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Effect of crosslinking in chitosan/aloe vera-based membranes for biomedical applications

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ABSTRACT

The positive interaction between polysaccharides with active phytochemicals found in medicinal plants may represent a strategy to create active wound dressing materials useful for skin repair. In the present work, blended membranes composed of chitosan (Cht) and aloe vera gel were prepared through the solvent casting, and were crosslinked with genipin to improve their properties. Topography, swelling, wettability, mechanical properties and *in vitro* cellular response of the membranes were investigated. With the incorporation of aloe vera gel into chitosan solution, the developed chitosan/aloe-based membranes displayed increased roughness and wettability; while the genipin crosslinking promoted the formation of stiffer membranes in comparison to those of the non-modified membranes. Moreover, *in vitro* cell culture studies evidenced that the L929 cells have high cell viability, confirmed by MTS test and calcein-AM staining. The findings suggested that both blend compositions and crosslinking affected the physico-chemical properties and cellular behavior of the developed membranes.

1. Introduction

The recognition of the pharmaceutical, medical and economical value of medicinal plants is still growing, although this varies widely between countries (Hoareau & Da Silva, 1999). Despite the use of herbal plants over many countries, only a relatively small number of plant species has been studied in a large range of applications. Aloe vera (AV), a tropical plant belonging to the *Liliacea* family (Reynolds & Dweck, 1999), can be found in the formulations of cosmetics, dietary supplements, lotions and beauty products (Eshun & He, 2004). The interest of this plant is associated to its heterogeneous composition. For instance, the AV gel is composed not only by many active compounds but also polysaccharides which express various health benefits (Hamman, 2008). Many studies have been shown that AV has anti-inflammatory, anti-tumor, immodulatory and antibacterial activity (Hamman, 2008; Reynolds & Dweck, 1999). Based on the mentioned properties, current research on AV gel had investigated its use conjugated with synthetic and natural polymers aiming the production of 2D and 3D matrices as, *e.g.* hydrogels, nanofibers and films (Inpanya, Faikrua, Ounaroon, Sittichokechaiwut, & Viyoch, 2012; Khoshgozaran-Abras, Azizi, Hamidy, & Bagheripoor-Fallah, 2012; Park & Nho, 2004; Pereira, Tojeira, Vaz, Mendes, & Bartolo, 2011). In those studies, positive effects on mechanical, biocompatibility and thermal degradation of the resulting matrices were observed due to the incorporation of AV gel.

On the other hand, chitosan, a natural polymer, has been recognized by its intrinsic properties such as biodegradability, solubility in weak acids, cationic nature, anti-bacterial and haemostatic properties (Alves & Mano, 2008; Pillai, Paul, & Sharma, 2009). Among the distinct chitosan-based matrices reported in the literature (Guibal, Milot, Eterradossi, Gauffier, & Domard, 1999; Muzzarelli, 2009; Reys et al., 2013; Silva et al., 2008) and proposed for biomedical applications, chitosan membranes have been widely investigated for the purpose of wound coverage because of their easy production and long shelf life (Altiok, Altiok, & Tihminlioglu, 2010; Silva et al., 2007). Nevertheless, the development of chitosan membranes with desirable properties sometimes requires modification at surface or bulk level by chemical and physical means (Silva, Santos, Coutinho, Mano, & Reis, 2005; Silva et al., 2007). Promising findings on wound healing have also achieved using combinations of chitosan and soy protein, alginate or gelatin (Santos et al., 2013; Silva, Mano, & Reis, 2010). It has been reported that chitosan-based matrices crosslinked using genipin, an effective natural crosslinking agent,

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have better stability in aqueous medium and mechanical properties as compared to non-modified materials (Alves & Mano, 2008). Even though the mechanism of reaction of genipin with chitosan is well documented (Muzzarelli, 2009), no information has been found involving reactions of genipin with AV gel. In the present work, blended membranes composed by chitosan and aloe vera gel were produced by solvent casting, and subsequently submitted to crosslinking using genipin. Both native AV gel and a commercial one (ACTIValoe®) were used in the formulation of the blended membranes in order to study its interactions with chitosan. We hypothesized that these membranes could be used as wound dressings. Then, they will facilitate the penetration of the AV gel onto the skin, and the membrane may act at different stages of the healing process, while protecting the injury from infection, constituting a more value added material as compared to synthetic ones used for this purpose. Additionally, chitosan will increase the stability of the polysaccharides and/or compounds present in the AV composition (Hamman, 2008), which can in turn keep their natural biological activity. By its turn, the chemical crosslinking on chitosan/aloe-based membranes using genipin will brings the possibility to control the leaching out of AV gel portion from the blended membranes. Herein, issues concerning topography, swelling, wettability, mechanical properties and cellular behavior of the developed membranes were evaluated.

2. Experimental

2.1. Materials

Fresh whole aloe vera (*Aloe barbadensis Miller*) leaves obtained in a Portuguese botanic shop, were used as the raw material in all experiments. The studied leaves have between 30 and 40 cm of length, corresponded to 4-year old plants. Aloe vera (Tai-Nin Chow, Williamson, Yates, & Goux, 2005) commercial powder (Aloe *barbadensis Miller*, ACTIValoe® Aloe Vera Gel Qmatrix 200X) from Aloecorp-Zeus Química Ltda (Portugal) was used as received. Reagent grade medium molecular weight chitosan – Cht (Sigma Aldrich, CAS 9012-76-4) was used, with a deacetylation degree of 76.6%, and molecular weight of 166kDa, determined by ^{1}H NMR and SEC-MALLS, respectively (Reys et al., 2013). Prior use, chitosan was purified using a re-precipitation method, as described previously (Signini & Filho, 2001). All other chemicals were reagent grade and were used as received.

2.2. Methods

2.2.1. Extraction of the aloe vera gel

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

2.2.2. Preparation of the membranes

Chitosan flakes were dissolved in an aqueous acetic acid 0.2 M at concentration of 2%wt% (w/v) to obtain a homogeneous solution. The chitosan/aloe vera gel blended films were prepared using two strategies. In the first approach, the extracted AV gel was added to Cht solution at a ratio of 1:2, 1:3 and 2:1 (v/v) Cht/AV. In the second approach, the AV powder (ACTIValoe®) was added to Cht solution at ratio, 1:2, 1:3 and 2:1 (w/v). Glycerol, a well-known plasticizer, was also added to blended mixture (water/glycerol 2.5%, v/v). Then, the blended systems were kept under stirring at 4 °C for at least 3 h. After homogenization, the blended solutions were casted into petri dishes and dried at room temperature for 4 days. Subsequently, the neutralization of the membranes was performed by soaking them in a NaOH 4%/ethanol 1:1 for 10 min, followed by washing with ethanol and then with distilled water until pH 7. For Cht membranes, the neutralization was performed using only NaOH 4% (w/v). The identification of the Cht/AV membranes was CA and CAQ, for those prepared using native and commercial AV, respectively.

2.2.2.1. Determination of aloe vera release. The aloe release from the membranes (CA and CAQ), during neutralization process, was calculated using a standard curve constructed using freeze-dried AV gel (concentrations ranging from 0.01 to 1%mg/ml) dissolved in NaOH/ethanol 1:1 (v/v). Then the optical absorbance of the solution was recorded with a spectrophotometer (Bio-Rad SmartSpec 3000, CA) at a wavelength of 400 nm. All tests were performed using six replicates.

2.2.3. Genipin crosslinking on membranes

Dried CA and CAQ membranes were crosslinked by immersion in a bath containing genipin 10%mM, previously dissolved in pure ethanol. The crosslinking reaction was performed at room temperature during 30 min and then, the membranes were washed with ethanol, followed by distilled water to remove non-reacted genipin. The membranes were dried at room temperature and stored at 4 °C until use. The identification of the crosslinked blended membranes was CAG and CAG1, prepared using native and commercial AV gel, respectively. The crosslinking degree of each test group of samples (non- and crosslinked membranes) was determined by the use of the ninhydrin assay (Eick, Good, & Neumann, 1975). In the ninhydrin assay, the sample was weighed (3%mg) and heated with a ninhydrin solution (2%wt%, v/v) at 100 °C for 20 min. Then the optical absorbance of the solution was recorded with a spectrophotometer (Bio-Rad SmartSpec 3000, CA) at a wavelength of 570 nm. Glycine solutions of various known concentrations were used as standards, and both CA and CAQ membranes that were prepared without genipin were used as control materials. After the sample was heated with ninhydrin, the number of free amino groups in the test sample was proportional to the optical absorbance of the solution. Each sample was made in triplicate. Then, the degree of cross-linking of the samples was calculated (Eq. (1)). Fresh and fixed are the mole fractions of free NH_2 remaining in non-crosslinked and crosslinked samples, respectively.

$$\text{Degree of crosslinking} = \left[\frac{(\text{NH reactive amine})\text{fresh} - (\text{NH reative amine})\text{fixed}}{(\text{NH reative amine})\text{fresh}}\right] \times 100 \quad (1)$$

2.3. Characterization

2.3.1. Atomic force microscopy (AFM)

The samples were measured on at least three spots using TappingMode™ with a MultiMode connected to a NanoScope, both supplied from Veeco, USA, with non-contacting silicon nanoprobes (*ca* 300 kHz, setpoint 2–3%V) from Nanosensors, Switzerland. All images (10 μm wide) were fitted to a plane using the 3rd degree flatten procedure included in the NanoScope software version 4.43r8. The surface roughness was calculated as Sq (root mean square from average flat surface) and Sa (average absolute distance from average flat surface). The values are presented as mean (standard deviation).