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New method to produce poly(vinyl alcohol)/cellulose acetate films with improved antibacterial action

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Abstract

New alternatives to the conventional wound dressings are being engineered. Here, we propose the processing of two biodegradable polymers, poly(vinyl alcohol) (PVA) and cellulose acetate (CA), in the form of films via a new method that combines principles of solvent casting and phase-inversion. Highly flexible and mechanically resistant films were obtained. PVA/CA films were then treated with the antibiotic vancomycin via two methods, blending and physisorption (via dopamine). Immobilization of vancomycin was proven efficient in promoting the films' antibacterial action against *Staphylococcus epidermidis* bacteria. Data demonstrates the potentialities of PVA/CA films for prospective wound healing applications.

Keywords: biodegradable polymers; solvent casting; phase inversion; antimicrobial peptides; antibacterial activity; wound-healing applications;

1. Introduction

Wound healing is an essential physiological process consisting in the collaboration of many cell strains, biological molecules and signal pathways, that engage in complex interactions in an attempt to restore the skin integrity. It consists of four major, overlapping phases, the hemostasis, inflammation, proliferation and tissue remodeling. In case of acute wounds, these phases occur in the proper sequence, at a specific time and with a specific duration. However, due to various potential stimuli, the healing process may stall in one of these phases (i.e. inflammatory) and contribute to the wound chronicity. Chronic wounds are the result of gradual tissue degradation becoming more difficult to treat and requiring multi-step therapies. Nevertheless, in both acute and chronic wounds, dressings are an essential part of the healing process; in fact, they are responsible not only to protect the wound but may also intervene actively in the healing process [1-3].

Biodegradable polymers, particularly those biocompatible, are highly desirable in biomedical applications, including wound healing. Many formulations of individual or blends of synthetic and natural-origin polymers have been investigated for the production of dressings with successful results, including poly(vinyl alcohol) (PVA) and cellulose [2,4,5]. PVA is a water-soluble, biodegradable, non-toxic, biocompatible polymer with excellent physical and mechanical properties [6]. Cellulose is one of the most abundant natural-origin biopolymers, which possesses remarkable physical-chemical properties, is biocompatible and low cost. Its acetate ester, the cellulose acetate (CA), due to its ease of process is many times used instead of cellulose [7]. Combinations of PVA and CA are very common in filtration membranes [8]. To the authors' knowledge little has been reported on the use of PVA/CA polymeric matrices for wound healing applications.

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In recent years, much research has been dedicated to the development of bioactive dressings. This is a fairly new class of wound dressings responsible for promoting wound healing (i.e. fighting infection) via the incorporation of biomolecules of interest within the matrix of the dressing, including antibiotics. Antibiotics can be naturally occurring or synthetically produced to act selectively against pathogens colonizing the wounded site. Vancomycin, for instance, is an actinobacteria-derived glycopeptide antibiotic that inhibits the proper cell wall synthesis in Gram-positive bacteria and is used to treat several infections, including skin infections. It was discovered in the 1950's but was soon discarded in favor of other antibiotics deemed more efficient, particularly against bacteria from enterococci and staphylococci strains [9,10]. However, recent studies have rekindled the interest on this antibiotic by studying new ways to administer the antibiotic that will reduce bacteria resistance [10,11].

In the present work, PVA/CA films processed at a ratio 80/20 v/v using a new method that combined principles of casting and phase inversion were fashioned and then functionalized with vancomycin (via blending or physisorption) with the purpose of generating a biodegradable alternative wound dressing with effective antibacterial action. The films' mechanical stability, flexibility, porosity and hydration capacity were evaluated, and their antibacterial activity was tested against *Staphylococcus epidermidis* bacteria (*S. epidermidis*, Gram-positive), the most common in the human skin.

2. Experimental Methods

2.1. Films Production

PVA (Mw 78,000, 88% hydrolyzed from Polysciences Europe GmbH) and CA (39.8 wt% acetyl content, Mw 30,000 from Sigma) were individually dissolved at 10 wt% in dimethylformamide (DMF, Fisher) for 6 h. PVA was dissolved at 130 ºC, while CA was dissolved at room temperature (RT) for the same period of time. Once completely dissolved, the temperature from the PVA solution was reduced to 60 ºC and the CA was combined, leaving the blend to homogenize for 2 h. PVA/CA films were prepared at the ratios 100/0, 80/20 and 0/100 v/v. The combination 80/20 was selected as desirable to generate a flexible and breathable film based on previous experiments, in which ratios of 90/10, 70/30, 60/40 and 50/50 were also examined [12,13]. The 100 PVA and 100 CA, respectively PVA/CA 100/0 and 0/100 v/v, were used as control. 40 mL of each solution were casted onto clean glass petri dishes of 140 mm of diameter. After 15 min of resting (films reached RT), 80 mL of a clotting bath, composed of 8 wt% sodium sulfate (Sigma) and 4 wt% sodium hydroxide (Sigma), were added and left in contact with the films for 24 h. This step is known as phase-inversion method. In the end, the films were washed several times in distilled water (dH_2O) to eliminate any trace of DMF or the clotting bath and maintained hydrated in dH_2O (exchanged twice a week) at 4 ºC before use (Fig.1).

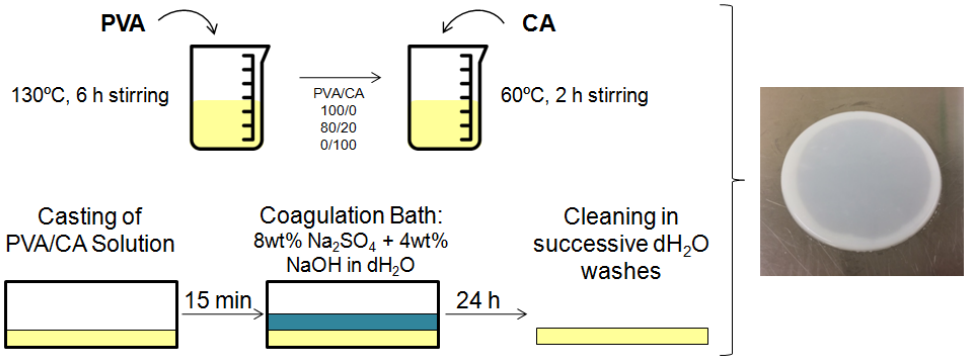

Fig.1. Representation of the PVA/CA films production process and respective final product.

2.2. Characterization

For characterization purposes, films were slowly dried for 5 days at 37 ºC before testing. This gradual dH_2O evaporation prevented any alteration to the film's structural integrity, introduced by elevated temperatures.

Attenuated Total Reflectance with Fourier-Transform Infrared Spectroscopy (ATR-FTIR) was used to record the PVA/CA films chemical groups spectra. Here, a Nicolet Avatar 360 FTIR spectrophotometer (Madison, USA) with

an ATR accessory was employed. For each film, a total of 45 scans were performed at a spectral resolution of 4 cm^{-1}, over the range of 400-4000 cm^{-1}.

The films tensile strength and elongation at break were evaluated using a Housfield H5KS dynamometer (Artilab) associated with the QMAT Materials Testing & Analysis software, following the standard ASTM D5035. Because of its fragile structural integrity (wet paper-like consistency), 100 CA films were not tested. Three rectangular-shaped specimens of 8 cm long and 2 cm width were cut from each film. The average thickness of the samples was determined at approximately 0.16 cm. The gauge length and grip distance were established at 4 cm. The crosshead speed was 50 mm/min and the selected load cell was of 250 N, with a load range of 8 N and pre-load of 0.1 N. Experiments were conducted at RT.

The porosity was determined by measuring the films' mass loss after drying. Films were weighted before and after drying for 5 days at 37 ºC, moment at which the films' mass reached a constant value. The porosity (ε) was calculated using the equation:

(1) $\varepsilon = \frac{(m_w - m_d)}{AL\rho}$

where m_w (g) is the weight of the wet film, m_d (g) is the weight of the dry film, and A, L and ρ are the wet film effective area (cm^2), the wet film thickness (cm) and the dH_2O density (g/cm^3), respectively.

The films' degree of swelling (DS) was also determined by measuring the weight of the samples before and after drying. It was calculated using the following equation:

(2) $DS\ (\%) = \frac{(m_w - m_d)}{m_w} \times 100.$

2.3. Vancomycin Immobilization

PVA/CA films of 1.1 cm diameter were functionalized with the antibiotic vancomycin hydrochloride (Sigma) using two methods: in the first, the antibiotic was directly embedded within the polymeric solution before casting, and in the second, dopamine hydrochloride (DOPA, Sigma) was used as a binding agent. The blended method required the combination of the antibiotic with the polymeric solution during the homogenization step. In this case, the temperature of that step was reduced to 40 ºC so the integrity of the antibiotic was not compromised. Considering the Minimum Inhibitory Concentration (MIC) of vancomycin (non-functionalized) against *Staphylococcus epidermidis* is inferior to 50 µg/mL [14], to prevent loss of action during functionalization, vancomycin was prepared at 100 µg/mL in PBS. After homogenization with vancomycin, the consecutive film production steps were followed as described in sub-section 2.2. In the second method, DOPA was prepared at 100 µg/mL in mM Tris-HCl at pH 8.5 and surfaces were immersed for 24 h at RT under 100 rpm (to prevent aggregates). For optimal polymerization conditions (conversion of DOPA into polydopamine or pDOPA), this step was conducted protected from light. pDOPA-coated films were then sonicated twice with Tris-HCl and once with dH_2O (2 min) to remove existing pDOPA precipitates, followed by several washes with dH_2O [15]. Then, films were placed in contact with the vancomycin solution for 12 h at 37 ºC, under static conditions. In the end, films were washed three times with PBS and unattached or weakly bound molecules were removed.

2.4. Antibacterial Action

The antibacterial efficacy of the vancomycin functionalized PVA/CA films was assessed quantitatively following the standard ASTM-E2149-01, which determines the antimicrobial efficacy of an antimicrobial agent under dynamic conditions. The Gram-positive bacteria *S. epidermidis* (ATCC 35984) was selected for this test since it is the most abundant bacteria present in the human skin. The experiment was conducted aseptically to ensure the absence of any contamination. Bacteria inoculum was prepared from a single colony and incubated overnight in tryptic soy broth (TSB, Merck) at 37 ºC and 120 rpm. Tests were carried out using an initial concentration of 1.5-3.0 x 10^7 CFUs/mL in PBS. 1.1 cm diameter films weighing approximately 0.12 g were immersed in 2 mL of bacteria suspension and incubated at 37ºC and 100 rpm. After 0 h (before contact with sample) and 24 h of culture, the bacteria were serially diluted, cultured onto tryptic soy agar (TSA, Merck) plates, and further incubated for another 24 h. Quantitative results were obtained by counting the colonies of surviving bacteria on the agar plates. Antibacterial activity was reported quantitatively in terms of % bacteria reduction calculated as the ratio between the number of surviving

bacteria colonies present on the TSA plates, before and after contact with film. Experiments were conducted in triplicate and statistical analysis was conducted using the GraphPad Prism 7.

3. Results and Discussion

3.1. PVA/CA Films Characterization

Films prepared of PVA and CA at an 80/20 v/v ratio were characterized in terms of functional groups, mechanical stability, porosity and hydration capacity. These factors are extremely important in wound healing. Indeed, the engineered dressing must meet specific goals to facilitate healing, including be a flexible dressing to adapt to different geometries, positions and angles, defined by the affected area; possess sufficient resistant strength to endure skin movements and mechanical stress without tearing apart; be porous to allow oxygen exchange (breathable); and have a polymeric matrix capable of adsorbing large amounts of wound exudates [1,2].

To confirm the presence of CA with the 80/20 PVA/CA mixture, ATR-FTIR spectra were collected and compared to the 100 PVA and 100 CA (Fig.2). Because of the overlapping of bands, and the predominance of PVA within the 80/20 film, little differences were detected among spectra. A strong band between 3350 and 3200 cm^{-1} was observed on all films and was associated with O-H stretching vibrations of hydroxyl groups and with intramolecular hydrogen bonds. This is an indicative that even after 5 days of drying at 37ºC, because of the hydration capacity of both PVA and CA, there are still few water molecules bonded to the films. At 1740 cm^{-1}, the first difference in the PVA/CA film compared to 100 PVA is observed. This peak refers to the C=O stretching vibrations and is characteristic of CA, thus proving the presence of CA within the 80/20 mixture. Another peak associated with CA that was observed in the 80/20 film was detected at 1240 cm^{-1} and relates to the C-O-C anti-symmetric stretching vibrations of the CA ester groups. Even though the presence of CA was evident, one of the most important peaks of PVA (1090 cm^{-1}), the C-O stretching of acetyl groups from the PVA back-bone, remained [16-18].

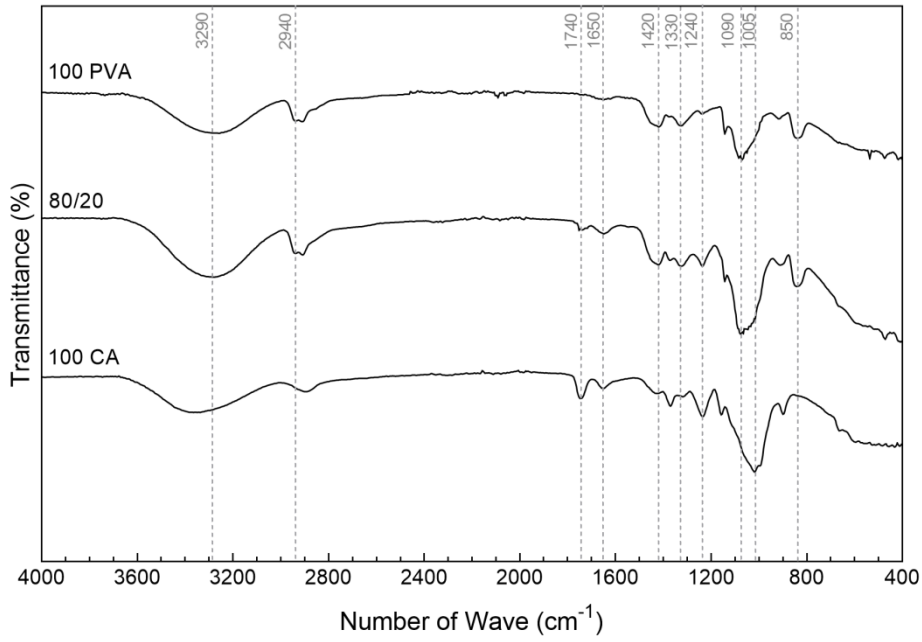

Fig.2. ATR-FTIR spectra of the PVA/CA films (4000-400 cm-1).

The films' flexibility and mechanical resistance were studied via elongation at break and tensile strength examinations, while the porosity and hydration capacity were determined based on the weight and dimension of the films before and after drying. Results are reported in Table 1 (mean ± standard deviation, with n = 3). It is clear from the data that the mechanical resistance of the films decreases with the inclusion of CA within the polymeric matrix. This is to be expected since 100 CA films were found very brittle with a consistency resembling mashed paper. It

was also because of this fragile structural integrity that no mechanical experiments were conducted for 100 CA. The elongation at break was as well smaller than 100 PVA; still, it allowed for the film to reach twice its size. Regarding the porosity, which is intimately related with oxygen transfer, the addition of CA increased. Again, this is to be expected if we look at the control data. The degree of swelling did not behave in a predictable manner as the other properties. In fact, both 100 PVA and 100 CA were able to bind to more water molecules than the 80/20 film. It is likely some hydroxyl groups available for water binding were being used for intermolecular interactions between the two polymers. Nevertheless, in all samples, the swelling or hydration capacity of the films was always superior to 80% of its dried state, which in terms of wound dressing can be translated in the absorption of a large amount of exudates.

Table 1. PVA/CA films tensile strength (TS), elongation at break (EB), porosity (ε) and degree of swelling (DS).

Films	TS (N ± SD)	EB (% ± SD)	ε (% ± SD)	DS (% ± SD)
100 PVA	5.33 ± 1.02	235.80 ± 38.19	26.88 ± 3.01	88.07 ± 0.36
80/20	4.87 ± 0.81	193.62 ± 18.22	30.27 ± 1.45	83.51 ± 0.90
100 CA	-	-	87.13 ± 7.81	87.42 ± 2.03

3.2. Antibacterial Activity

The antibacterial efficacy of the PVA/CA films functionalized with vancomycin was tested against *S. epidermidis* bacteria, using the standard shake flask method. Vancomycin was immobilized onto the PVA/CA surfaces via two methods, blending and physisorption (with pDOPA). Results from Fig.3 demonstrate that the immobilization via blending was unsuccessful for most of the tested samples. The antibacterial activity of vancomycin was lost within the polymeric matrix. It is likely that instead of being available at the surface, the antibiotic got entrapped within the film having little impact on the bacteria surrounding it. Still, the antibacterial activity of the functionalized 80/20 film was superior to that of the bare film ($p<0.05$) and the control groups. As the hydration capacity of 100 PVA and 100 CA is superior to the 80/20 films, perhaps this has enabled the entrapment of more antibiotic molecules.

Regarding the data from the physisorbed vancomycin, the antibiotic presence at the films' surface was confirmed with an elimination of bacteria in the order of 50%, a performance that was significantly above the non-functionalized surfaces. It is also important to notice that because of the large surface area of both 80/20 and 100 CA, the antibiotic was more available to the bacteria and as such these films' activity was well above 55%. However, it is important to refer that the almost 30% of bacterial inhibition promoted by the bare films was the result of the bacteria immobilization onto the porous surface or entrapment within the films' matrix due to their exceptional hydration capacity. In the future we expect to overcome this situation by improving the immobilization of the antibiotic, allowing for a homogeneous distribution along the surface of the dressings, by resorting to grafting processes and using antimicrobial peptides by themselves or in combination with vancomycin [19,20].

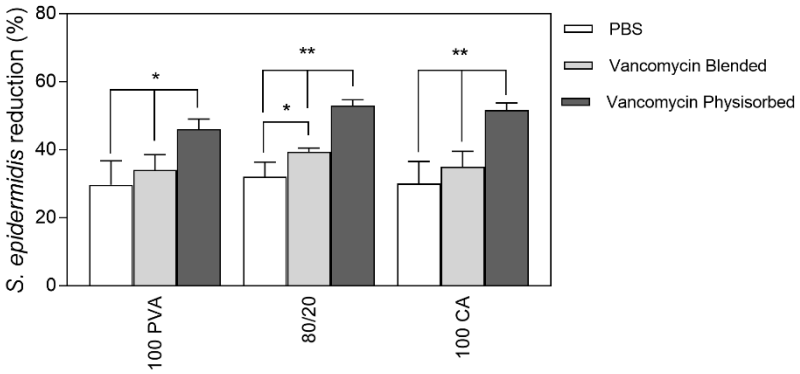

Fig.3. Antibacterial action of PVA/CA films (100/0, 80/20 and 0/100) bare and functionalized with vancomycin, via blending and physisorption (pDOPA), against *S. epidermidis* bacteria. Statistical significance between films is indicated by * (*$p<0.05$, **$p<0.001$, One-Way ANOVA following Tukey's Posttest).

4. Conclusions

The incorporation of vancomycin within the 80/20 PVA/CA films was proven successful in fighting bacteria in a dynamic environment. Addition of CA to the PVA matrix was confirmed and was seen to increase the porosity of the film, and with it its surface area. As expected, the flexibility and resistance strength were slightly compromised with the addition of CA, even though the final result displayed optimal properties for wound healing applications. The hydration capacity of the films was superior to 80%, indicating the films were capable to welling almost twice their size to retain wound exudates. Functionalization of vancomycin via physisorption, using pDOPA as a binding agent, was proven more effective than blending, with a killing rate of ≈ 55%, significantly superior than the bare films. These results were very promising. However, in the future, new surface functionalization methods will be tested, and new biomolecules will also be functionalized, so the overall antimicrobial activity of the film is improved.

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