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PVA hydrogels loaded with a Brazilian propolis for burn wound healing applications

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ABSTRACT: PVA hydrogels offer many suitable characteristics for burn wound dressings. However, unmodified PVA gels do not act against infection. Propolis is a natural antimicrobial agent suitable for incorporation into PVA gels. PVA–propolis gels were produced by freeze–thawing method, and their microstructure, mechanical, and swelling properties (in standard PBS and a PBS-based solution with pH 4.0) were characterized. The propolis release profiles and the gel's antibacterial and cytotoxicity properties were also investigated. The presence of propolis in the gels interfered with the PVA crystallization profile and with the mechanical properties. All samples swelled at least 400% in both media. The propolis was mostly released to the media in the first day of immersion. PVA–propolis gels with concentrations of 15% propolis or more were active against the gram-positive bacterium *Staphylococcus aureus*, which is associated with initial colonization of the wound. All PVA–propolis samples acted as barriers to microbial penetration. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 42129.

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INTRODUCTION

Burns are considered as being the cause of death of approximately 265,000 people per year globally.[1] Infection is the main cause of deaths related to burns.[2] Typically, burns are colonized by microorganisms (fungi, e.g. *Candida albicans*, and bacteria). They are initially colonized by gram-positive bacteria, e.g., *Staphylococcus aureus*, and, after a week, these may be replaced by gram-negative organisms, e.g., *Escherichia coli*.[3,4]

The treatment of partial-thickness burns requires the use of dressings.[5] The ideal burn dressing should maintain a moisturized environment, be transparent, absorb excess exudates, eliminate empty space, be pain-free, promote thermal insulation, be a barrier to microorganisms, be conformable, elastic, sterile, nontoxic, and exhibit water vapor transmissibility.[6–8]

Hydrogels are 3D networks, based on cross-linked hydrophilic polymers that swell in contact with aqueous solutions keeping their structural integrity.[8] Polyvinyl alcohol (PVA) hydrogels are transparent, malleable, bio-inert, and biocompatible. They present many characteristics of the ideal dressing. They have shown potential for biomedical applications such as contact lens, artificial hearts, drug release systems, articular cartilage, catheters, dialysis membranes, burn dressings, and temporary skin substitutes.[9]

PVA hydrogels can be cross-linked via chemical or physical routes. Chemical routes include irradiation processes and processes that use cross-linking agents, e.g. glutaraldehyde and formaldehyde. These react with PVA chains connecting them via covalent bonds.[9] However, residual chemicals remaining within the matrix may be potentially hazardous.[11] PVA can be physically cross-linked by freeze–thawing, where adjacent PVA chains form crystallites that act as physical cross-links. PVA cryogels are non-toxic, and present high mechanical strength and high swelling capacity in aqueous solutions.[12,13]

However, PVA hydrogels do not possess any intrinsic antimicrobial property. To overcome this limitation, PVA hydrogels have

Table I. Samples Named According to the Amount of Propolis in Each Sample/in Each Petri Dish Regarding Their Total Weight

	Samples weight per dish			Solution volume per dish (ϕ140 mm)		
Samples (w/w) (%)	PVA (g)	Propolis (g)	Total (g)	10% (w/v) aqueous solution of PVA (mL)	12% (w/v) solution of propolis in alcohol (mL)	Total volume (mL)
PVA	2.00	0.00	2.00	20.00	0.00	20.00
8	2.00	0.18	2.18	20.00	1.50	21.50
15	2.00	0.36	2.36	20.00	3.00	23.00
35	2.00	1.08	3.08	20.00	9.00	29.00
52	2.00	2.16	4.16	20.00	18.00	38.00

been combined with antimicrobial agents, with examples including PVA/dextran hydrogels loaded with gentamicin[14] and PVA hydrogels loaded with Ag nanoparticles.[15,16] One of the advantages of silver nanoparticles being embedded in polymeric matrices is that they can have an antimicrobial effect without being cytotoxic to fibroblasts.[17] However, Ag nanoparticles stabilized by PVA for biosensors have been observed to accumulate in the brains of rats.[18] To overcome this issue, natural antimicrobial agents could be an alternative.

Propolis is a resinous substance produced by bees with antibacterial, antifungal, antiviral, and anti-inflammatory activities.[19,20] There are more than 300 compounds in propolis. The characteristics and properties of propolis are related to its geographical origin and to the local flora.[21,22] Propolis antibacterial activity is bacteriostatic and, in high concentration, bactericidal.[19] Propolis has antimicrobial activity against gram-positive bacteria, e.g., *S. aureus*, but limited action against gram-negative bacteria and also against some fungi, e.g., *C. albicans*.[23,24]

Some membranes incorporating propolis have been developed recently for woundcare. Biocellulose membranes loaded with propolis were effective against *Staphylococcus* species and also promoted a better tissue repair in the early periods of skin healing.[24] Latex membranes loaded with propolis inhibited the growth of *C. albicans* colonies.[23] Collagen films loaded with green and red propolis decreased the severity of inflammation at the burn site and improved the epithelialization rates.[25]

PVA hydrogels loaded with propolis have not been reported in the literature. Compared to the other propolis-loaded matrix materials (latex, collagen, and biocellulose), PVA hydrogels present some possible advantages. As well as the required biocompatibility, they present low or no skin cell adhesion and they have the mechanical strength and resilience characteristics necessary for wound dressing applications.[9,10,26] The goal of the present work was to develop PVA–propolis gels and to analyze their behavior in simulated body conditions. Microstructure, thermal, and mechanical properties after immersion in simulated body fluids (PBS and a PBS-based solution with pH 4.0) and other *in vitro* properties were investigated.

EXPERIMENTAL

Poly(vinyl alcohol) (PVA), Mw 85.000–124.000 g mol^{-1} and degree of hydrolysis >99%, was purchased from Sigma-Aldrich and used without further purification. Green propolis extract (Extrato de Própolis Makrovit), 12% propolis/alcohol, was produced by W. Wenzel Ind. e Com. de Produtos Apícolas Ltda, São Paulo, Brazil.

Preparation of Hydrogels

Ten percent w/v PVA aqueous solutions were prepared (90°C for 6 h, under mechanical stirring) and the solutions remained under stirring until they reached environmental temperature. For the PVA–propolis samples, when the PVA solution reached room temperature, specific amounts of propolis extract were added to the PVA aqueous solution under mechanical stirring. The volume of solution in each petri dish (ϕ140 mm) was fixed at 20 mL of PVA solution plus the added volume of propolis per dish. The samples were prepared according to Table I. The samples were then freeze–thawed for 16 h at −18°C followed by 5 cycles of 30 min at room temperature and 1 h at −18°C. The samples were dried in room conditions and then they were exposed to 30 min of UVB radiation to sterilize them.

Microstructural Analysis

Microstructural analysis of the samples was performed by X-ray diffraction (XRD) using the XRD 6000 Shimadzu Diffractometer with Cu$\kappa\alpha$ at 30 kV and 30 mA and step length of 0.02° with step time of 1 s. The diffraction angle was set between 5° and 50°. Fourier-Transform Infrared Spectroscopy (FTIR) was carried out using the NICOLET 6700 Spectrometer with 64 scans per sample in the region of 550–4000 cm^{-1}.

The samples were also analyzed via differential scanning calorimetry (DSC) using the Perkin Elmer, DSC 8000. Approximately 10 mg of each sample was submitted to a heating rate of 10°C min^{-1} from room temperature to 250°C. To overcome the thermal history of the samples, the second heating cycle was used to obtain the gels properties: glass temperature (T_g) and melting temperature (T_m). The degree of crystallinity (X_c) was calculated according to eq. (1), where ΔH is the melting enthalpy, φ is the weight fraction of the filler, and the $\Delta H_{100\%}$ is the melting enthalpy of 100% crystalline PVA,[27] 138.6 J g^{-1}.

$$Xc = 100\ \frac{\Delta H}{(1-\varphi)\Delta H_{100}\%}(\%) \quad (1)$$

Swelling/Weight Loss Tests and Propolis Release

The swelling/weight loss tests were performed when triplicates of each samples composition (~2 cm^2, weight normalized) were

immersed in 2 mL of different fluids at 37°C for each time interval studied (1, 2, 4, 24, and 96 h). Two different media were used in accordance with the ISO 10993-9 standard. The first media was Phosphate Buffered Saline (PBS, Sigma Aldrich), intended to mimic the inorganic phase of human plasma. The other media was PBS with a reduced pH which was intended to simulate the local inflammatory environment of the wounds. This is termed Solution pH 4.0. The pH was lowered using Lactic Acid (Sigma Aldrich). The fluid absorption of each sample was calculated according to eq. (2) to obtain their swelling degree (SD). W_S is the weight of the sample at each time interval (swollen weight) and W_D is the dry weight before swelling.[28] After 4 days of immersion, the samples were dried and weighed in order to calculate their weight loss (WL) [eq. (3)], where W_D and W_{DS} are the weight of the dried samples before and after swelling tests, respectively.

$$SD = 100\ \frac{W_S - W_D}{W_D}\ (\%) \tag{2}$$

$$WL\ =\ 100\ \frac{W_D - W_{DS}}{W_D}\ (\%) \tag{3}$$

To analyze the propolis release, the swelling media was analyzed after 1, 2, 24, and 96 h of immersion via UV–Vis spectrometer, from 300 to 800 nm, using polystyrene cuvettes. For quantification of the amount of propolis released, a standard curve was created by diluting the original propolis in isopropanol resulting in several aliquots of known concentration, which were then analyzed in the same wavelength range. The area of the peak of these aliquots (of known concentration of propolis) was calculated and used to compare with those of the propolis released by the samples.

Mechanical Properties

For the tensile tests, at least 10 samples of each composition (in accordance with ASTM D882-00 standard) after 1 day of swelling in one of the two different media at room temperature, were cut into dog-bone shapes (length of (26 ± 2) mm, width of (3.1 ± 0.2) mm, and thickness of (0.4 ± 0.5) mm). Three measurements of the cross-section area of each sample were made. The samples were then attached to the grips (Zwick Z005 Tensile Test Machine) with the help of sand paper. The tests were performed using a 5 kN load cell, and a cross-head rate of 10 mm/min until failure at room temperature. The fracture strength values (σ_F) and the Secant modulus values at a strain of 50% (E) were calculated.

In Vitro Analysis

Antimicrobial activity of the PVA and of PVA–propolis hydrogels (swollen in PBS) against *E. coli* (ATCC 25992), *S. aureus* (ATCC 6538), and *C. albicans* (ATCC 10231) were evaluated using the disc diffusion method.[29] Overnight grown cultures of *E. coli*, *S. aureus*, and *C. albicans* were individually diluted and plated on Mueller–Hinton agar inoculated with approximately 10^8 colony forming units mL^{-1}. The hydrogels were cut (circular, ϕ1.80 cm), kept in 5 mL of PBS overnight, and then placed on the plates. The plates were incubated at 37°C for 18 h and the zones of inhibition were measured. For the antimicrobial penetration test, autoclaved test tubes with 10 mL of nutrient broth were covered with the hydrogels, a sealed tube was used as the negative control, and an open tube was used as the positive control. The turbidity of the media was observed for up to 1 month.

The cytotoxicity test was performed according to the Alamar Blue Assay,[30] to evaluate the cell viability. The Alamar Blue Assay incorporates a fluorometric/colorimetric growth indicator based on detection of metabolic activity. Specifically, the system incorporates an oxidation–reduction (REDOX) indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth. Human keratinocytes, HACAT cells, were obtained from the cell bank of Dublin City University (DCU), Dublin, Ireland. The cells were seeded in a sterile 48-well plate at a density of 5×10^4 cells mL^{-1} of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and grown in a humidified atmosphere of 5% CO_2 for 24 h at 37°C until they reached 70% confluency. The media was then removed and 1 mL of the sample's extract fluid (from the swelling media after 1 day of immersion) was added to a corresponding well. The negative controls were wells in which 1 mL of DMEM had been added, and the positive controls were empty wells. Sample extracts were prepared after immersion of 2 cm^2 of each sample (triplicates) in 2 mL of DMEM for 24 h at 37°C in incubator. The HACAT cells remained in contact with 1 mL of each samples' extract in a humidified 5% CO_2 atmosphere for 24 h at 37°C. After incubation, 1 mL of solution 10% Alamar Blue in DMEM was added to each well and the plate remained for 5 h in the incubator. Two hundred microliters of each media in each well was placed in the wells of a 96-well plate (no centrifugation) and they were analyzed in a UV–Vis spectrometer (Nanoquant Infinite m200, Tecan). Absorbance was measured at 570 and 600 nm to evaluate the cell viability. Cell growth maintains a reduced environment (red color), while inhibition is associated with an oxidized environment (blue color). A material is considered nontoxic if at least 70% of the cells survived (ISO 10993-5).

Statistical Analysis

ANOVA two-way analysis, $\alpha = 0.05$, was used to analyze the significance of two factors: (i) type of media, with two levels: PBS and Solution pH 4.0, and (ii) amount of propolis, with five levels: 0 (PVA), 8, 15, 35, and 52% of propolis, on the gels' swelling capacity, drug release, weight loss, and mechanical properties, using the Origin Pro 8® program. ANOVA one-way analysis, $\alpha = 0.05$, was used to analyze the significance of the amount of propolis on the gels' antimicrobial activity, with five levels: 0 (PVA), 8, 15, 35, and 52% of propolis, and on the gels cytotoxicity, where the levels of the factor amount of propolis were the same as the previous levels but also included the negative control (-) and the positive control (+).

RESULTS AND DISCUSSION

Microstructural Analysis

The XRD profiles of the PVA–propolis samples can be observed in Figure 1(a). PVA hydrogel is an amorphous material with a narrow diffraction peak[31] around $2\theta = 20°$ and a shoulder between $2\theta = 20$–$25°$. According to Drapak and collaborators,[32] propolis presents a narrow peak around $2\theta = 22°$. For high

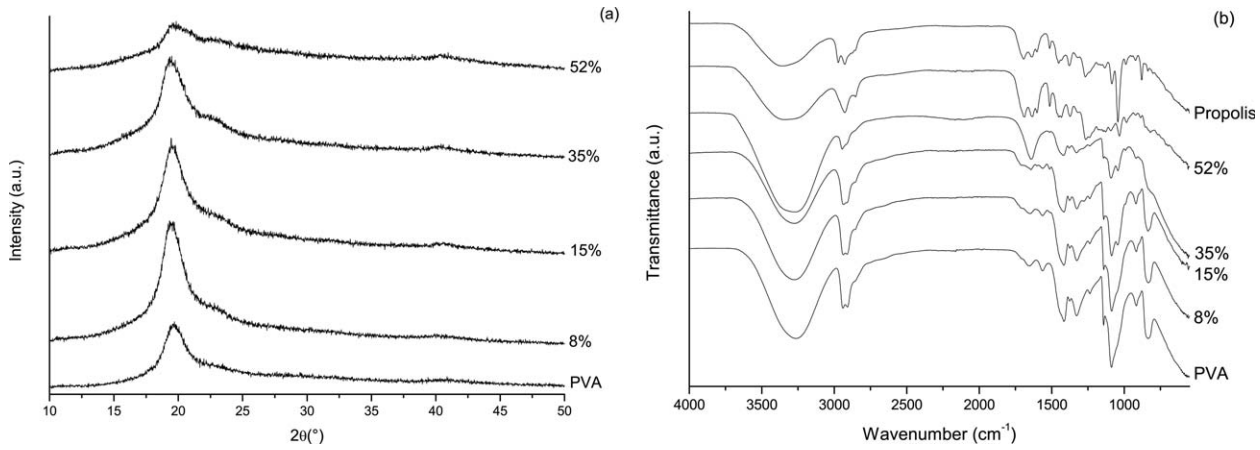

Figure 1. Microstructural analysis of the PVA and of the PVA–propolis hydrogels: (a) XRD pattern of the original samples; (b) FTIR spectra of the original samples and of the propolis itself.

propolis concentrations, the PVA peak's intensity is considerably lower, suggesting that propolis could be a physical barrier to PVA chains packing. The FTIR bands of the samples and the propolis bands are displayed in Figure 1(b). PVA[11,33–35] and propolis[23,24,36,37] characteristic bands and vibration modes from the literature are displayed in Table II.

Considering FTIR spectra [Figure 1(b)], all PVA bands can be found in all samples, although some of them present lower intensity with the presence of increased amounts of propolis. No band related only to propolis was encountered in the sample with 8% propolis, although some PVA bands in these samples could overlap some of the propolis bands. Samples with 15% of propolis or more presented both PVA and propolis bands. In some cases, some shift of the PVA bands towards the propolis bands' position could mean that PVA and propolis bands overlap.

Figure 2 shows the spectra of the dried samples after swelling in both media for 4 days. The main bands of PVA were present in all pure PVA samples. Nonetheless, the band at 1564 cm^{-1}, related to nonhydrolyzed acetate groups, was absent after swelling in all media, probably indicating some interaction of these groups with the media which could inhibit their vibration. In addition, after swelling in Solution pH 4.0, a band at 1713 cm^{-1} emerged. This band was related to stretching of C=O of lactic acid, indicating that the acid used to prepare the Solution pH 4.0 interacted with the PVA chains.[38,39]

Samples with 8% propolis, before and after swelling in both media, presented similar FTIR spectra to those of pure PVA. Samples with 15% of propolis or more were the ones in which the bands of PVA and the bands of propolis could be distinguished. In the 15% samples, the band at 1564 cm^{-1} (nonhydrolyzed acetate groups) was again absent after swelling. The PVA band at 2909 cm^{-1}, that overlapped the propolis band at 2928 cm^{-1}, was shifted toward the propolis band wavenumber after immersion of the 15% samples in Solution pH 4.0. Some bands related to propolis only were also present in the 15% samples spectra after immersion in Solution pH 4.0: between 1602 and 1456 cm^{-1}, related to aromatic ring vibration, and the band between 1276 and 1270 cm^{-1}, related to the vibration of C–O groups of polyols, e.g., hydroxyflavonoids. For 15% samples swollen in PBS, there was a band between 1276 and 1270 cm^{-1}, but the other bands, related to aromatic ring vibration, were absent after immersion. This fact could be related to the release of these compounds to the media since propolis release was higher in PBS.[40]

Besides the PVA bands in the spectra of 35% propolis samples after swelling, it can be noticed that there were some bands related to the propolis itself, the bands between 1602 and 1425 cm^{-1}, between 1330 and 1272 cm^{-1}, a band at ~990 cm^{-1}, bands between 1030 and 1042 cm^{-1} and between 890 and 833 cm^{-1}. Some of these bands could be observed only after swelling, possibly due to reorganization of PVA chains that enabled these groups' vibrations. It is worth noting that the original (preswelling) spectra of the 35% propolis samples presented the PVA bands with low intensity and the propolis bands seemed to be poorly defined or dislocated, which could have suggested some chemical interactions between the propolis and the PVA. Since the propolis bands vibration modes were enabled after swelling, it can be deduced that this was not a covalent interaction between the PVA and propolis. The effect was likely due to a physical impairment to some vibration modes prior to the swelling or due to the breaking of electrostatic interactions when gels were immersed in media, enabling the vibration of some propolis groups after swelling.[24,37] In addition, some propolis compounds were absent after immersion of the 35% samples, indicating possible release of these compounds to the media.

In the 52% propolis samples, after swelling, the main bands of the PVA and the propolis can be distinguished. Some of the original PVA bands that could potentially overlap the propolis ones were shifted toward the propolis bands.

The glass transition temperature (T_g), the melting temperature (T_m), and the degree of crystallinity (X_c) of the samples, before and after 4 days of swelling, are displayed in Table III. Regarding the original (preswelling) samples, there was a decrease in the T_g with the increase of propolis for amount of propolis up

Table II. FTIR Bands and Vibrational Groups Present in Each Original Sample

PVA (cm^{-1})	PVA groups' vibration modes	8% (cm^{-1})	15%	35%	52%	Propolis	Propolis groups' vibration modes
3259	Stretching (O-H)	3280	3278	3284	3326	3353	Wagging (OH) of phenolic compounds; stretching (OH) groups
-	-	-	-	-	-	2973	Aliphatic stretching (CH_2), C-H bands of aromatic compounds
2942	Stretching (C-H) - alkyl groups	2939	2939	2943	2931	2928	C-H bands of aromatic compounds
2909		2911	2909(s)	2912(s)	-	2873(s)	
2850(s)		2853(s)	2854(s)	2854(s)	2854	2856(s)	
-	-	-	1715(s)	2112	2637	-	-
-	-	-	-	-	1689	1694	Stretching of carboxyl groups
1655	Stretching (C=O) of unhydrolyzed acetate groups, stretching (C=C)	1652	1645	1642	1634	1640	Stretching (C=O) of CAPE and its derivatives; stretching (C=C), Aromatic ring bands
-	-	-	1602	-	1602	1602	Aromatic ring bands
1564	Stretching (C=C)	1563	1565	-	-	-	
-	-	-	1516	1516	1514	1514	
-	-	-	-	-	1465	-	
-	-	-	-	-	1451	1452	
1415	Bending, wagging, in plane (C-H in CH_2 groups); stretching (C-O-C) of unhydrolyzed acetate groups, in plane(O-H)	1417	1417	1419	1434	1435	N.I.
1378	Coupling of in plane(O-H) wagging(C-H)	1378	1378	1380	1374	1378	
1329	Bending(CH + OH), fan and twist(-CH_2-)	1326	1327	1330	1318	1320	
-	-	-	1276	1280	1265	1270	C-O groups of polyols, e.g., hydroxyflavonoids
1236	Stretching (C-C), fan and twist(-CH_2-)	1236	1238	1236	1239	-	-
-	-	-	-	-	1179	1177	N.I.
1142	Stretching (C-O-C), stretching (C-C) crystalline sensitive band	1142	1142	1143	1144	1159	
-	-	-	-	-	1128	1131	
1088	Stretching (C-O), bending (O-H)	1089	1087	1091	1092	1087	stretching (C-O) of ester groups
-	-	-	1045	1044	1033	1043	
		-	-	991	984	989	N.I.
917	Stretching, [bending out of plane] (C-H)	917	919	918	921	919	
-	-	-	-	-	887	880	Aromatic ring vibration
836	Stretching, pendular(C-C)	835	836	-	817	837	

(s), shoulder.

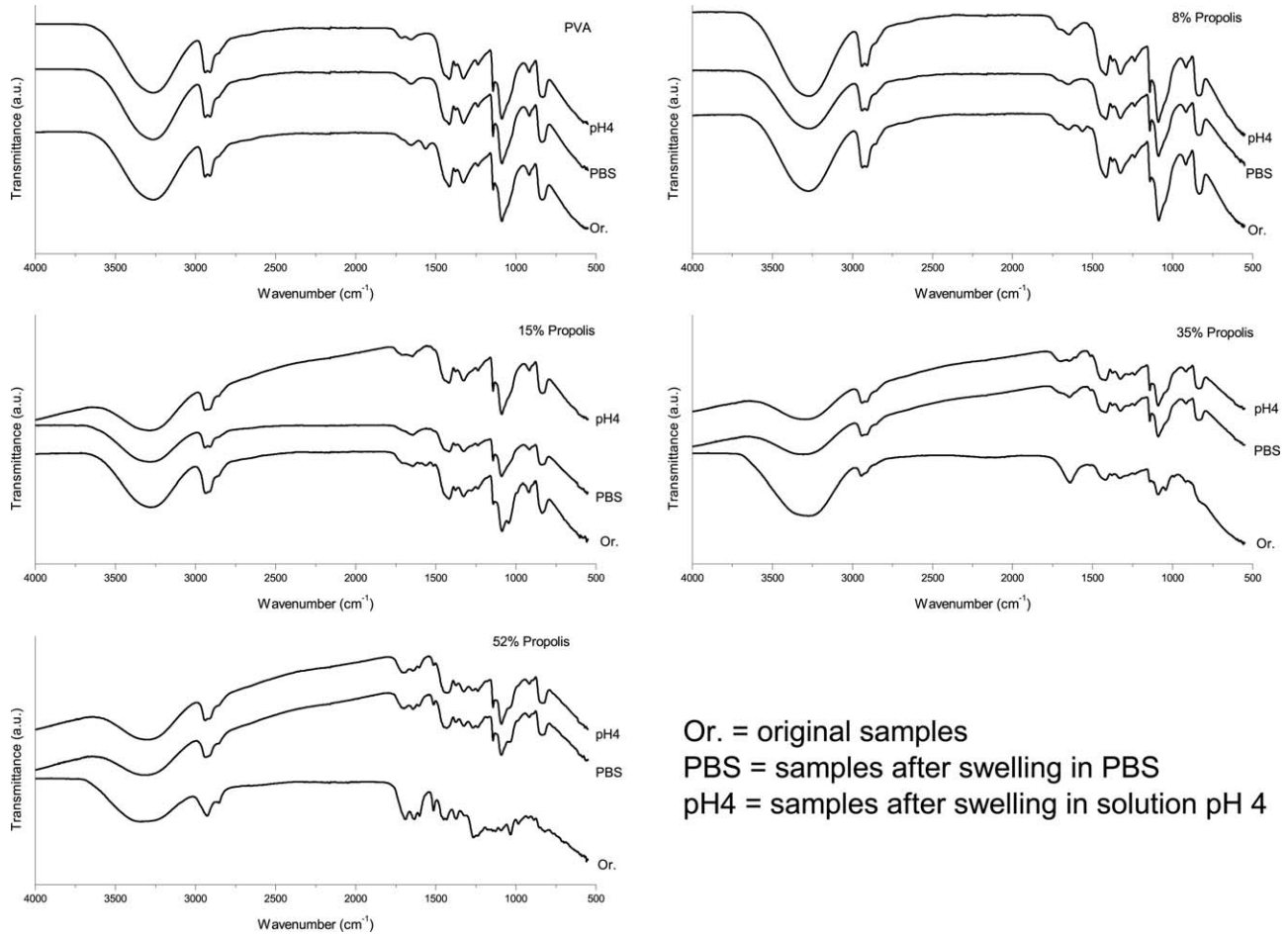

Figure 2. FTIR spectra of PVA–propolis original samples (Or.) and FTIR spectra of PVA–propolis samples after 4 days of immersion in PBS (PBS) and in Solution pH 4.0 (pH 4).

to and including 15%. Thirty five percent of propolis samples exhibited a T_g rise and no T_g was identified for the 52% propolis samples. These data indicate that the presence of more propolis diminished the mobility of the chains in the PVA amorphous phase, probably due to electrostatic interactions between the propolis and the PVA, as visualized in FTIR spectra.

Regarding the PVA crystalline phase, the melting temperature (T_m) and the degree of crystallinity (X_c) decreased with the increase of the propolis amount in the original samples. The propolis could impair the PVA chains' movement, so increased amounts of propolis in the samples could hinder the chains packing and inhibit crystals growth, diminishing X_c and T_m, respectively. The presence of loaded substances/drugs can alter the polymeric matrix's characteristics. For example, PVA/PAA hydrogels presented lower T_g and T_m when loaded with aspirin,[41] and PVA hydrogels presented no T_m when loaded with liposomes.[42]

The PVA–propolis samples were also analyzed after the swelling test. After immersion in PBS, the 8 and 15% propolis samples presented higher T_m and lower X_c compared to the original 8 and 15% propolis samples. Nonetheless, the 35 and 52% propolis samples after immersion presented higher T_m and higher X_c compared to the corresponding original samples. After immersion in Solution pH 4.0, it was observed that there was a decrease in the T_m and X_c for the 8% propolis samples. After immersion, there were no considerable changes in the T_m and X_c of 15% propolis samples. For 35% propolis samples after swelling, the T_m was lower and the X_c was higher compared to the 35% propolis original samples. In addition, the 52% propolis samples after immersion presented higher T_m and higher X_c compared to the original samples.

The X_c values of the samples 35 and 52% after immersion were higher than the ones of the respective original samples, indicating some crystallization after immersion in both media. These samples had considerable amounts of propolis in the network and released the highest amounts of propolis to the PBS. The propolis release as well as the effect of the media on the PVA chains mobility could lead to late crystallization. After swelling in Solution pH 4.0, an increase in the X_c of the 35 and 52% propolis samples was observed and also an increase in the T_m of the 52% propolis samples (suggesting that the crystals were well formed). Some crystallization of the samples with high amounts of propolis after immersion in both media was observed, where the chains mobility could lead to chains packing.[43,44]

Table III. Thermal Analysis (DSC) Results of the Original Samples and T_m and X_c of the Dried Samples After Swelling for 4 Days in the Different Media (PBS and Solution pH 4.0)

Sample	Pre-swelling samples			After swelling in PBS		After swelling in solution pH 4.0	
	T_g (°C)	T_m (°C)	X_c (%)	T_m (°C)	X_c (%)	T_m (°C)	X_c (%)
PVA	71	222	36	-	-	-	-
8%	69	218	32	223	29	210	21
15%	63	215	30	222	27	215	31
35%	74	208	14	218	23	202	16
52%	-	201	2	214	17	205	13

T_g, glass transition temperature; T_m, melting temperature; X_c, degree of crystallinity.

Swelling Tests and Propolis Release

The swelling tests in PBS and in Solution pH 4.0 for 4 days revealed that when reaching the equilibrium swelling degree (ESD), all samples swelled to ~400% (Figure 3). Based on the ESD values, the gels can be considered as superabsorbent.[45] A peak of media uptake was observed at the beginning of all curves, and after 1 day of swelling there was a plateau—the ESD. The ESD occurs when the hydration forces (the network stretching by the initial fluid uptake) and the elastic force of the cross-linkages reach the equilibrium.[46,47]

The ANOVA analysis, $\alpha = 0.05$, on the ESD revealed that neither the amount of propolis nor the composition of the media was significant to the ESD (for all factors analyzed and for their interaction, p value > 0.30). The fluid uptake by all samples in the different media was approximately the same.

The samples weight loss (WL) was higher for samples with more propolis (Table IV). The amount of propolis was significant to the samples weight loss (p value $= 2 \times 10^{-5}$), and there was a significant difference between the 15% propolis samples and the others: $WL_{15\%} > WL_{PVA,\ 8\%}$ and $WL_{15\%} < WL_{35\%,\ 52\%}$.

The samples weight loss was higher for the samples with more propolis. However, the weight loss cannot be fully accounted for by propolis release and must also involve loss of polymer. As discussed before, high amounts of propolis led to low degree of crystallinity and low percentage of crystalline phase. When the samples swell, the amorphous chains could have more freedom to move and, if they were not cross-linked (or in a crystallite), with the help of the media, they could detach from the network to the media, increasing the weight loss.[48]

The amount of propolis release in swelling media was analyzed after 1, 2, 24, and 96 h of immersion (Figure 4). The propolis release by polymeric systems usually occurs in two steps: the release of certain amounts of propolis in the first day of swelling as well as a prolonged release in some cases.[49] A trend

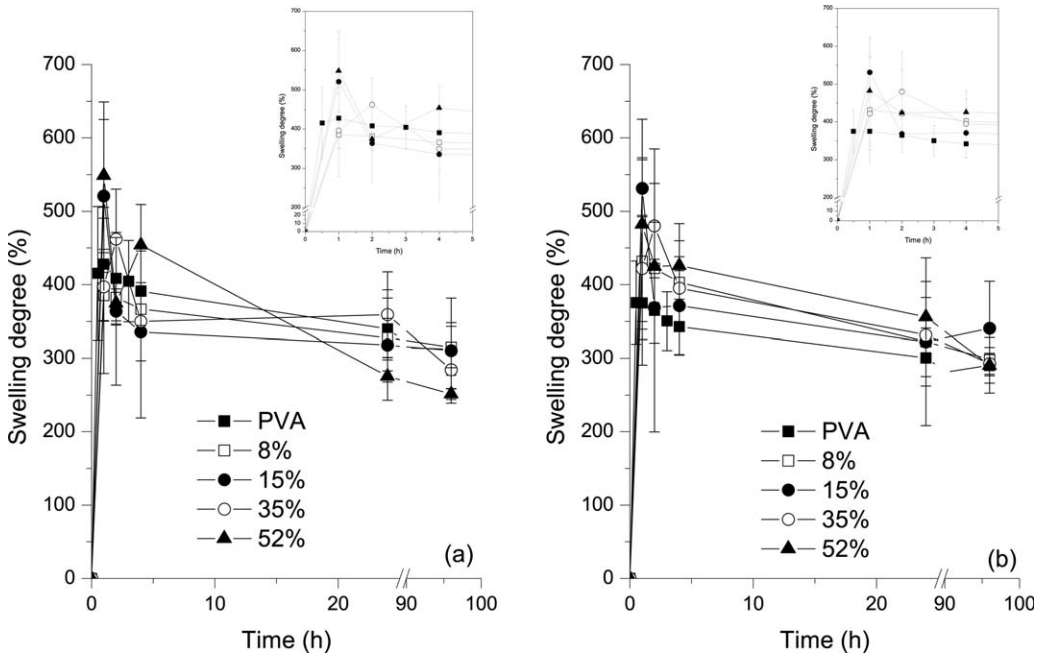

Figure 3. Swelling degree (SD) results of PVA and of PVA–propolis samples, after regular time intervals (1, 2, 4, 24, 96 h), when immersed in (a) PBS and (b) Solution pH 4.0 for 4 days.

Table IV. Total Weight Loss of the PVA and the PVA–Propolis Samples After 4 days of Immersion in PBS and in Solution pH 4.0

Samples	Weight loss (%)	
	PBS	Solution pH 4.0
PVA	11.5 ± 4.1	11.5 ± 2.6
8%	18.7 ± 6.7	7.7 ± 2.5
15%	18.5 ± 13.4	21.1 ± 10.2
35%	33.8 ± 4.3	26.2 ± 0.3
52%	31.0 ± 2.6	27.9 ± 1.8

could be observed in all curves after 4 days of immersion: there was a high propolis release in the initial hours and the cumulative release reached constant values up to 1 day of immersion. No prolonged release was observed. Layered hydrogels, in which PVA layers with different concentrations of propolis are produced, could be an alternative in order to control/sustain the propolis delivery for longer periods of time.[50] In addition, the trend was for higher amounts of propolis in the samples to result in higher release.

The ANOVA analysis on the total propolis released by the samples (for all factors analyzed and for their interaction, p value $< 1 \times 10^{-9}$) revealed that the type of media and the amount of propolis in the original samples, as well as their interaction, were significant to the release. The higher the propolis amount in the samples, the higher was the release in both media. Nonetheless, the samples released more propolis to PBS than to Solution pH 4.0, probably indicating that the propolis release can be influenced by the media pH.

Brazilian propolis types are rich in phenolic compounds.[51] Phenolic compounds' solubility varies with the solution pH. The ionization and solubility of phenols increase in response to increase in solution pH.[52] The low propolis delivery to acid pH could be related to the low solubility of the phenolic compounds compared to their solubility in neutral pH (PBS).

Mechanical Properties

The tensile curves of 10 samples of each condition were used to plot average stress versus strain curves (Figure 5). The secant modulus (E) was calculated (at strain of 50%) and the fracture strength (σ_F) was also obtained.

Both the type of media and the amount of propolis and their interaction were significant to the E values (p values $< 3 \times 10^{-5}$). It can be observed that in PBS, the secant modulus (E) values increased with the content of propolis up to 35% of propolis, after which there was a considerable decrease (Table V). In Solution pH 4.0, the E value decreased with the content of propolis from 15% propolis upward.

The samples' secant modulus (E) varied according to the samples composition in the different media, and the secant modulus values ranged from ~0.03 to ~1.17 MPa, which are considered adequate for woundcare applications.[53]

In PBS, the σ_F values increased with the content of propolis until a maximum at 35% of propolis, after which there was a considerable decrease. In Solution pH 4.0, higher amounts of propolis lead to lower fracture strengths (σ_F). In both media, the lowest fracture strength was that of the 52% propolis samples. The ANOVA analysis, $\alpha = 0.05$, on the fracture strength revealed that the type of media was not significant (p value = 0.33). Nonetheless the amount of propolis was significant to the fracture strength as well as the interaction between media and amount of propolis (p value < 2 x 10^{-6}).

The mechanical properties (E and σ_F) of the samples immersed for 1 day in PBS increased with the content of propolis up to the 35% sample. Since the propolis release to PBS was quite high, less propolis remained in the PVA network after 1 day.

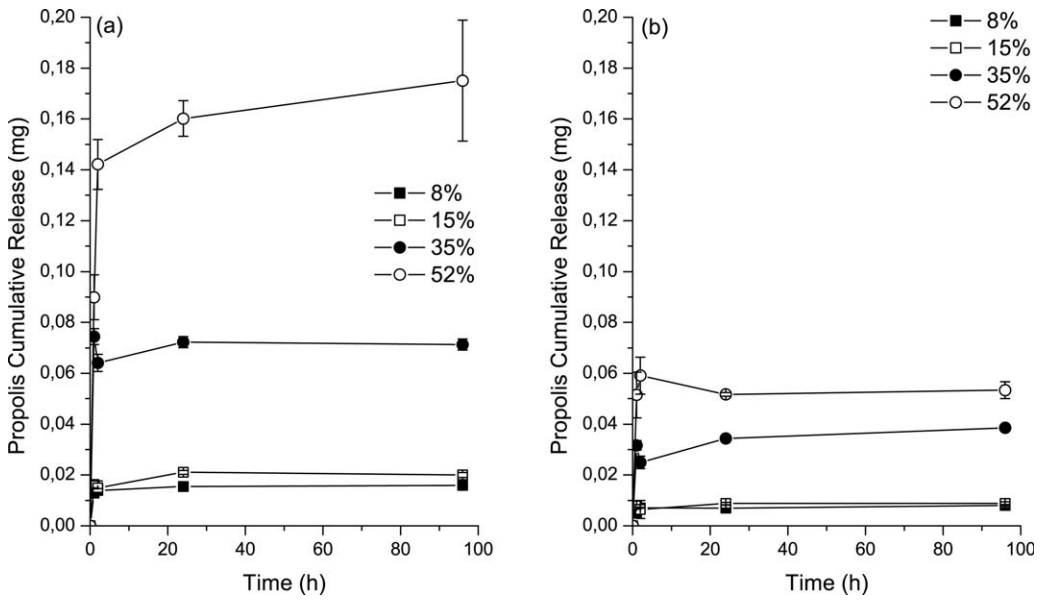

Figure 4. Propolis cumulative release profile of PVA–propolis samples. The PVA–propolis samples were immersed in (a) PBS and (b) Solution pH 4.0 and the propolis delivered was quantified after regular intervals of time for 4 days.

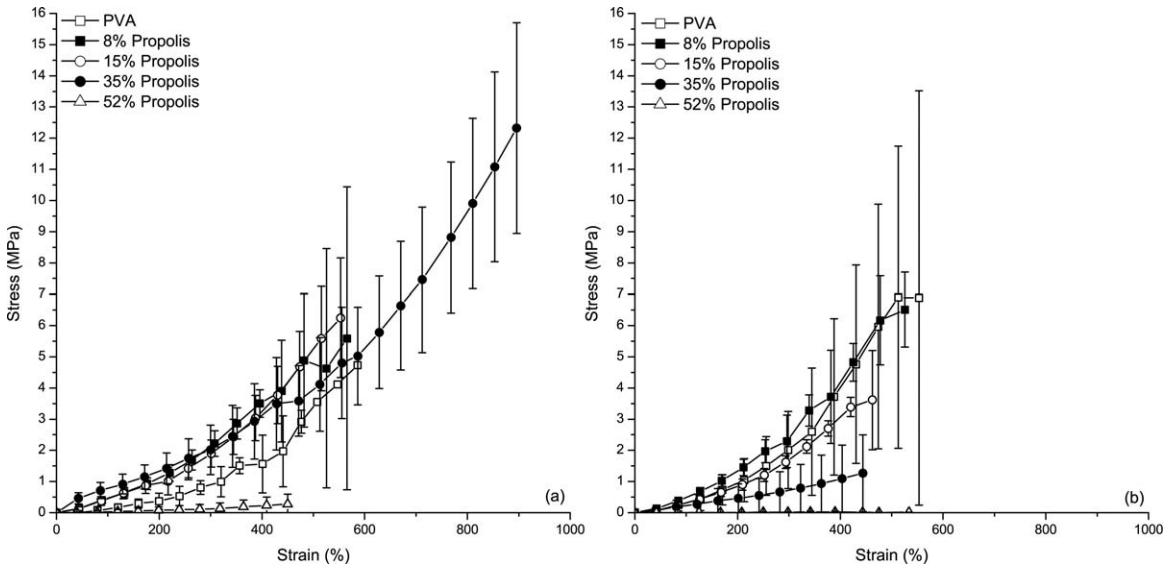

Figure 5. Tensile tests of all swollen samples, PVA, and PVA–propolis samples, after 1 day of immersion in (a) PBS and (b) Solution pH 4.0.

Less propolis could mean less impairment to the PVA chains interactions, increasing the mechanical properties values. The gelsÇ mechanical properties after 1 day of immersion in solution pH 4.0 decreased with the increase of the amount of propolis in the samples. Since the propolis release to solution pH 4.0 was low, more propolis could remain in the PVA network and more impairment to the chains approach could be present.

The presence of an additive to PVA hydrogels usually alters the PVA gels mechanical properties. If the added components bind to PVA chains or act as physical barriers to crystallite formation, the cross-linking/chain packing are hampered and the mechanical properties deteriorate.[54] The thermal analysis of the samples after immersion revealed that PVA amorphous chains gain movement when in media and the gels can crystallize after swelling/propolis release.[44] In addition, under tensile strain, the chains could pack, leading to high modulus and fracture strength.[55] In Solution pH 4.0, less propolis was released (compared to PBS), and more propolis remained in the network diminishing the possibility of crystallization during tensile tests.

The samples with 52% propolis presented the lowest values of the mechanical tests, independent of the media. Nonetheless, the amount of propolis in these samples was so high that, even releasing propolis, a considerable amount of propolis could still remain trapped in the network, as can be observed in the FTIR spectra of the samples after swelling, Figure 2, and be responsible for the poor mechanical properties. Nonetheless, the PVA hydrogels loaded with propolis in the swollen state would have enough mechanical strength to be used as dressings.[56]

In Vitro Analysis

The antimicrobial tests revealed that the samples with amounts of propolis equal or superior to 15% of propolis presented antimicrobial activity against *S. aureus*, one of the most common gram-positive bacteria in burn wounds.[57] The gram-positive bacteria are responsible for the initial colonization of infected burns and, after a week, these may be replaced by gram-negative organisms.[3,58] According to different reports, the main substances present in propolis related to its anti-inflamatory/antimicrobial activity are flavonoids, phenolic substances, and cinnamic acid derivatives.[59–61] Propolis is well known to have antibacterial activity against gram-positive bacteria and to have limited activity against gram-negative ones.[62–64] Most of the samples with propolis were effective againt gram-positive bacteria (*S. aureus*), as seen in Figure 6, but no activity was observed against gram-negative bacteria (*E. coli*) or against fungi (*C. albicans*).

The amount of propolis was significant to the *S. aureus* inhibition (p value $= 1 \times 10^{-11}$). Neither PVA nor 8% propolis were active against *S. aureus*. The other samples were active against *S. aureus*, where the 52% propolis samples presented the highest inhibition zone. Higher amount of propolis led to higher

Table V. Mechanical Properties of the PVA and of the PVA–Propolis Samples After 1 Day of Immersion in PBS and in Solution pH 4.0

Samples		PVA	8%	15%	35%	52%
PBS	*E* (MPa)	0.19 ± 0.06	0.43 ± 0.08	0.50 ± 0.11	1.17 ± 0.49	0.03 ± 0.03
	Failure strength (MPa)	1.85 ± 1.45	6.69 ± 3.42	5.75 ± 3.04	8.42 ± 5.23	0.41 ± 0.65
pH4	*E* (MPa)	0.41 ± 0.21	0.43 ± 0.11	0.37 ± 0.10	0.24 ± 0.26	0.03 ± 0.03
	Failure strength (MPa)	7.21 ± 5.57	5.77 ± 1.44	4.71 ± 2.79	2.10 ± 2.32	0.37 ± 0.63

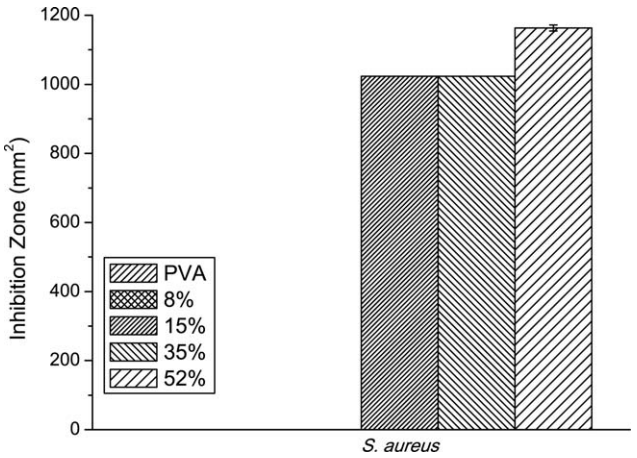

Figure 6. Antimicrobial activity of the PVA–propolis samples against *S. aureus*.

inhibition of *S. aureus*. The inhibition zones of the membranes of the present work (>300 mm^2) can be considered effective to be used as bactericide dressings.[24]

The microbial penetration test revealed that all samples were barriers to the penetration of organisms. After 1 month of exposure to the environment, the nutrient broth in the test tubes covered by the samples showed no change in turbidity indicating the absence of any growth of microorganisms while the nutrient broth in the control tube exposed to the environment was turbid indicating contamination from the atmosphere. All the samples (PVA and PVA–propolis samples) were barrier to microorganisms' growth (Figure 7) and the gel networks were responsible for acting as a physical barrier to microorganisms.[8]

In the cytotoxicity test, the viability of the HACAT cells in contact with the samples extracts was ~78% for PVA samples, ~5% for 8% samples, and 0% for the other samples (not shown). The cell viability was lower than 70% for PVA–propolis samples, indicating that these samples were cytotoxic for human keratinocytes. According to Funari and collaborators,[65] propolis can be antimicrobial, but also toxic to fibroblasts. Nonetheless, burns treated with propolis can present accelerated tissue repair and decreased local inflammation.[66] Even cytotoxical levels of propolis could still stimulate the reepithalization, which could improve healing.

Among the PVA–propolis samples studied, the ones with 15 and 35% propolis present the most effective properties for the woundcare application.

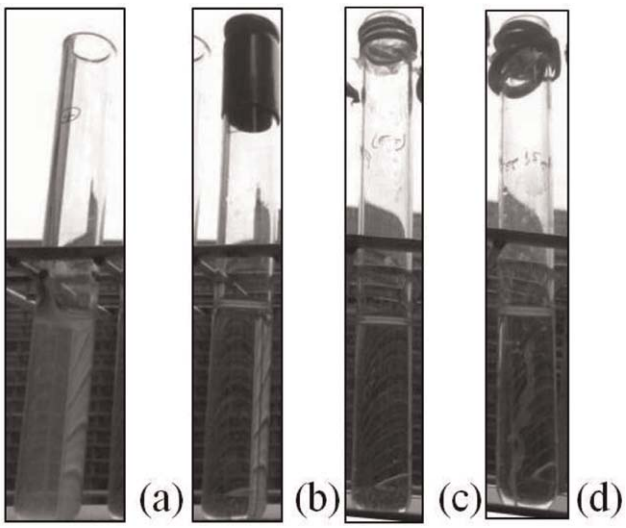

Figure 7. Antimicrobial test results of (a) positive control, showing the media turbidity (b) negative control, (c) PVA sample covering the tube, and (d) PVA–8%propolis sample covering the tube, the last ones with no media turbidity.

CONCLUSIONS

The PVA–propolis gels presented high fluid uptake (~400% in all media); the propolis was all released to the media in the first day, which would be suitable for dressings that are changed daily. No chemical interaction between the propolis and the PVA was observed. However, propolis impairs the PVA chain packing, inhibiting the polymer crystallization, and alters the gels' mechanical properties. The gels with high amounts of propolis (≥ 35%) presented higher crystallinity after immersion in all media (late crystallization), indicating that the propolis release coupled with the presence of media (which facilitates the amorphous chains movement) led to increased PVA chain packing. All swollen PVA–propolis gels presented mechanical properties (in all media) considered to be adequate for the application. The PVA–propolis samples (those with an amount of propolis > 15%) were active against the gram-positive bacteria *S. aureus*, which are the first to colonize the wound. The PVA and PVA–propolis samples also acted as barriers to the penetration of microorganisms, although the samples were cytotoxic to human keratinocytes.

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