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Development of biodegradable antibacterial cellulose based hydrogel membranes for wound healing

Nelisa Türkoğlu Laçin*

Science and Technology Application and Research Center, Yıldız Technical University, 34349 Istanbul, Turkey

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ABSTRACT

Cellulose-based hydrogels have wide applications in tissue engineering and controlled delivery systems. In this study, chloramphenicol (CAP) loaded 2,3 dialdehyde cellulose (DABC) hydrogel membranes were prepared, characterized and their antibacterial efficacy was evaluated. Bacterial cellulose (BC) secreted by *Acetobacter xylinum* was modified to become DABC by oxidation via the sodium metaperiodate method. CAP–BC and CAP–DABC interactions were illustrated via ATR–FTIR analysis. Water retention capacity of BC and DABC membranes were determined as $65.6 \pm 1.6\%$ and $5.3 \pm 0.3\%$, respectively. CAP release profiles were determined via HPLC analysis. The drug-loading capacities of BC and DABC membranes were 5 mg/cm^2 and 0.1 mg/cm^2, respectively. Membranes released 99–99.5% of the contained CAP within 24 h and an initial burst release effect was not observed. In vitro antibacterial tests of BC and DABC, both CAP-loaded, demonstrated their ability to inhibit bacterial growth for a prolonged duration. Antimicrobial effect against bacteria was still prevalent after 3 days of incubation period with disc diffusion tests. The MTT test results reveal that fibroblast adhesion and proliferation on CAP-loaded DABC membranes were noticeably higher than CAP-loaded BC membrane. This newly developed drug containing DABC membranes seem to be highly suitable for wound healing due to its unique properties of biodegradability, biocompatibility, and antimicrobial effectiveness.

1. Introduction

Three-dimensional wound dressing substances must cover the wound, serve as a physical barrier against external infection, and provide cellular support. A successful tissue scaffold should possess the appropriate physical and mechanical characteristics, in addition to possessing an appropriate surface chemistry to facilitate cellular attachment, proliferation, and differentiation [1]. Cellulose, a linear polysaccharide, is one of the most abundant organic materials in nature with a variety of useful applications. A few bacterial species, taxonomically closely related to the genus *Acetobacter xylinum* (*A. Xylinum*), produce and extracellularly secrete cellulose in the form of fibre called BC [2]. It is a natural polymer with potential due to its unique structural and mechanical properties [3]. *A. xylinum* is a simple gram-negative bacterium that has an ability to synthesize high-quality cellulose composed of twisting ribbons of microfibrillar bundles [4]. The thick, gelatinous membrane formed in static culture conditions as a result of these processes is characterized by a 3D structure consisting of an ultrafine network of cellulose nanofibres (3–8 nm), which are highly uniaxially oriented [5].

BC is a natural hydrogel. Hydrogels are defined as three-dimensional polymer networks swollen by large amounts of a solvent. BC is a hydrogel that can take water up to 99% its own weight, mainly due to its amorphous structure [6]. Owing to its unique nano-scaled three-dimensional network structure, BC has high water retention, high mechanical strength, and outstanding biocompatibility, which enable it to serve as a natural scaffolding material for the regeneration of a wide variety of tissues [7]. Dialdehyde cellulose (DABC) is a cellulose derivate produced by regioselective oxidation of cellulose with the use of periodate as an oxidation agent. It is biodegradable and biocompatible and has a large potential to be used in many applications [8,9]. Wei et al. have prepared a type of new functional dry BC film containing benzalkonium chloride as a potential antimicrobial wound dressing material and reported that it has strong antibacterial properties that especially resist both *Staphylococcus aureus* and *Bacillus subtilis* (gram-positive bacteria). Also, a stable and lasting release of the antimicrobial agent for at least 24 h was reported [10]. There are also commercial cellulose-based wound healing systems such as Biofill, Gengiflex, and XCell. Cellulose-based hydrogels are biocompatible, have low production costs, and are non-toxic. Therefore,

* Tel.: +90 532 2257368; fax: +90 212 3832974.
E-mail address: melisalacin@yahoo.com

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cellulose-based hydrogels have wide applications in tissue engineering and controllable delivery systems [11,12]. The high water absorption ability of the antimicrobial BC dry film is crucial for wound dressing to absorb blood and tissue fluid on acute traumas and would promote wound healing [10]. CAP was chosen as a model biologically active agent because it presents a wide antibacterial spectrum with a bactericide activity on gram-negative and gram-positive bacteria [13].

The aim of this study was to develop CAP loaded membranes as antimicrobial wound healing materials, investigate the antimicrobial activities against model gram-positive and gram-negative bacteria (*S. aureus*, *Streptococcus pneumoniae* and *Escherichia coli*), and to observe the proliferation and attachment of fibroblasts on drug-loaded membranes.

2. Experimental procedure

2.1. Materials

E. coli (ATCC 25293), *S. aureus* (ATCC 25925) and *S. pneumoniae* (ATCC6301) were purchased from Institute of Microbiology Chinese Academy of Science.

2.2. Production of BC and preparation of DABC membranes

The production of bacterial cellulose membranes (BCMs) was achieved by growing *A. xylinum* (ATCC 10245) in Hestrin–Schramm medium, pH 5.1(adjusted by 1 M HCl). The medium containing 20 g/L glucose, 10 g/L bactopeptone, 10 g/L yeast extract, 4 mM KH_2PO_4, and 6 mM K_2HPO_4 was used to produce cellulose pellicles in static culture. The inoculum was prepared by growing *A. xylinum* at 30 °C using a rotary shaker for 3 days. The BC nanofibre formation was allowed to occur in a period of 7 days after inoculating the subculture in the proportion of 1:10 in petri dishes statically.

The harvested BC membranes were washed with distilled water to remove s medium components and then incubated in 1 M NaOH solution at 80 °C for 2 h to eliminate attached cells and other impurity. After that, the BC membranes were further purified to remove other residues by distilled water washing until the pH of the washing liquid was neutral. Finally, the bacterial cellulose membranes were cut into 1 cm^2 size [14]. The morphology and microstructure of membranes were characterized by scanning electron microscopy (FE-SEM, Zeiss supra 55) and AFM (Park systems/XE-100E advance scanning probe microscope). Prior to the FE-SEM observation, all samples were sputter coated with a thin layer of platinum to avoid electrical charging. Periodate oxidized cellulose is often referred to as dialdehyde cellulose (DABC) [2]. Oxidation of cellulose using sodium metaperiodate has been extensively investigated in the literature and it is found to lead to selective cleavage at the C-2 and C-3 vicinal hydroxyl groups to yield a product with 2,3-dialdehyde units along the polymer chain [15,16]. The dialdehyde groups also serve as reactive chemical anchors for further reactions, conducive for the chemical derivatization of cellulose. The presence of dialdehyde groups in bacterial cellulose also improves biodegradability and they are discussed in relation to tissue-scaffold engineering [17,18]. Periodate oxidate BCMs were prepared by placing the membranes in DI water, adding 1.3 times the weight sodium metaperiodate and gently stirring at 55 °C in dark for 4 h. After the excess periodate was decomposed with ethylene glycol, the DABC membranes were washed by DI.

2.2.1. Determination of dialdehyde content

Determination of the aldehyde content of DABC was based on the oxime reaction between aldehyde group and $NH_2OH{\cdot}HCl$. The periodate oxidized cellulose, which never underwent drying procedures, has been placed in a 250 mL beaker containing 1.39 g of $NH_2OH{\cdot}HCl$ dissolved in 100 mL of 0.1 M acetate buffer (pH = 4.5). The beaker was covered with a thin rubber foil and the mixture was stirred for 48 h at room temperature with a magnetic stirrer. The product was filtrated and washed with 600 mL of deionized water after which it was dried in a freeze-dryer. For the determination of the aldehyde content of DABC, five membranes were periodate oxidized under the same conditions and the nitrogen content of the oxime derivate of DABCs was determined by using a Leco truSpec series elemental analyzer [19]. The results are expressed as the average values of five samples.

2.2.2. Water retention capacity of hydrogels

In order to dry the membranes without harming the 3D nanonetwork structure, the porous membranes were frozen overnight in a freeze dryer (Christ Alpha 2-4 LD) at −80 °C for 24 h. To determine the water retention capacity of membranes, the freeze dried 1 cm^2 BC and DABC membranes were immersed in phosphate-buffered saline (PBS) at room temperature until equilibration. After that, the membranes were removed from the PBS and excess PBS at the surface of the membranes was blotted out with Kim wipes paper. The weights of the freeze dried membranes were measured, and then the membranes were swollen until no further weight change was observed and the procedure was repeated. The results are expressed as the average values of five samples. The water retention of membranes was calculated with the following formula: W_h: Weight of hydrate membrane, W_d: weight of dry membrane: $WAC(\%) = (W_h - W_d)/W_d \times 100$ [2].

2.3. Preparation of antimicrobial membranes

CAP was loaded into the hydrogels using the swelling-diffusion method. The interaction of CAP with the membranes was investigated by attenuated total reflectance—Fourier transform infrared (Perkin-Elmer). The 1 cm^2 sized freeze-dried membranes were immersed in CAP stock solution (0.05 g/mL) ($CAP_{stock\ sol.}$) for 8 h. Membranes were removed from the CAP stock solution ($CAP_{residual}$) and immersed in 1 mL distilled water for 10 s to remove the non-absorbed CAP ($CAP_{washing\ sol.}$). The amount of CAP loaded into membranes was calculated with the following formula:

$$CAP_{membrane} = (CAP_{stock\ sol.}) - \left(CAP_{residual} + CAP_{washing\ sol.}\right)$$

Afterwards, CAP-loaded membranes were placed in PBS (10 mL) and incubated at 37 °C, 50 rpm for 24 h. CAP amount released from membranes was determined by high performance liquid chromatography (HPLC) to verify the calculation above. The same treatment was applied to each membrane and another freeze–drying step was administered. Finally, 30 min of UV sterilization was performed.

2.4. Determination of antibacterial activity

The antimicrobial activities were investigated against *E. coli*, *S. pneumoniae*, and *S. aureus* using two different methods explained below.

2.4.1. Growth curve method

Single colonies of *E. coli*, *S. Aureus*, and *S. pneumoniae* were grown on nutrient agar. Cell suspensions for inoculum were arranged according to standard no 0.5 as 1–1.5 × 10^8 CFU/mL. The positive control was the bacteria in nutrient broth, the negative control the culture with the pure membrane in nutrient broth, and the blank was the test tube only containing nutrient broth. Cultures were incubated at 37 °C for 24 h. Samples drawn from the system every 2 h were analyzed spectrophotometrically by measuring the