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Article

Formation of hydrophilic fibers containing polyhexamethylene biguanide and hyaluronic acid by electrospinning to wound healing

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For good wound management, simultaneous phases during the wound healing process must be considered, including protection against infection, modulation of the inflammatory process, and proliferation and acceleration of cell migration. The aim of this study was to obtain Poly (Vinyl Pyrrolidone) (PVP) hydrophilic fibers containing polyhexamethylene biguanide (PHMB) and hyaluronic acid (HA) using the electrospinning technique to improve chronic wound treatment. Polymers mean hydrodynamic radius and polydispersity index (PdI) were measured by dynamic light scattering (DLS), whereas fiber morphology and diameter were evaluated by scanning electron microscopy (SEM). Also, Fourier transform infrared spectroscopy (FTIR) and contact angle measurements were performed. Fibers were obtained and presented homogenous morphology with a smooth surface. The FTIR study demonstrated an interaction between C=O groups of PVP and HA/PHMB in the PVP + HA/PHMB fibrous composites. Contact angle analysis has shown a hydrophilic characteristic of fibers produced. These findings demonstrate the great potential of electrospinning for developing drug-delivery systems with antimicrobial properties for wound healing treatment.

Keywords: Electrospinning. Biomaterial. Chemical stability. PHMB. Hyaluronic acid. Wound dressing.

INTRODUCTION

Wound treatment is an important issue for healthcare systems. People affected by vascular diseases, neuropathies, diabetes mellitus, and burns commonly have a high prevalence and incidence of chronic wounds, which have particular challenges to manage (Borda *et al.*, 2018) since their healing process is not expected to have a normal trajectory (Whitney, 2005).

Hemostasis, inflammatory, proliferative cellular responses (Ellis, Lin, Tartar, 2018; Whitney, 2005), and wound remodeling (Ellis, Lin, Tartar, 2018) are impaired in these cases. In addition, chronic wounds have bacteria on their surface that can form biofilms, distributed non-

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uniformly in the wound bed (Rhoads, Wolcott, Percival, 2008), and contribute to an unsatisfactory response to wound treatment (Mendoza *et al*., 2019).

The biofilm comprises pathogenic bacteria surrounded by a self-produced extracellular polymeric matrix composed of nucleic acids, proteins, polysaccharides, and lipids (Hall-Stoodley *et al*., 2012). It stimulates a chronic inflammatory response by increasing the concentration of reactive oxygen species (ROS) and proteases that can damage normal tissues. Also, there is an increase in exudate production, which provides a source of nutrition and helps to perpetuate the biofilm (Lawrence *et al*., 2007). Its formation can delay the healing time since it can concentrate on a variety of environmental nutrients and be able to develop antimicrobial resistance (Gompelman *et al*., 2016).

Some outcomes connected with the unsuccessful treatment of chronic wounds include amputations, decreased quality of life, high costs to health systems, and increased mortality (Escandon *et al*., 2011). Thus, effective wound treatment must consider repairing injured tissue by reducing biofilm formation, if any, and recovering the healing normal trajectory.

Fiber produced by electrospinning demonstrated benefits in healing properties. The approach of this technique is based on the creation of polymeric fibers by using an electric field. The pore sizes between the interstices of the membrane fibers in micro or nanometric scales prevent the penetration of bacteria. In addition, the high surface area is extremely efficient for absorbing exudative fluids from wounds (Pezzin *et al*., 2018), which suggests that it is an excellent dressing to treat chronic wounds to be used alone or as a delivery system for other wound healing agents (Lima *et al*., 2020, 2019).

Hyaluronic acid (HA) is a polyanionic polysaccharide found in the intercellular matrix of connective tissues, especially in the skin, and plays a protective, structural stabilizing, and impact-absorbing role. The natural and unique viscoelasticity, biocompatibility, and non-immunogenic HA provided its wide clinical applications, such as facilitating the healing and regeneration of surgical wounds (Snetkov *et al*., 2020). HA can actively modulate tissue regeneration on multiple levels, which is especially relevant in chronic wounds. HA demonstrates angiogenic and antioxidant effects actioning on the acute inflammatory, repair, and remodeling phases of healing (Gao *et al*., 2019; Gonçalves *et al*., 2016; Oliveira *et al*., 2017).

Dressings and topical agents containing polyhexamethylene biguanide (PHMB), also known as polyhexanide, have been used to avoid biofilm formation and reduce exudate, pain, and wound size (Santos, 2017).

The PHMB is a fast-acting biguanide compound composed of a synthetic polymer blend structurally similar to antimicrobial peptides produced by many cells (i.e., keratinocytes and neutrophils) within the wound to protect healthy tissue against infection (Ousey, McIntosh, 2009). Antimicrobial peptides have a broad spectrum of antimicrobial action (bacteria, viruses, and fungi), inducing cell death through disruption of cell membrane without affecting the host cells (Butcher, 2012; Ikeda *et al*., 1984) and the efflux pump, a mechanism used by many bacterial cells to remove toxins. This means that intracellular bactericide concentrations are maintained (Kingley *et al*., 2009). Once inside the cell, PHMB binds to DNA and other nucleic acids, suggesting it can also damage or inactivate bacterial DNA (Allen, Morby, White, 2004). The PHMB affects both planktonic bacteria (free and dispersed) and biofilms (Butcher, 2012).

Usually, HA and PHMB are applied to treat wounds in isolated formulations (Berce *et al*., 2018) with suitable clinical results. Therefore, it is hoped that the conjugate of these bioactive agents can improve the therapeutic activity. However, due to the anionic character of HA and the cationic character of PHMB (Sowlati-Hashjin, Carbone, Karttunen, 2020), producing a stable formulation with HA and PHMB is challenging.

The electrospinning technique has been used to join incompatible liquid bioactive agents and improve the formulation's chemical stability. Besides that, electrospun microstructure enhances the PHMB and HA solubility in the wound site (Quan *et al*., 2011). Therefore, electrospun microstructure improves the bioavailability, affecting the pharmacological activity.

Based on the abovementioned, the aim of this study is to produce a hydrophilic fiber containing PHMB and HA in a single product by using the electrospinning technique. The fibers will act like a delivery system

for HA, as a regenerative agent, and for PHMB as an antimicrobial and antibiofilm.

MATERIAL AND METHODS

Material

Poly (vinyl pyrrolidone) (PVP) (MW = 1,300,00) was purchased from Sigma-Aldrich (USA); Cosmocil® CQ (aqueous solution based on 20% (w/w) of PHMB) was kindly provided by Lonza Co. (Switzerland); 1.0% (w/w) of HA solution was provided by TRB Pharma Ltda (Brazil), absolute ethyl alcohol PA was supplied from Dinâmica Co. (Brazil). The microorganisms *Escherichia coli* (ATCC: 8739), *Staphylococcus aureus* (ATCC 6538), and *Candida albicans* (ATCC: 10231) were used for performing the antimicrobial assay.

Solution preparation

A 15.0% (w/v) PVP solution was prepared by dispersion of PVP in 20 mL ethyl alcohol at room temperature and stirred for 24h to ensure complete dissolution of the polymers. The solution was sealed to avoid solvent evaporation. After, 2.0 mL of the 1.0% (w/w) HA solution was added to the PVP solution. The PHMB solution was obtained by dilution of 100 µL of PHMB commercial solution 20.0% (w/w) in 1.0 mL ethyl alcohol followed by the addition and dissolution of the PVP and HA solution. Four different preparations were obtained: 1) PVP; 2) PVP + HA; 3) PVP + PHMB, and 4) PVP and association of HA + PHMB. All preparation was immediately used for electrospinning.

Dynamic light scattering (DLS)

Polymeric mean hydrodynamic radius (z-AVE) and polydispersity index (PdI) were determined by dynamic light scattering (DLS, Zetasizer Nano NS, Malvern Instruments, Malvern, UK). The samples were diluted in ultra-purified water (10 µL/mL) and placed in the scintillation vials. All the analyses were performed in triplicate (n = 3), and data were presented as mean values and standard deviations (SD).

Electrospinning

Each solution was loaded into a 20 mL plastic syringe with a metallic needle attached (0.70 x 20 mm). The syringe pump (KD Scientific 100, USA) delivered a polymer solution at a controlled flow rate of 0.1-20 mL/h. The electrospinning was conducted at 5-15 kV and 25°C and relative humidity at 45%. The distance between the capillary tip and the collector was about 10–25 cm.

Morphology and diameter of fiber analysis

The fiber's morphology was performed by scanning electron microscopy (SEM) (EVO MA15, ZEISS, Germany). The samples were fixed on a silicon substrate, and to create a conductive layer on the sample surface, they were covered with gold (BALTEC SCD 050, Liechtenstein). The fiber diameters and their distributions were determined for each electrospun mat. Fifty fibers were randomly selected from each SEM image using image analysis software Image-J (National Institutes of Health (NIH)). Firstly, the images were calibrated using the Image-J calibration tool, and then using the measurement tool, the fiber diameters were determined to perform the mean and standard deviation. After, the data were exported as a text file, and the histograms with the distribution curves were performed using the Origin Pro 8.0 software (Leal *et al.*, 2019; Salles *et al.*, 2020).

Infrared spectroscopy

The Fourier transform infrared (FTIR) spectra were recorded at room temperature in an infrared spectrophotometer with attenuated total reflectance (Spectrum 100, Perkin Elmer, USA). All spectra were taken in the 4000–500 cm^{-1} spectral range with a 4.0 cm^{-1} resolution. Spectrum instrument software (Perkin Elmer, USA) was used to operate the FTIR spectrometer and collect all the data. After, the spectra were exported as a text file and analyzed using the Origin Pro 8.0 software. Measurements were performed on HA and PHMB powder, fiber PVP, fiber PVP + HA, fiber PVP + PHMB, and fiber PVP + HA/PHMB.

Contact angle (CA)

The fibers' wettability was calculated by the sessile drop technique using the contact angle goniometer (Contact angle meter OCA 15, DataPhysics Instruments GmbH, Germany). The samples at room temperature were fixed on a stage of the CA device. The water droplets (50.0 µL) were dropped onto the material surface at a rate of 0.5 µL/second, and the SCA 20 software was used to obtain the CA value. The mean CA value of three measurements was performed at different positions on the same sample.

Antimicrobial assay - Agar diffusion assay

The agar diffusion assay was performed as described by dos Santos *et al.* (2023) and modified according to this study. A 300 µL of inoculum at 10^6 cells/mL was dispersed on the plate surface of Tryptic Soy Agar (for *Staphylococcus aureus* and *Escherichia coli*) or Sabouraud Dextrose Agar (for *Candida albicans*). Circular discs of 10 mm of PVP, PVP + HA, PVP + PHMB, and PVP/ HA + PHMB were used. In addition, filter paper discs of 10 mm were used to incorporate 0.33 µL of PHMB solution 20% to have a similar concentration of PVP + PHMB and PVP/ HA + PHMB, which is around 0.066 µg/cm^2. The samples were exposed to UV light (110 V, 254 nm) for 30 minutes on each side before the test as a sterilization procedure. Each disc was carefully placed on the agar plate and then incubated at 37°C for 24h. The test was performed in triplicate (dos Santos *et al.*, 2023).

Statistical analysis

All data were expressed as mean ± SD. Statistical analysis of the data was performed by One Way ANOVA with post hoc Tukey HSD test, with a confidence level of 95% ($p < 0.05$).

RESULTS

Hydrodynamic diameter and Polydispersity index (PdI)

Table I presents the hydrodynamic diameter, the PdI, and the zeta potential evaluated, demonstrating the homogeneity of the solutions formed.

TABLE I - The hydrodynamic ratio (Z-Ave), the polydispersity index (PdI), and the zeta potential of the solutions obtained

Solutions	Z-Ave (d.nm) ± SD	PdI ± SD
PVP	167.10 ± 18.80	0.7863 ± 0.0920
PVP + HA	143.00 ± 12.60	0.5507 ± 0.1636
PVP + PHMB	359.00 ± 97.05	0.4760 ± 0.1321*
PVP + HA/PHMB	167.60 ± 42.82	0.4077 ± 0.1851**

SD means Standard Deviation.

* Statistically significant ($p < 0.05$); ** p-value summary ($p = 0.0072$); *** p-value summary ($p = 0.0001$).

Morphology and diameter of fiber analysis

Figure 1 demonstrates the fiber morphology and the histogram of the diameter distribution of fibers.

The four histograms of fiber distribution showed different features. The PVP histogram distribution varies between 2µm and 6µm, with the most frequency around 4.2 µm. PVP + HA histogram distribution varies between 1µm and 3µm, with the most frequency around 1µm and 2µm. PVP + PHMB histogram distribution varies between 1µm and 3µm; however, the most frequency is around 1.5µm and 1.8µm. PVP + HA/PHMB histogram distribution varies between 1µm and 4µm, with the most frequency around 2.5µm.

Therefore, the chemical formulations produced histogram distribution with distinct characteristics. PVP alone has the widest value distribution, and PVP + HA showed the narrowed value distribution.

Mean fiber diameters with statistical significance are shown in Table II.

TABLE II - Fiber diameters

Fibers	Diameters (µm) ± SD
PVP	4.199 ± 0.870^{*A}
PVP + HA	1.443 ± 0.599^{B}
PVP + PHMB	1.222 ± 0.669^{B}
PVP + HA/PHMB	1.893 ± 0.667^{B}

SD means Standard Deviation.

* Statistically significant ($p < 0.05$). Same letter indicates no significant differences between values (Tukey test, $p < 0.05$).

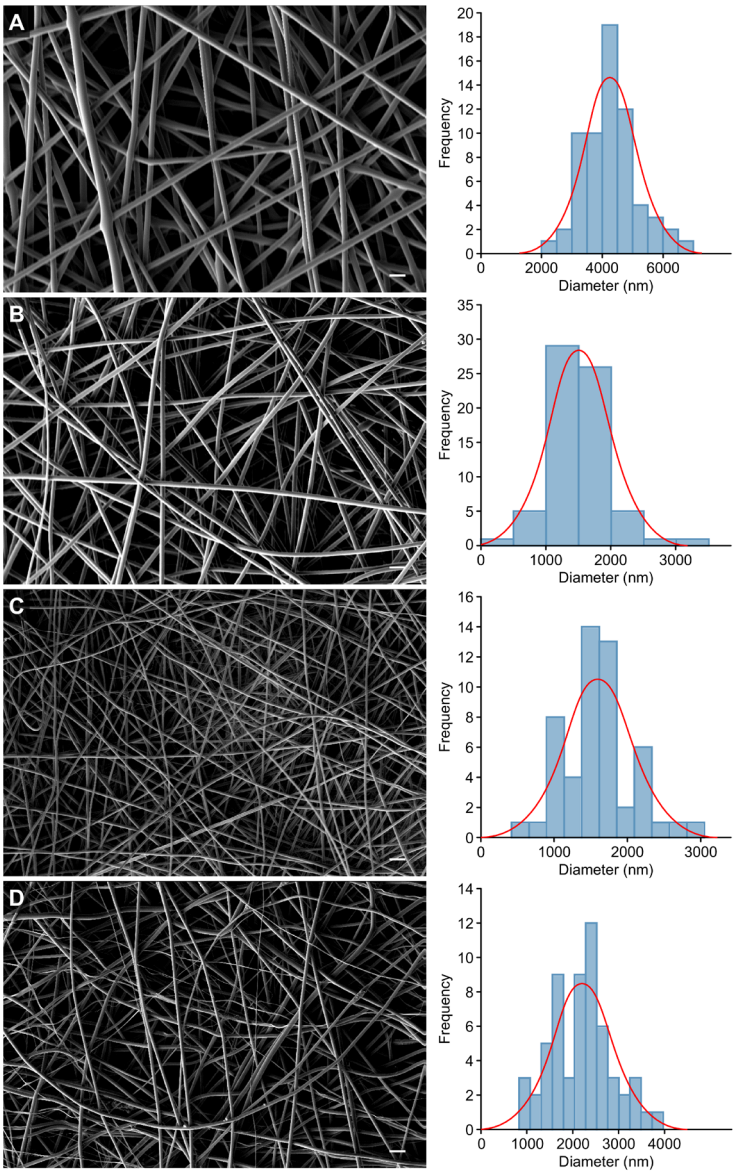

FIGURE 1 - SEM micrographs (left) and histogram of diameter distribution of fibers obtained (right). A) Fiber PVP; B) Fibers PVP + HA; C) Fiber PVP + PHMB; and D) Fiber PVP + HA/PHMB. Scale bar 10 µm.

Infrared spectroscopy

The FTIR study investigated the possible interactions between the PVP matrix and the HA, PHMB, or HA/PHMB addition. The FTIR spectra of fibers are compared in Figure 2, and Table III shows the main functional groups assignment.

TABLE III - Wavenumber values and functional groups assignment in the FTIR spectra

Wavenumber (cm^{-1})	Group
1644; 1651; 1660	C=O
1440	CH_n; OH
1370	CH
1290	CN

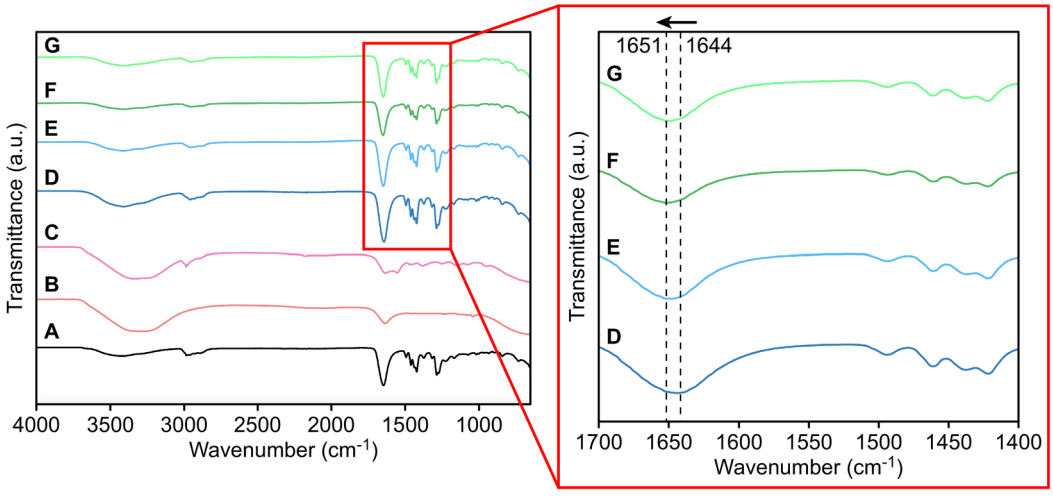

FIGURE 2 - Infrared analysis. A) PVP; B) HA; C) PHMB; D) Fiber PVP; E) PVP + HA; F) PVP + PHMB; and G) PVP + HA/PHMB.

Contact angle (CA) and permeability rate

The sessile drop technique was used to study the CA and water permeability rate on the flat, smooth, and non-porous surfaces, also on electrospun mat surfaces.

The CA of thin films with different compositions was investigated to evaluate the water-favorable character of the produced membranes. The results of the wetting angles in degrees (°) are shown in Figure 3 and Figure 4.

The mean water CA values were lower than 90°, representing the good wetting surface of the formulations. The typical aspect of the water drop on a non-porous surface and the statistical values are shown in Figure 3.

There is statistical significance between the formulations; in the ANOVA with the Tukey test, the p-value reached 0.008148.

The pairwise comparison between the PVP + HA and PVP + HA/PHMB formulations shows a significant difference, with p = 0.01845. Similarly, the pairwise comparison between the PVP + PHMB and PVP + HA/PHMB was statistically significant, with p = 0.01461.

The conjugation between HA and PHMB presents a synergistic effect, creating a non-porous film with a more hydrophilic character, which is interesting to cell adhesion and proliferation (Salles *et al.*, 2020).

The results of water permeation in the electrospun mat are shown in Figure 4. Similarly, the PVP + HA/PHMB showed a faster water permeability rate, around 1.5 seconds. Therefore, PVP + HA/PHMB has a more hydrophilic character between the four formulations.

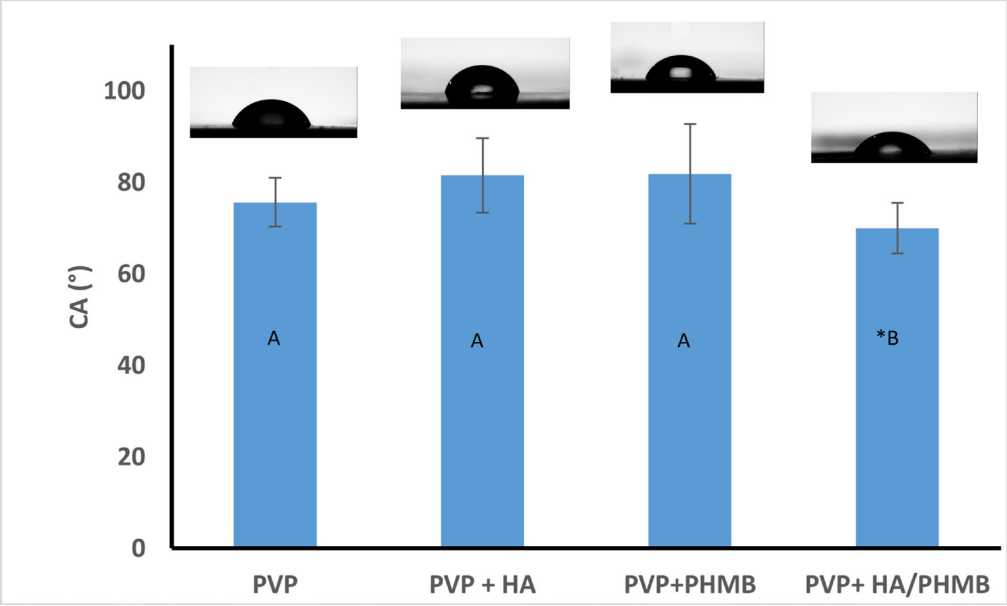

FIGURE 3 - Typical aspect of the water drop on the film surface and the statistical values. Images of the water droplets were taken at 2 seconds. Data are mean ± standard error of the mean, n = 3. * Statistically significant ($p < 0.05$). Same letter indicates no significant differences between values (Tukey test, $p < 0.05$).

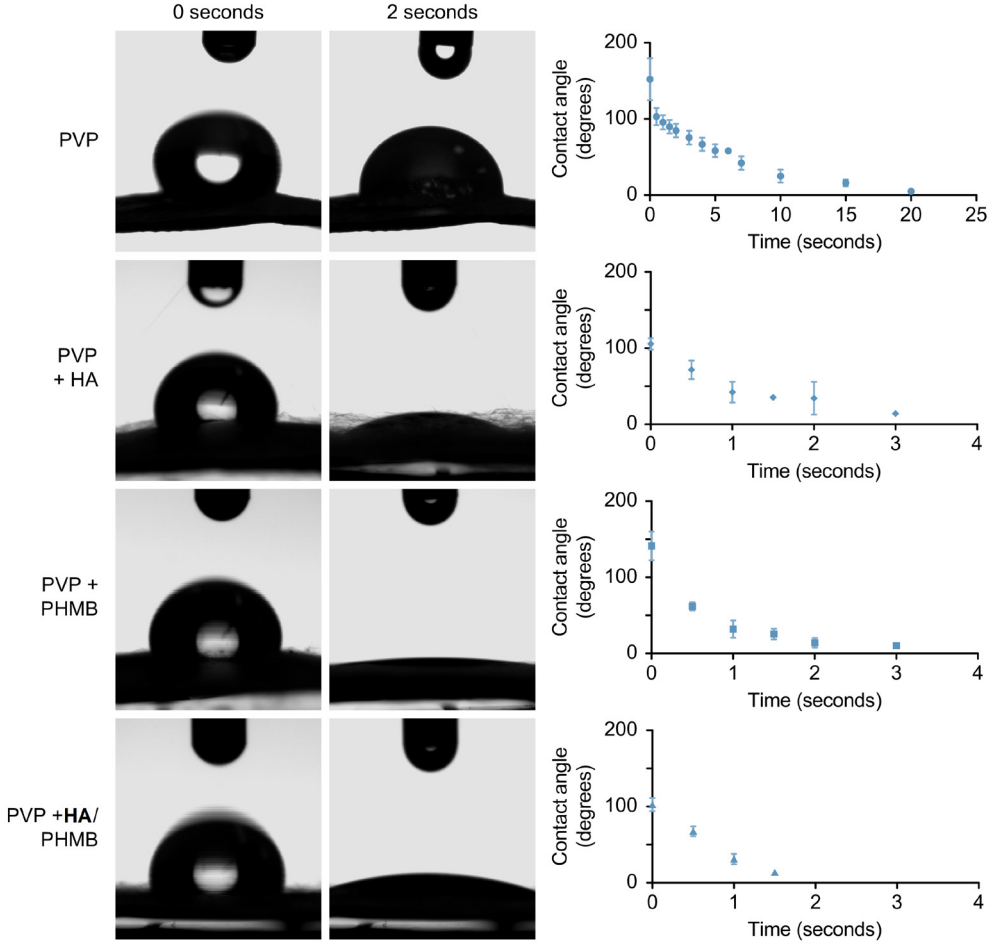

FIGURE 4 - Images of the water droplets were taken at 0 and 2 seconds (left). Changes in the contact angles before the water droplets on the fibers membranes (right). Data are mean ± standard error of the mean, n = 3.

Antimicrobial test - Agar diffusion assay

Figures 5 and 6 shows the results of the microbiological study performed with three microorganisms. The performance of the fibers against the microorganisms was different and there is statistical significance in the Anova with Tukey's test ($p < 0.05$).

Poly (vinyl pyrrolidone) and HA alone did not inhibit the proliferation of microorganisms, and no zone of inhibition halos were observed. However, the association with PHMB proved positive, and the inhibition halos were observed in these formulations.

Escherichia coli proved more resistant to PHMB, and the inhibition halos measures were the smallest. In contrast, PHMB proved more effective in treating contamination with *Candida albicans*, and the inhibition halos reached higher values with this microorganism.

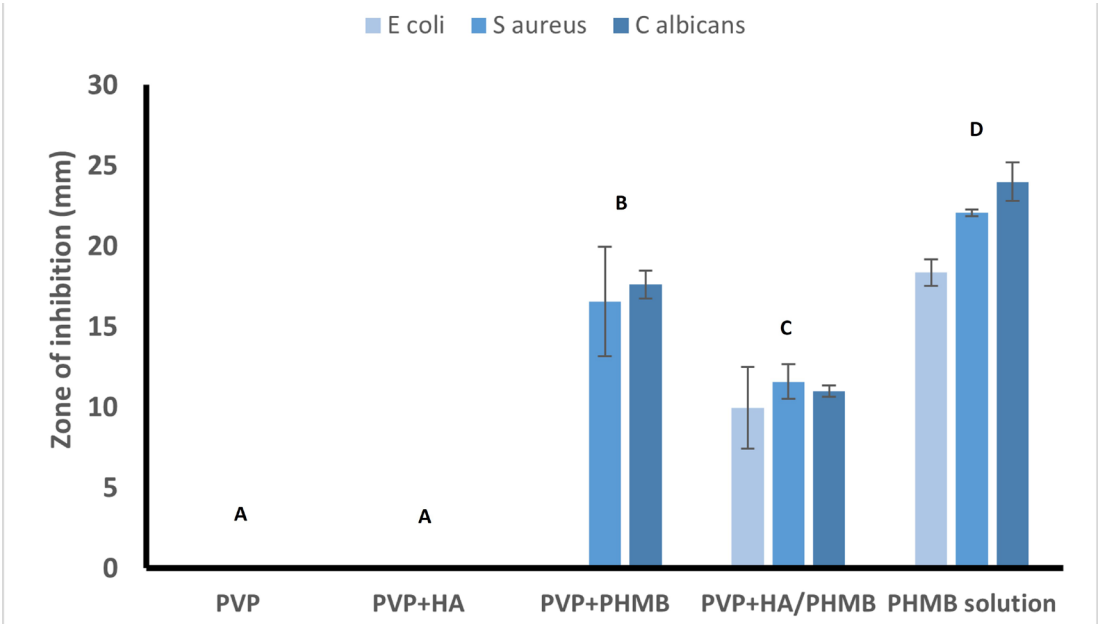

FIGURE 5 - Zone of inhibition of three microorganisms tested. Data are mean ± standard error of the mean, n = 3. Same letter indicates no significant differences between values (Tukey test, $p < 0.05$).

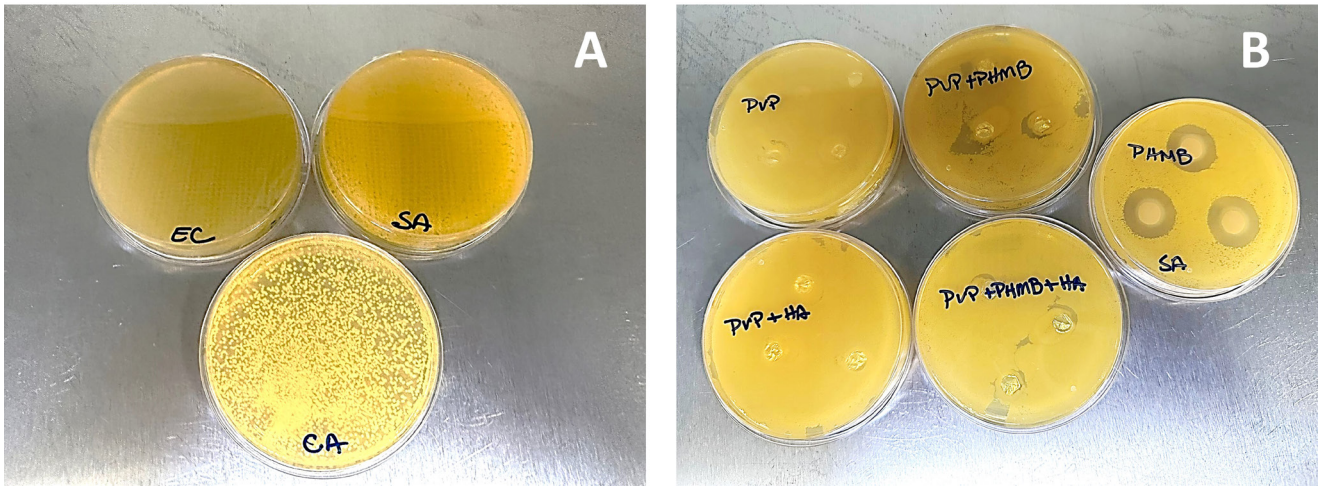

FIGURE 6 - (A) Images of microorganisms cultivated in the petri dishes, and (B) zone of inhibition observed during the study with *Staphylococcus aureus*.

4. DISCUSSION

Recent studies demonstrate that simultaneous phases are involved in the wound healing process and need to be considered for good wound management. Protection against infection and free radicals, modulation of the inflammation process, and support for the proliferation and acceleration of cell migration must be respected for successful healing (Baron, Glatz, Proksch, 2020).

The correct choice of ingredients to compose the biodegradable membrane that promotes the ideal benefits to maintain and treat wounds and contributes to managing the phases that make up the healing process is a great challenge.

However, in the mid-1960s, polymers were believed to be a passive material with minimal action in the healing process. Nowadays, it is demonstrated that hydrogels are a very interesting alternative for biomedical applications due to their peculiar properties like hydrophilic nature, high sensitivity to physiological environments, soft tissue-like water content, and adequate flexibility (Kamoun, Kenawy, Chen, 2017).

Poly (Vinyl Pyrrolidone) is a biocompatible, biodegradable synthetic polymer with low toxicity. It is impermeable to bacteria and is water-soluble, which has been used as a hydrogel membrane (Kamoun, Kenawy, Chen,, 2017).

Hydration and prevention of excessive collagen deposition by reducing inflammation are HA functions especially important in the wound healing process. Besides that, more HA benefits in the healing process have already been described, such as the framework through which cells migrate, blood vessel formation, space-filling capacity, and modulation of acute and chronic inflammation (Ialenti, Di Rosa, 1994; Papakonstantinou, Roth, Karakiulakis, 2012). Combining such benefits with the antimicrobial and biofilm-reducing properties of PHMB, apart from being challenging, can offer numerous advantages for wound healing treatment. Therefore, biodegradable membranes obtained by electrospinning may be interesting to help the healing process.

Polymer solution homogeneity is a critical and important parameter for an electrospinning process (Salas, 2017). Cationic surfactants like PHMB can cause polyanion precipitation despite the electrostatic interaction of HA negative charge (Fischer, Harosh, 2008; Serrano-Sevilla *et al.*, 2019). Therefore, to avoid polyanion precipitation, the PVP was incorporated as a stabilizer. PVP is a nonionic amphiphilic polymer (Ali, Ghosh, Kabir-ud-Din, 2010) with a complexing ability to proton donors, thereby controlling the electrostatic interaction between PHMB and HA, producing a stable viscous solution able to electrospinning.

Furthermore, PVP was studied as a substitute for surfactants in cosmetics and detergent formulations because surfactants can lead to skin irritations and allergic reactions and, additionally, impair the epidermal barrier function. In the PVP formulation, an increase in the stability during storage and an improvement in the safety of product use by reducing the irritant potential was observed (Bujak, Wasilewski, Nizioł-Łukaszewska, 2015).

As shown in Table I, the mean diameter increased by adding PHMB to the PVP solution. This might be due to PVP–PHMB interactions, resulting in the molecular coil increase. Also, the HA/PHMB association in the polymeric PVP solution promoted a significant decrease in the polydispersity index ($p = 0.0072$) compared to the PVP fiber.

The fiber morphology and diameter depend on polymeric solution properties such as viscosity, electrical conductivity, and surface tension. Also, electrospinning parameters such as voltage, flow rate, and distance between the needle and collector plate affect the properties of the fiber produced (Colmenares Roldán *et al.*, 2017).

The obtained fiber morphology can be observed using the SEM image in Figure 1, from (A) to (D), indicating that all produced fibers showed smooth morphology and continuous characteristics without bead formation or major secondary artifacts. For all groups, fibers appeared to be uniformly distributed, which means that the process used had good quality, and the fibers maintained their structural integrity even after adding HA and PHMB solutions. Macroscopically, the films are milky white and easy to handle. Microscopically, the surface of film biomimicry the extracellular matrix (Figure 7).

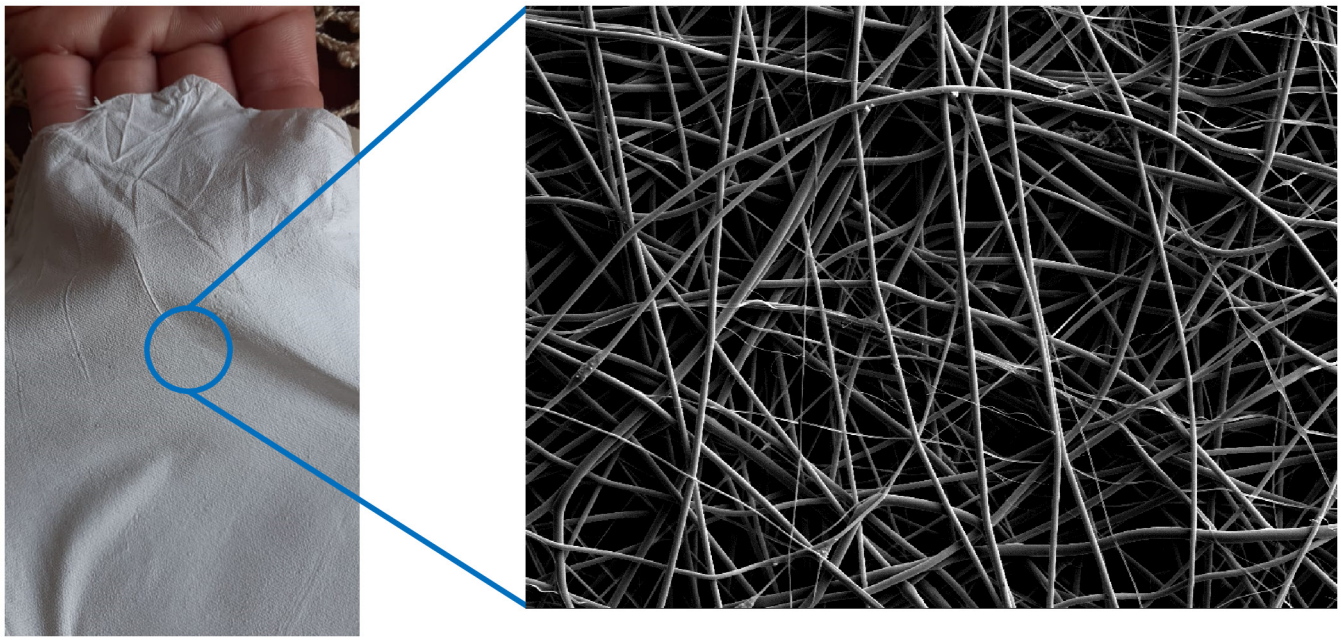

FIGURE 7 - Macroscopic and microscopic morphology of electrospun fiber.

Remarkably, as seen in Figure 1 (C) and (D), all polymer solutions formulated with PHMB could form fibers. The addition of the HA solution promoted a reduction in mean fiber diameter, from 4199 ± 870 nm on PVP fiber (Figure 1 (A)) to 1443 ± 599 nm on PVP + HA fiber (Figure 1(B)). The same characteristic can be observed in the fiber PVP + PHMB (1222 ± 669 nm) and fiber PVP + HA/PHMB (1893 ± 667 nm), which are demonstrated in Figure 1 (C) and (D), respectively. The smaller fiber diameter observed by adding PHMB can indicate higher charge densities at the fibers' surface, consequently stretching the polymer jet (Dilamian, Montazer, Masoumi, 2013).

The FTIR study demonstrated in Figure 2 showed the characteristic PVP bands observed at 1660, 1440, 1370, and 1290 cm^{-1}, which are attributed to the vibration of the carbonyl group (C=O); C-H_n, O-H bending; lactone structure; and -C-N stretching, respectively; with similar intensities for all bands, as expected for samples of similar crystallinity (Deniz *et al.*, 2011).

Biguanide groups have five conjugated amines that can bind with neighboring molecules by establishing multiple hydrogen-bonding interactions (Lebel, Maris T, Wuest, 2006), promoting interesting supramolecular chemistry.

The absorption band at 1644 cm^{-1} for PVP fibers has shifted slightly to 1651 cm^{-1} in the PVP + HA/PHMB fibers, indicating the presence of interaction between C=O groups of PVP and HA/PHMB in the PVP + HA/PHMB nanofibrous composites. However, the modest interaction observed may be due to the low HA and PHMB concentrations used and the low sensitivity of the technique.

Figure 4 demonstrated that after placing a water droplet on PVP fiber membrane, the water droplet was completely absorbed at 20 seconds by this fiber membrane because of the hydrophilic hydroxyl groups in PVP and its porous structures. After adding HA or PHMB, the absorption of the water droplet was even higher, immediately absorbed by the fiber membrane after its placement. Hyaluronic acid and PHMB are hydrophilic compounds that increase their hydrophilic properties after being included. This test can be correlated with dissolution time at the wound site and showed the fast-dissolving ability of wound dressing. The fast dissolving causes no pain and new trauma to replace the wound dressing during the treatment.

Pre-clinical and clinical studies demonstrated that histologically and histomorphometrically measurements

present a significant superiority at healing when a hydrophilic biomaterial is used (Sartoretto *et al*., 2017) because the hydrophilic material can efficiently load and deliver hydrophilic growth factors and stimulate epithelial cell proliferation for guided tissue regeneration (Oh, Lee, 2019).

In this study, a novel fiber-based antimicrobial-regenerative system was successfully developed, demonstrating the homogeneity of solutions prepared with a low polydispersity index, showing a monomodal distribution, especially for the PVP solution plus AH/PHMB. However, we reinforce the importance of conducting clinical studies to assess the physiological benefits of wound treatment. The fibers created presented homogenous morphology, with a smooth surface and no beads or major secondary artifacts formation, maintaining their structural integrity even after adding HA and PHMB solutions. The FTIR study demonstrated an interaction between C=O groups of PVP and HA/PHMB in the PVP + HA/PHMB nanofibrous composites. The wettability analysis showed a hydrophilic characteristic of fibers produced, which could benefit tissue regeneration.

Here, an elegant electrospinning approach is presented to produce a hydrophilic fiber dressing containing PHMB and HA. The electrospinning technique was chosen because it has potential to be industrially scalable to mass production and commercialization of the wound dressing. Moreover, electrospun microstructure increases the PHMB and HA release, and the regenerative agents are combined to synergistically treat the wound. The dressing was formulated with nontoxic solvents without chemical additives like preservatives, colorants, surfactants, and artificial fragrances. The electrospinning process was performed in a simple one-step under mild temperature conditions.

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