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An investigation of the potential application of chitosan/aloe-based membranes for regenerative medicine

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ABSTRACT

A significant number of therapeutics derived from natural polymers and plants have been developed to replace or to be used in conjunction with existing dressing products. The use of the therapeutic properties of aloe vera could be very useful in the creation of active wound dressing materials. The present work was undertaken to examine issues concerning structural features, topography, enzymatic degradation behavior, antibacterial activity and cellular response of chitosan/aloe vera-based membranes. The chitosan/aloe vera-based membranes that were developed displayed satisfactory degradation, roughness, wettability and mechanical properties. A higher antibacterial potency was displayed by the blended membranes. Moreover, in vitro assays demonstrated that these blended membranes have good cell compatibility with primary human dermal fibroblasts. The chitosan/aloe vera-based membranes might be promising wound dressing materials.

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1. Introduction

In recent years the inclusion of medicinal plants in alternative medicine has been increasing in our society as a way to improve people's quality of life [1]. This interest arises from the therapeutic properties of plants, which are useful in healing various diseases, with the advantage of being 100% natural. A good example is the use of aloe vera (AV), a tropical plant belonging to the family Liliaceae, as a healing accelerator on small wounds and burns because of its antimicrobial effect and epithelizing action on damaged skin tissue [2]. The gel extracted from AV leaf has also been used as a healing agent in cosmetic products and drugs [3]. The health benefits associated with AV have been attributed to its compositional heterogeneity, containing amino acids, enzymes, vitamins, polysaccharides (pectins, cellulose, hemicellulose, glucomannan, acemannan and mannose derivatives) and other low molecular weight substances [2]. An analysis of the effects of AV regarding the requirements for wound healing, such as the maintenance of nutrients, moisture, oxygenation, control of inflammation, immunodulatory activity, epithelialization and fibroblast proliferation [4] indicated that AV products fulfill almost all these necessities. For example, a whole gel extract was found to have anti-inflammatory activity on carrageenan-induced edema in rat paws [5]. Other studies have shown that AV increases collagen content within the wound, supporting faster wound healing [6]. On the other hand, chitosan, a natural polymer derived by the deacetylation of chitin [7], has been widely used as a base material in the production of matrices for wound management [8]. Chitosan-based membranes have, for instance, been widely investigated as wound dressings due to their easy production and long shelf life [9] as well as the intrinsic properties of this polymer [7]. In this work the synergistic association of chitosan and native AV gel was explored as an approach to create blended membranes which could be useful as active wound dressings. Within this context issues concerning topography, structural features, degradation behavior, antibacterial activity and cellular response of chitosan/AV-based membranes were evaluated.

2. Materials and methods

2.1. Materials

Fresh whole AV (*Aloe barbadensis* Miller) leaves obtained from a Portuguese botanic shop were used as the raw material in all experiments. The studied leaves, between 30 and 40 cm long, came

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from to 4-year-old plants. Reagent grade medium molecular weight chitosan (Cht) (Sigma Aldrich, CAS 9012-76-4) was used, with a 23.4% degree of acetylation and molecular weight of 166 kDa, determined by ^{1}H NMR and SEC-MALLS, respectively. Prior to use Cht was purified using a reprecipitation method, as described previously [10]. All other chemicals were reagent grade and were used as received.

2.2. Methods

2.2.1. Extraction of the gel

Whole leaves were washed with distilled water to remove dirt from the surface. The skin was carefully separated from the parenchyma using a knife. The samples were extensively washed with distilled water to remove exudate from their surfaces. The samples were then homogenized in a blender and the homogenized mass filtered. After that the AV gel was stabilized at 65 °C for 15 min and stored at 4 °C prior to use.

2.2.2. Preparation of the membranes

Chitosan flakes were dissolved in 0.2 M aqueous acetic acid at a concentration of 2 wt.% to obtain a homogeneous solution. Then the solution was filtered to remove impurities. The extracted AV gel was added to the Cht solution at ratios of 2:1 and 1:1 v/w Cht/AV, termed CAV and CAV1, respectively. Glycerol, a known plasticizer (water/glycerol 2.5 vol.%) was also added to the blended mixture. The blended systems were kept under stirring at 4 °C for at least 3 h. After homogenization the blended solutions were cast in Petri dishes and dried at room temperature for 4 days. Subsequently neutralization of the membranes was performed by soaking them in 4% NaOH/ethanol 1:1 for 10 min, followed by washing with ethanol and then with distilled water until pH 7 was reached. This neutralization process was used in order to avoid leaching out of the AV due to its high solubility in water. The Cht membranes were neutralized using only 4% NaOH [11].

2.3. Characterization

2.3.1. Fourier transform infrared spectroscopy

The infrared spectra of the powdered membranes were recorded in a FTIR spectrometer (Perkin-Elmer 1600 series). Prior to analysis the powdered membranes were mixed with potassium bromide at a ratio of 1:100 (by weight), followed by uniaxially pressing into a disk. All spectra were obtained between 4000 and 400 cm^{-1}.

2.3.2. Atomic force microscopy

The samples were observed at at least three spots using tapping mode with a MultiMode sensor connected to a NanoScope, both supplied by Veeco, with non-contacting silicon nanoprobes (~300 kHz, set point 2–3 V) from Nanosensors. All images (10 μm wide) were fitted to a plane using the third degree flatten procedure included in the NanoScope software v. 4.43r8. The surface roughness was calculated as S_q (root mean square from an average flat surface) and S_a (average absolute distance from an average flat surface). The values are presented as means ± standard deviations.

2.3.3. Contact angle measurements

The surface properties of the membranes were also investigated by means of static contact angle (θ) measurements using the sessile drop method with glycerol (polar) and diiodomethane (non-polar) (OCA, with SCA-20 software). Six measurements were carried out for each sample. The presented data is the average of six measurements. The surface energy was calculated using the Owens, Wendt, Rabel and Kaelble (OWRK) equation [12].

2.3.4. Dynamical mechanical analysis (DMA)

Viscoelastic measurements were performed using a TRITEC8000B dynamic mechanical analyzer (Triton Technology) in tensile mode. The measurements were carried out at 37 °C. Samples were cut into 1 cm square × 1.5 cm thick (measured with a micrometer) blocks. The membranes were analyzed while immersed in a liquid bath in a Teflon reservoir. The samples were clamped in the DMA apparatus and immersed in phosphate-buffered saline (PBS). The DMA spectra were obtained during a frequency scan between 0.1 and 10 Hz at 37 °C. The experiments were performed under constant strain amplitude (50 μm). A minimum of three samples was used for each condition.

2.3.5. Swelling and enzymatic degradation

Swelling and degradation tests were performed by immersing all membranes in PBS containing 13.6 mg l^{-1} lysozyme (Sigma Aldrich) and PBS without enzyme at 37 °C for up to 30 days. All experiments were conducted in triplicate and the solutions were changed every 7 days to guarantee enzyme activity during the study period. The swollen sample weights were measured after removing excess surface water by gently tapping the surface with filter paper. Water uptake was determined from the swollen state (after equilibration and eventual degradation or partial solubilization) w_s and the final dried weight w_f using Eq. (1). Each experiment was repeated three times, and the average value was considered to be the water uptake value.

$$\text{water uptake}(\%) = ((w_s - w_f)/w_f) \times 100 \quad (1)$$

The weight loss was calculated from the initial dried weight w_i and final dried weight w_f, using Eq. (2).

$$\text{weight loss}(\%) = ((w_i - w_f)/w_i) \times 100 \quad (2)$$

2.3.6. Antibacterial activity

Staphylococcus aureus ATCC 25923 were first grown for 24 h in Tryptic Soy Agar (TSA) (Merck, Darmstadt, Germany) at 37 °C. After this period 50 μl of cell suspension were transferred to 30 ml of fresh Tryptic Soy Broth (TSB) and incubated for 18 h (late exponential phase) at 37 °C and 120 r.p.m. Then the cells were centrifuged (Sigma 4K10, B. Braun) for 5 min at 8000 r.p.m. and 4 °C and washed twice with saline solution (0.9% NaCl) in distilled water. The cell suspension was adjusted to a final concentration of approximately 1×10^8 *S. aureus* cells ml^{-1}, determined from the optical density at 640 nm. Prior to use the membranes were sterilized with ethylene oxide. Each membrane was placed in an individual well of a 24-well tissue culture plate (Sarstedt, Newton, NC) containing 1 ml of the cell suspension with 1×10^8 cells ml^{-1} in each well. The plates were incubated for 24 h at 37 °C in an orbital shaker (120 r.p.m.). Assays were performed in triplicate and repeated three times. The number of colony-forming units (CFU ml^{-1}) in suspension (cells not adherent on the membranes) was determined by the serial dilution method. Viable cell numbers were determined by performing 10-fold serial dilutions of the cell suspension in each well in saline blanks and plating on TSA. Colonies were counted after 24 h at 37 °C. Only plates containing between 30 and 300 colonies were counted. The results are presented as $\log_{10}$ CFU ml^{-1} after challenge. Bactericidal activity was defined as a 3 $\log_{10}$ CFU ml^{-1} (−99.9%) reduction in bacterial numbers.

2.3.6.1. Agar disk diffusion method. In the agar diffusion technique 30 ml of Plate Count Agar (PCA) was added to a Petri dish and then 0.1 ml of bacterial solution (1×10^8 cells ml^{-1}) was placed in the center the dish and spread. After drying the membranes were punched into the agar at the center of each Petri dish. After 24,

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