Polymer Testing 63 (2017) 360–366

Contents lists available at ScienceDirect

Polymer Testing

journal homepage: www.elsevier.com/locate/polytest

Antimicrobial activity of collagen material with thymol addition for potential application as wound dressing

Marta Michalska-Sionkowska [a, *], Maciej Walczak [a], Alina Sionkowska [b]

[a] Nicolaus Copernicus University in Torun, Faculty of Biology and Environment Protection, Department of Environmental Microbiology and Biotechnology, Lwowska 1, Toruń, Poland
[b] Nicolaus Copernicus University in Torun, Faculty of Chemistry, Department of Chemistry of Biomaterials and Cosmetics, Gagarina 7, 87-100 Toruń, Poland

ARTICLE INFO

Article history:
Received 17 July 2017
Received in revised form
18 August 2017
Accepted 27 August 2017
Available online 30 August 2017

Keywords:
Collagen
Thymol
Bactericidal properties
Biofilm formation

ABSTRACT

Recently, special attention has been paid to the development of active wound dressing materials based on biopolymers. Collagen is a natural polymer, which meets the requirements of modern materials for medical applications. However, despite its unique properties, collagen has no antimicrobial activity. In this work thymol was incorporated into collagen films to meet antimicrobial properties of the material. Thymol is a naturally occurring phenolic compound recognized as an antimicrobial agent. Collagen/thymol thin films were obtained through solvent evaporation using collagen solutions containing different amounts of thymol. The structure of the obtained materials was studied using FTIR-ATR spectroscopy. The inhibition ability on the growth of several strains of microorganisms was tested. The standard ISO 22196:2007 was used to define the bactericidal properties of the material. The growth of the following bacteria on the collagen/thymol films was studied: *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis*, *Enterobacter aerogenes, Candida albicans*. The results showed that the growth of *Staphylococcus aureus* was the most inhibited compared to the other tested strains. Collagen/thymol material is more efficient against pathogens through direct contact compared to the diffusion of thymol from the material. In general, the thymol addition inhibits biofilm formation on the collagen surface.

1. Introduction

Traditional wound dressing materials such as cotton wool, gauze and bandages were aimed at maintaining a dry wound environment, enabling the evaporation of wound exudate and protecting the wound bed against the introduction of pathogenic microorganisms [1]. In recent years, there has been a new approach concerning wound dressing materials. Currently, it is believed that an ideal wound dressing should keep the wound environment moist [2], allow gas exchange, protect the wound against microorganisms and absorb excess fluids and secretions from the wounds [3]. Therefore, intensive work on the development of an active material (containing an active agent) has been taken. Among the active agents there may be antimicrobial compounds, growth factors and living cells [4]. For centuries, plant–derived products have been used as treatments for many ailments [5]. Essential oils and other plant origin compounds are very popular and they are considered as an alternative to using antibiotics. They are characterized by many features useful for medical applications. Essential oils can act as anti-inflammatory, anticancer, antifungal or bactericidal agents [6]. In addition, there is a low risk of developing bacteria resistance because of the presence of the complex chemical composition in plant origin substances [7]. Unfortunately, the composition of essential oil is variable due to external factors, such as the latitude in which plants grow, stages of development in which plants were collected, drying methods and even their storage conditions [8]. Therefore, it appears appropriate for the use of pure compounds present in the composition of the essential oils. Thymol (2-isopropyl-5-methylphenol) naturally occur in thyme (*Thymus vulgaris*) and oregano (*Origanum vulgare*) as one of the main components of essential oils composition [9,10]. Thymol shows antimicrobial activity against both Gram-positive and Gram-negative bacteria strains as well as against fungi and yeast [11]. The mechanism of the action of thymol against microorganisms rely on the disruption of the cytoplasmic membrane, which increases its permeability and depolarizes its potential [12].

* Corresponding author.
E-mail address: michalskamar@gmail.com (M. Michalska-Sionkowska).

http://dx.doi.org/10.1016/j.polymertesting.2017.08.036
0142-9418/

Membrane damage causes the leakage of intracellular components and disregulation of cell function [13]. Moreover, the U.S. Food and Drug Administration (FDA) classified thymol as substance "generally recognized as safe" (http://www.accessdata.fda.gov/scripts/fcn/fcnnavigation.cfm?rpt=eafuslisting accessed 21.03.2017).

Collagen is one of the most abundant natural polymers in mammalians bodies and is widely used in the biomedical and cosmetics field. Collagen has several properties required in the biomedical field such as biocompability, biodegrability and non-toxicity [14]. Furthermore, this protein interacts with cells and regulates cell anchorage, migration, proliferation and survival [15,16]. Additionally, collagen is able to promote fibroblast production and stimulates faster wound healing. Therefore, collagen is a suitable polymer for use as material for medical applications [17]. The aim of this work was to design, prepare and characterize collagen membranes with a different concentration of thymol addition. Collagen films with thymol have previously been prepared by Riella et al. (2012) and anti-inflammatory action and cicatrizing property on skin lesions of collagen films containing thymol in rodents was studied [18].

The aim of our work was to study the antibacterial properties of collagen/thymol materials as well as inhibition properties of such materials against biofilm formation.

2. Materials and methods

2.1. Samples preparation

Collagen (Coll) was obtained in the Faculty of Chemistry at Nicolaus Copernicus University in Torun from young rat tail tendons according to the procedure described earlier [19]. Tendons were washed in distilled water and dissolved in 0.1 M acetic acid for three days in 4 °C, the undissolved parts were removed by centrifugation for 10 min at 10 000 rpm. The completely frozen mixtures were lyophilized at − 55 °C and 5 Pa for 48 h (ALPHA 1–2 LD plus, CHRIST, Germany). 1% wt solution was prepared by dissolving collagen in 0.1 M acetic acid.

Thymol (T) pure >99% was purchased from Warchem. 10% thymol solution was prepared using ethanol as a solvent.

Solution of thymol (T) in ethanol was added to collagen to obtain the following amount of T: 4; 2; 1.5; 1; 0.75; 0.5 and 0.25 mg per cm^2 of collagen film. The concentrations were chosen based on previous results based on the disc diffusion method (unpublished data). Nonionic surfactant Polysorbate 80 (NS) was added to the collagen/thymol mixture to obtain better miscibility. Thin films made with collagen and thymol were obtained by solvent evaporation method. Thin film based on collagen and nonionic surfactant was left as a control sample.

2.2. ATR-FTIR spectroscopy

The structure of collagen as well as the interaction between collagen and thymol were confirmed by attenuated total reflection infrared spectroscopy using Nicolet iS10 equipment. All spectra were recorded by absorption mode at 4 cm^{-1} intervals and 64-times scanning [20].

2.3. The study of antimicrobial activity of collagen/thymol films using the diffusion method

The following microorganisms: *Escherichia coli* ATCC8739, *Pseudomonas aeruginosa* ATCC15442, *Staphylococcus aureus* ATCC6538, *Bacillus subtilis* ATCC6633, *Enterobacter aerogenes* ATCC13048, *Candida albicans* ATCC10231 were used for the study.

The determination of the inhibition effect of the collagen/thymol films on tested microorganisms was carried out using the diffusion method. The bacteria were cultured in flasks containing 50 ml of medium composed of (g/L): bacteriological peptone (5) and yeast extract (3), pH = 6.8–7.2. The yeast were cultured on a Sabouraud medium composed of (g/L): bacteriological peptone (10), glucose (40), agar (15), pH = 5.5–5.8. Bacteria and *Candida albicans* grown at 37 °C for 24 h. After cultivation, all the microbial cultures were diluted with saline salt to obtain optical density 0.5 in McFarland [21]. Petri plates containing 20 mL of Mueller-Hinton culture medium were inoculated with 100 μL of microbial suspension. Collagen films with square size 1 × 1cm containing different amounts of thymol, were placed on the surface of the medium with microorganisms. The plates were placed in a fridge for 2 h to allow diffusion of the thymol to the medium, and then were incubated at 37 °C for 24 h. The antimicrobial activity was evaluated by measuring the inhibition zone against the tested microorganisms. Each sample were tested in triplicate and the results (mm of zone of inhibition) were expressed as average values.

2.4. The assessment of bactericidal activity of collagen films containing different amount of thymol

Bactericidal properties of collagen films containing different amounts of thymol were assessed according to standard ISO 22196:2007. The research was based on *Escherichia coli* ATCC8739 and *Staphylococcus aureus* ATCC6538 strains. The analysis was performed in triplicate. As a control sample, collagen film with nonionic surfactant was used (Coll/NS) and as test samples collagen films with 4; 1; 0.75; 0.5; 0. 25 mg of thymol were chosen based on our previous study of thymol on paper disc-unpublished (respectively Coll/NS/T4; Coll/NS/T2; Coll/NS/T1.5; Coll/NS/T1; Coll/NS/T0.75; Coll/NS/T0.5; Coll/NS/T0.25). The control film and all tested samples were covered with the suspensions of bacterial strains investigated in the research with a specified number of cells having been left for a specified time (0 h—validation of recovery efficiency and 24 h). After this time, bacterial cells were recovered from the surface and suspended in a solution containing neutralizer (Soybean casein digest broth with leticin and polyoxyetylene sorbitan monoleate). Subsequently, the number of cells viable and capable of growth was determined by the inoculation on Plate Count Agar in triplicate [22]. The plates were incubated for 24 h at 37 °C.

The reduction of the number of living and viable cells of tested bacteria (R) was calculated using the following equation:

$$R = (U_t - U_o) - (W - U_o);$$

where U_o is the average of the common logarithm of the number of viable bacteria recovered from the control samples (Coll/NS) immediately after inoculation (validation of recovery efficiency); U_t is the average of the common logarithm of the number of viable bacteria recovered from the control samples (Coll/NS) after 24 h (controls of survival in time); W is the average of the common logarithm of the number of viable bacteria recovered from the test samples after 24 h.

According to standard ISO 22196, the reduction of the number of cells capable of growth by two orders of magnitude ($R \geq 2$) was interpreted as a bactericidal effect of the investigated composite.

2.5. The evaluation of bacterial biofilm formation on the collagen/thymol films surface

Flasks with 20 cm^3 of sterile nutrient broth (composition [g/dm^3]: peptone–5.0, meat extract–3.0, pH 7.4) were inoculated with *Staphylococcus aureus* and incubated at 37 °C for 24 h. Fragments 0.5 × 1.5 cm of the control and test films (with 0.75 and 0.5 thymol