3C_6H_5O_7$: 15, KH_2PO_4: 25, NH_4NO_3: 10, $MgSO_4$. $7H_2O$: 1, $CaCl_2$. $2H_2O$: 0.5. Glucose (Merck) was added to the prepared mediums to make 2% solution. Before sterilization (110°C for 25 min), the pH of the medium was adjusted to 4.7 by addition of 1 N HCl. Then it was cooled and filtered-sterilized 0.1% of Thiamin stock solution was added to this medium to make 0.1 % (v/v) solution [17].

Stock cultures of *T.versicolor* fungus were suspended in 5 ml of distilled water. For pre-culture this suspension was transferred to 250-ml Erlenmeyer flask containing 100 ml of Malt liquid medium and incubated on a rotary shaker at 140 rpm and 30°C for 72 h. The pre-culture was then homogenized and 2 ml of this homogenate was inoculated in 100 ml of Vogel's liquid medium in 250-ml Erlenmeyer flasks. Incubation was performed at 30°C on a rotary shaker at 140 rpm for 240 h.

Biopolymer Recovery from Culture Supernatant

Following incubation, Fungal biomass was filtered and supernatant was frozen overnight at -20°C. Frozen supernatant was left to be melted at room temperature. Polymer in the form of gel was obtained in the medium. Collected gel polymer was frozen overnight at -20°C. Then frozen sample was freeze-dried [18].

Preparation of Ciprofloxacin Loaded Fungal Biopolymer as Wound Dressing

After separation of fungal biomass from supernatant as explained in the previous subsection, a certain amount of ciprofloxacin was dissolved in 50 ml of supernatant. Next, this supernatant was frozen overnight at -20°C. Frozen supernatant was left to be melted at room temperature. Biopolymer in the form of gel was obtained in the medium. Collected gel biopolymer was frozen overnight at -20°C in petri dishes. Then frozen samples was freeze-dried and ciprofloxacin loaded fungal wound dressings in the form of circular discs was obtained.

Characterization of Fungi-derived Wound Dressings

Morphological Evaluations

Morphological properties of the surface and cross-section of freeze-dried wound dressing was investigated by using scanning electron microscopy (SEM, JEOL, Japan).

Swelling Behavior

Swelling Behavior of wound dressing was determined by the gravimetric method. In this method freeze-dried dry block polymer was placed in a buffer solution (pH: 7.4) for a required period of time. The swollen biopolymer was collected and its weight was determined by first blotting the polymer with filter paper and weighing immediately on an electronic balance (Mettler, Switzerland). The weight of the swollen polymer was recorded at a predetermined time period. Percent swelling was calculated from the following equation;

$$S = \frac{w_t - w_0}{w_o} \times 100 \qquad (1)$$

where S is the swelling (%), w_t denotes the weight of the polymer at time t and w_0 is the initial weight of the polymer. Effect of temperature and pH on swelling behavior were investigated.

In Vitro Ciprofloxacin Release Studies

Ciprofloxacin, as an antibacterial agent was loaded into the wound dressings during the preparation procedure. Different amounts of ciprofloxacin (0,4 mg/ml of supernatant, 0,8 mg/ml of supernatant and 1,6 mg/ml of supernatant) was used to prepare fungal wound dressings. Ciprofloxacin release from the dressings was followed in batch experiments. In these studies, ciprofloxacin loaded dressings were incubated with 20 mL of phosphate buffer solution (pH: 7.4) within a shaker at constant temperature (37°C). The supernatant were pipetted out periodically (i.e., 1, 2, 4, 12, 24, 48, 72, and 96 h) and the concentration of ciprofloxacin was measured spectrophotometrically (Schimadzu, Mini UV 1240, Japan) at 275 nm. During the release experiments, about 20% of the supernatant was replenished with a fresh buffer solution to obtain warm sink conditions and the removed ciprofloxacin from the release medium was also taken into consideration in the calculations.

In Vitro Degradation Studies

First the absolute-dry weight (w_0) of the fungal wound dressing samples were measured and then immersed in phosphate buffered saline solution (PBS, pH 7.4) including 5 μg/ml of Lysozyme at 37°C for 6 weeks with daily solution exchange and gentle shaking in order to determine their weight loss profiles. Samples (also those broken into small pieces) were removed every week, dried in a vacuum oven and their weight was recorded.

The weight loss was calculated using the following equation:

$$\text{Weight Loss (\%)} = \frac{w_0 - w_f}{w_o} \times 100 \qquad (2)$$

where w_0 and w_f are the weights of the dried samples before and after exposure to water, respectively. Five samples were tested for each point. The mean values are presented in the graphs.

RESULTS AND DISCUSSION

Morphological Evaluations

Surface structure and cross section of freeze-dried biopolymer was analyzed by using scanning electron microscopy. SEM micrographs of the biopolymer as cross-section and surface were shown in Figure 1A and 1B. As can be seen in Figure both surface and cross-section of the biopolymer were highly porous and interconnected with pore size around 150-200 μm. Porosity of the polymer was in the form of lamellar structure.

Swelling Behavior

Effective parameters (i.e. pH and temperature of the swelling medium) on swelling behaviour of the biopolymer were evaluated and related results were given in the following sub-sections with details.

Effect of pH on Swelling Behavior

pH of the swelling medium was varied as 4.5, 7.0 and 9.0 in order to investigate the effect of pH on swelling ratio of the biopolymer. The obtained results were shown in Figure 2. As can be seen from the graph the swelling ratio was increased

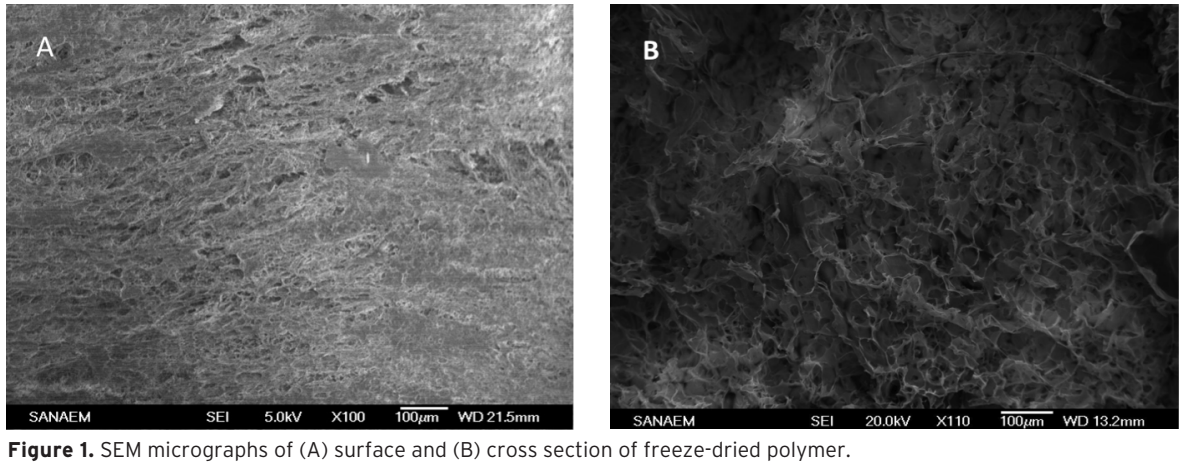

Figure 1. SEM micrographs of (A) surface and (B) cross section of freeze-dried polymer.

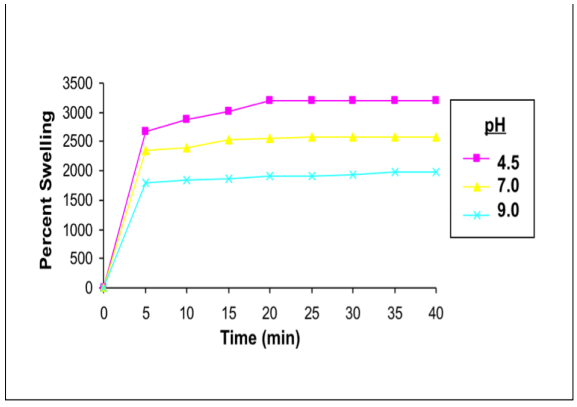

Figure 2. Effect of pH on swelling behavior

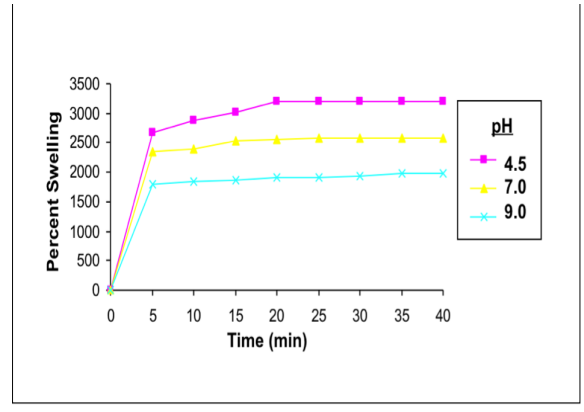

Figure 3. Effect of temperature on swelling behavior.

by decreasing pH. The highest swelling ratio was obtained with the lowest pH as 3210 % and the lowest swelling ratio was obtained with the highest pH as 1975 %. Swelling ratio at pH 7.0 was found as 2585 %. Biopolymers reached the saturation swelling ratio after 5-10 min.

Effect of Temperature on Swelling Behavior

Temperature of the swelling medium was varied as 4°C, 25°C, 30°C and 37°C in order to investigate the effect of temperature on swelling ratio of the biopolymer. The obtained results were shown in Figure 3. Biopolymers reached the saturation (or maximum) swelling ratio after 5-10 min and the swelling ratios changed approximately between 2500 % and 3400 % according to the temperature of the swelling medium.

The highest swelling ratio was obtained with the highest temperature. Swelling ratio was increased by the increase in temperature.

In-vitro Ciprofloxacin Release Studies

The active agent/polymer ratio is a well known effective parameter on the active agent release mechanism for many different types of polymer [19, 20]. Figure 4 shows the effect of initial drug loading amount (0,4 mg/ml of supernatant, 0,8 mg/ml of supernatant and 1,6 mg/ml of supernatant) on release profile of ciprofloxacin from wound dressings. Three stages can be observed.

The first stage, occurring during the first 2 h, exhibited a fast release due to a rapid water uptake of the wound dressings and dissolution of the ciprofloxacin at the surface. At the second stage, the release rate was observed to be decreased and the curve turned into a corner. At the last stage, the release rate was lower and became saturated. The release of ciprofloxacin increased with an increase in drug loading as shown in Figure 4. A higher concentration of the drug embedded in the dressings structured

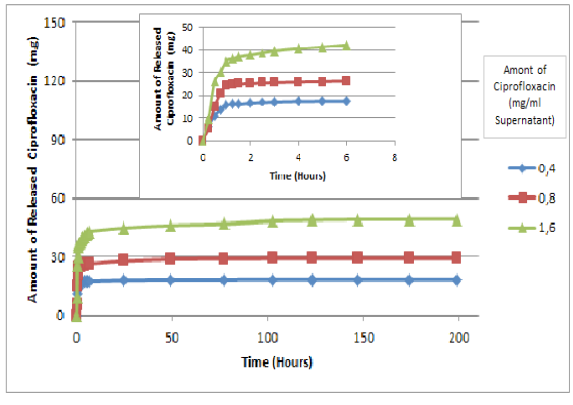

Figure 4. Effect of initial drug loading amount on release profile of ciprofloxacin from wound dressings.

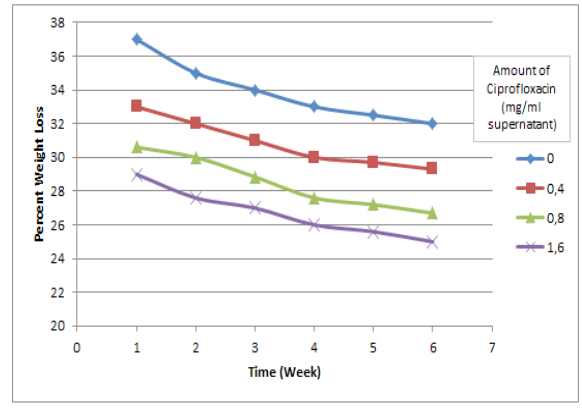

Figure 5. Effect of drug content on ciprofloxacin release

in the matrix led to a higher concentration gradient between the dressing and dissolution medium, so the drug diffusion from the matrix into the external phase was promoted [21]. The increase in drug release at a high concentration of drug loading could also be due to the relatively hydrophylic properties of the drug. The higher the drug concentration is in a delivery system, the faster is the release of hydrophilic model drugs, whereas the release profiles of hydrophobic model drugs are slower [22,23].

In Vitro Degradation Studies

In vitro degradation of the wound dressings having different drug loading was investigated by monitoring the change of the weight loss during degradation in phosphate buffer solution including lysozyme. Drug content was varied in the range of 0.4-1.6 mg/ml supernatant to investigate the effect of drug content on degradation. Figure 5 shows the obtained weight losses. It was observed that the weight loss of drug-loaded wound dressings over the incubation period was larger than that of the membrane without the drug. Also as seen in Figure 5 wound dressings having higher drug loading degraded faster than those with lower drug loading. The dissolution of ciprofloxacin in the dressing increased the contacted surface area between the polymer and water, consequently accelerated the matrix breakdown [24].

CONCLUSIONS

It is expected that the active agent loaded fungi-derived wound dressing will serve as a suitable three-dimensional scaffold for fibroblast populations and providing great potential in the treatment of dermal wounds as a new kind of wound dressing material. Further *in vitro* and *in vivo* studies are planned to demonstrate the efficacy of these antibacterial agent loaded wound dressings as a novel wound/burn dressing system.

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