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DERİ YARA KAPLAMALARINDAKİ UYGULAMALAR İÇİN ELEKTRİK ALAN LİF ÇEKİM İLE ÜRETİLMİŞ TIGER 17 PEPTİT YÜKLÜ POLİKAPROLAKTON/SELÜLOZ ASETAT NANO LİFLİ MATLAR

TIGER 17 PEPTIDE LOADED POLYCAPROLACTONE/CELLULOSE ACETATE ELECTROSPUN NANOFIBROUS MATS FOR APPLICATIONS IN CUTANEOUS WOUND DRESSINGS

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TIGER 17 PEPTIDE LOADED POLYCAPROLACTONE/CELLULOSE ACETATE ELECTROSPUN NANOFIBROUS MATS FOR APPLICATIONS IN CUTANEOUS WOUND DRESSINGS

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***ABSTRACT**:* A skin wound if not properly treated can result in a chronic wound susceptible to widespread infections, which can result in the patient's death. Currently, tissue engineering is described as an interdisciplinary field that combines principles of engineering, chemistry and biology to generate solutions that allow to repair, restore and/or improve the functions of injured tissues. In the same sense, the textile area addresses solutions based on polymeric fibers, produced from a wide range of polymers, which allow the generation of structures with a large surface area, porosity and mechanical resistance that can be used as bioactive dressings that promote a healing and efficient antimicrobial activity. This research work focused on the synthesis of Tiger 17, through microwave-assisted solid-phase synthesis methodologies, and Tiger 17 commercially obtained, respective structural characterization and evaluation of the antimicrobial capacity. Simultaneously, nanofibrous polymer matrices were produced using the electrospinning technique with the aim of immobilizing the developed biomolecule and thus creating potential vehicles for a local and sustainable antimicrobial action (controlled release). In order to verify its physical and chemical properties, advanced characterization techniques were used: proton nuclear magnetic resonance, high performance liquid chromatography, optical microscopy, scanning electron microscopy, fourier transform infrared spectroscopy–attenuated total reflectance, thermogravimetry, differential scanning calorimetry, contact angle and surface energy and determination of porosity and hydration.

Keywords: Antimicrobial peptides; electrospun nanofibers; peptide synthesis; polymeric mats

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ÖZ: Düzgün tedavi edilmeyen bir cilt yarası, hastanın ölümüyle sonuçlanabilecek yaygın enfeksiyonlara duyarlı kronik bir yaraya neden olabilir. Günümüzde doku mühendisliği, hasarlı dokuların işlevlerini onarmaya, eski haline getirmeye ve/veya iyileştirmeye olanak tanıyan çözümler üretmek için mühendislik, kimya ve biyolojinin ilkelerini birleştiren disiplinler arası bir alan olarak tanımlanmaktadır. Bu kapsamda, tekstil, iyileşmeyi ve etkili antimikrobiyal aktiviteyi teşvik eden biyoaktif pansuman olarak kullanılabilecek, geniş yüzey alanlı, gözenekli ve mekanik dirençli yapıların oluşturulmasına izin veren, geniş bir polimer yelpazesinden üretilen liflere dayalı çözümler sunmaktadır. Bu araştırma çalışması, mikrodalga destekli katı faz sentez metodolojileri aracılığıyla Tiger 17'nin sentezine ve ticari olarak temin edilen Tiger 17 ile birlikte yapısal karakterizasyonunun ve antimikrobiyal kapasitenin değerlendirilmesine odaklanmıştır. Aynı zamanda, geliştirilen biyomolekülü hareketsiz hale getirmek ve böylece lokal ve sürdürülebilir bir antimikrobiyal etki (kontrollü salım) için potansiyel çözümler yaratmak amacıyla elektrik alan lif üretim tekniği kullanılarak nanolifli polimer matrisler üretildi. Yapının fiziksel ve kimyasal özelliklerini doğrulamak için, proton nükleer manyetik rezonans, yüksek performanslı sıvı kromatografisi, optik mikroskopi, taramalı elektron mikroskobu, fourier dönüşümü kızılötesi spektroskopisi-zayıflatılmış toplam yansıma, termogravimetri, diferansiyel taramalı kalorimetri, temas açısı, yüzey enerjisi, gözeneklilik ve su tutma ölçümleri gibi ileri karakterizasyon teknikleri kullanıldı:

Anahtar Kelimeler: Antimikrobiyal peptitler; Elektrik alan lif çekimi, nanolifler, peptit sentezi, polimerik matlar

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1. INTRODUCTION

During the healing process of a skin wound, dressings are used to protect the integrity of the dermis and epidermal tissues. Currently, the manufacture of dressings has reached a high level of quality in order to create a suitable environment for a healing process and skin regeneration to occur under optimal conditions in order to accelerate the process and avoid microbial colonization. In recent years, bioactive dressings have become systems with a relevant importance as they combine the physical and biochemical properties of natural and synthetic polymers, with active compounds that have beneficial properties for the healing and regeneration of skin wounds [1,2]. AMPs are the most recent acquisition of these systems, where controlling the proliferation and microbial colonization of bacteria, promoting the synthesis of proteins essential for wound healing, enhancing cell migration and proliferation and modulating the host's immune response are the main objectives.

These AMPs are biomolecules involved in a variety of biochemical processes and physiological functions because they are, for the most part, selective for a given target, act at low concentrations, have a high degree of efficacy, specificity, activity, and low toxicity, avoiding undesirable side effects. by being biologically accepted by human metabolism [3,4].

Tiger17 is a peptide based on tigerinins with a great ability to heal deep skin wounds, ensuring the re-epithelialization of the affected site and accelerating the healing process. In some studies, it has been shown to exert significant effects on the three most crucial stages of the wound healing process. These steps include (1) inducing the recruitment of macrophages to the wound site upon the inflammatory reaction; (2) promotion of migration and proliferation of keratinocytes and fibroblasts, leading to re-epithelialization and formation of new tissue; and (3) tissue remodeling phase, returning its structure and functionality [5]. However, there were no records about its potential application as an antimicrobial peptide and its effectiveness in preventing microbial colonization in chronic wounds.

The polymer of natural origin, cellulose acetate (CA) is widely used for the production of nanofibers because it has excellent moisture retention properties, which in chronic wounds is seen as a positive factor for inducing faster healing due to the adequate supply of growth factors and other molecules essential for healing tissues. [6]. At the same time, it helps in the absorption of exudates, is a low-cost material, resistant to heat and has a porous structure, mimicking the extracellular matrix of the skin [7]. Combined with polycaprolactone (PCL), a polymer of synthetic origin, it allows the production of nanofibers using the electrospinning technique [8,9]. This simple, cost-effective and straightforward method has attracted much attention in biomedical research by allowing the production of nanoscale fibers with a larger surface area than fibers obtained from conventional spinning processes with tunable mechanical, thermal, hydrophobic and viscoelastic properties [10-12]. The process involves the ejection of a polymer solution through a needle, that under an electric field is attracted towards a collector, converting the initial solution into nanofibers with exceptional features for applications in dressings for chronic skin wound care [6,13].

2. MATERIALS AND METHODS

2.1 Tiger 17 Peptide Synthesis c[Trp-Cys-(Lys-Pro)3 -Arg-Cys-His-NH_2]

The microwave-assisted solid-phase synthesis peptide synthesis method (MW-SPPS) was used for its convenience, speed and greater probability of obtaining higher values of mass and yield, taking into account that it is a peptide consisting of 11 amino acid residues. The deprotection reactions of the Fmoc group took place under microwave irradiation at a power of 50 W at 75°C, in two cycles, one for 30 seconds and the other for 3 minutes with a 20% solution of piperidine in DMF. Coupling reactions were also carried out under microwave irradiation for 5 minutes at a power of 25 W at 75°C using a coupling mixture consisting of Fmoc-amino acid, DIC and Oxime in dry DMF with 6; 7.5 and 10 eq. excess (depending on amino acid), relative to resin loading. The resin was Fmoc-Rink-Amide with a degree of functionalization of 0.53 mmol/g. The choice of this resin is based on the fact that it already has the Rink linker, which allows obtaining peptides in the C-terminal amide form. Amino acids were coupled sequentially as follows: Fmoc-His(Trt)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Arg(Pbf)-OH, 3x(Fmoc-Pro-OH, Fmoc-Lys(Boc) -OH), Fmoc-Cys(Trt)-OH and finally, Fmoc-Trp(Boc)-OH.

2.2 Tiger 17 Peptide Characterization: Proton Nuclear Magnetic Resonance (^{1}H NMR)

To confirm the presence of all amino acids and their structure on Tiger 17 peptide, 5 mg of peptide were dissolved in 500 µL of DMSO-d_6 and transferred to an NMR tube. The ^{1}H RMN spectrum was recorded at 400 megahertz (MHz) using the solvent peak as internal reference.

2.3 Tiger 17 Peptide Characterization: High Performance Liquid Chromatography (HPLC)

To confirm the purity of Tiger 17 peptide, various eluent solutions were prepared: ACN/H_2O (1:9), ACN/H_2O (1:3), ACN/H_2O (1:2), ACN/H_2O (1:1), ACN/H_2O (2:1), ACN/H_2O (3:1), ACN/H_2O (5:1) and 100% ACN to which 0.1% TFA was added. All solutions were placed in an ultrasound bath for 10 minutes for degassing. The equipment conditions tested were: flow of 1 mL/h, 0.8 mL/h, 0.4 mL/h, wavelength of 215 and 280 nm, and range of 0.32 and 0.64.

2.4 Tiger 17 commercially obtained (Tiger 17C) Minimum Inhibitory Concentration (MIC)

Tiger 17C MIC was determined using the broth microdilution method [14]. The 4 mg/mL working solution for the commercial Tiger 17 was prepared using the most suitable solvent and 50 µL of the bacteria suspensions prepared at 2×10^7 colony forming units (CFUs)/mL in MHB was used.

2.5 Preparation of PCL and PCL/CA Polymeric Mixtures and Production of Polymeric Mats by Electrospinning

PCL (Mw: 78,000, 88% hydrolyzed, Polysciences Europe GmbH) and CA (Mw: 30,000 with 39.8% wt of acetyl content, Sigma). CA was dissolved in 10% (w/v) acetic acid, while PCL was dissolved in an $CDCl_3$/DMF mixture at 300 rpm and 90°C for 1 h. In the electrospinning equipment, PCL/CA was processed at 14%:10%wt (3:1) with 24,7 kV, 3,2 mL/h and 21 cm, and PCL 14%wt with 12 kV, 0,7 mL/h and 17 cm distance.

2.6 Fiber Characterization

Scanning electron microscopy (SEM) images were taken from PCL and PCL/CA films (NOVA 200 Nano SEM FEI Company) with an acceleration voltage of 10 kV. The fiber diameter distribution was determined using images used were 10,000x magnitude, processed with ImageJ software.

2.7 Chemical Composition: Fourier Transform Infrared Spectroscopy–Attenuated Total Reflectance (ATR-FTIR)

ATR-FTIR spectra were collected using an IRAffinity-1S spectrophotometer, SHIMADZU (Kyoto, Japan), with a diamond crystal. Here, a spectral resolution of 8 cm^{-1} was applied, for 45 scans, in a range of 4000-400 cm^{-1}.

2.8 Thermal Stability: Thermogravimetry (TGA) and Differential Scanning Calorimetry (DSC) analysis

Thermal properties, including thermal degradation of PCL and PCL/CA films, were analyzed by TGA and DSC. The TGA evaluated weight loss variations with temperature increase in the range of 25-600°C, at a heating rate of 10°C/min and a flow rate of 200 mL/min (inert nitrogen atmosphere) in a STA 7200 Hitachi® (Fukuoka, Japan), using aluminum pan. DSC was also used to evaluate the thermal stability of the films. Here, a DSC 822 Mettler Toledo (Columbus, USA) was used. The films were subjected to a single heating step from 0-250°C at a heating rate of 10°C/min, under a nitrogen atmosphere and a flow rate of 20 mL/min (inert environment).

2.9 Contact Angle and Surface Energy Measurement

The contact angle and surface energy of PCL and PCL/CA films for dH_2O (polar liquid), benzene (nonpolar liquid) and polyethylene glycol (nonpolar and polar) were evaluated using an OCA 200 equipment together with Software OCA15 plus (version 1.2), following the sessile drop method. Volume was set at 5 µL. Angle values were retrieved 8 sec after contact with the surface. The surface energy values were obtained by the mathematical model Owens-Wendt-Rabel & Kaelble, and using the polar and dispersive components.

2.10 Fiber Functionalization with Tiger 17C

PCL and PCL/CA films were functionalized with Tiger 17C-NH_2 through physical adsorption. 6 mm diameter samples were immersed in a 2xMIC peptide solution, prepared in dH_2O, for 24 h at 37°C and 100 rpm. Loosely bound peptide molecules were removed by washing in dH_2O.

2.11 Microbial Growth Kinetics Test with Fibers Functionalized with Tiger 17C

6 mm diameter film samples functionalized with the peptide at 2xMIC were tested against *S. aureus* at $1x10^5$ CFU/mL in TSB, following the guidelines of ASTM-E2149-01. After 0 (before the action of the agent), 1, 2, 4, 6 and 24 h of incubation, the bacteria were serially diluted (101 to 104 in PBS), cultured on TSA plates and the number of colonies counted after 24 h of incubation at 37°C.

3. RESULTS AND DISCUSSION

AMP Tiger 17 has reappeared in recent years thanks to its potential as a healing promoter. Due to its recent investigation, there are no records of its synthesis in an academic laboratory environment, which means that the entire process of peptide synthesis and characterization required optimization. Two syntheses of Tiger 17 were carried out under the same conditions, with only the process of cleavage of the peptide from the resin varying. According to some literature [15], cleavage of the peptide would be more effective using a scavenger in the cleavage mixture (TFA:H_2O (95:5)). That said, cleavage was tested using a scavenger, triisopropyl silane (TIPS), and without using it. Different exposure periods, 2h and 4h were also investigated.

It was concluded that increasing the exposure period did not benefit the cleavage of the peptide from the resin, where with only 2h a reaction yield of approximately 40% was obtained and with 4h of exposure, the yield was only 11%.

In the synthesis using a scavenger, the amount of solid obtained was higher compared to the same synthesis without the aid of a scavenger, allowing a better yield to be obtained.

The ^1H NMR spectrum of both syntheses proved to be quite complex, due to peptide composition of 11 amino acids and, possibly, to the presence of impurities. However, there are some signals that theoretically correspond to elements present in the peptide (aromatic zone and aliphatic zone). HPLC results with the ACN/H_2O (1:1) +0.1% TFA eluent confirmed the result, the peptide was impure.

In view of this result, the peptide was not subjected to microbiological tests to assess its antimicrobial activity. Alternatively, the antimicrobial activity of Tiger 17C peptide was evaluated, in order to establish the basic characteristics of the peptide in the fight against microorganisms, never before verified.

The Tiger 17C MIC was established against *S. aureus* bacteria, one of the most prevalent and difficult to eliminate in skin infections. After 24 h of incubation, the MIC was determined to be 250 µg/mL. Considering that its most important effect is recorded in the induction of tissue regeneration and healing of chronic wounds, the addition of antimicrobial resources to its

action is extremely promising in tissue engineering and biomedical applications, particularly in wound healing.

Considering the above data, only Tiger 17C peptide was considered for fiber immobilization. However, until reaching this stage, an extensive optimization of both the polymer solution and the processing parameters required for the production of nanofibers by the electrospinning technique was necessary. A range of polymer solutions with different ratios of PCL and CA in different solvents were studied. With the PCL solution at 14%wt in $CDCl_3$/DMF, nanofibers were obtained through eletrospinning and that as the percentage of PCL in the PCL/CA mixture was increased, more regular fibers were obtained, having reached the conclusion that PCL/CA 14%:10%wt (3:1) in acetone/DMF (for CA) and $CDCl_3$/DMF (for PCL) was the best option. It was decided to produce films using both solutions so that PCL 14%wt fibers served as a control. CA only fibers were not processable due to the instability of the electrical beam and the inability to produce fibers continuously and without defects.

In an initial phase, the polymeric mats were analyzed by optical microscopy (OM), to confirm the existence of fibers. Only the mats where it was possible to detect the presence of fibers went on to capture SEM images. By analyzing the images (Figure 1), the isolated PCL formed fibers with larger diameters, with an average of 1085±333 nm. However, it was expected that the diameter distribution would be as uniform as possible. This did not happen since the standard deviation obtained represents a very high value. This event can be explained by several factors of the chemical nature of the polymer, such as the possible occurrence of chemical and electrostatic interactions with the solvent.

Analyzing the results obtained in (b), taken from the polymeric combination of PCL/CA, the SEM image shows the presence of fibers with high diameters and others with much smaller diameters. The average of diameters was 728±341 nm. The interaction between CA and PCL through hydrogen bonds is established between the hydroxyl group of CA and the carbonyl group of PCL. However, as the standard deviation has a high value this suggests that there is only partial miscibility between the CA and the PCL.

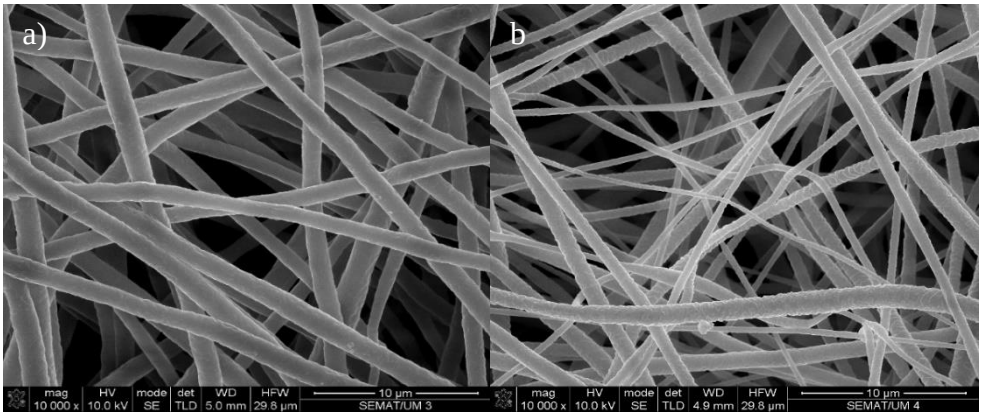

Figure 1. SEM images at 10,000x ampliation. In a) PCL 14%wt in $CDCl_3$/DMF under conditions of 12 kV, 0.7 mL/h and 17 cm and b) PCL/CA 14%:10%wt (3:1) under conditions of 24.7 kV, 3.2 mL/h and 21 cm.

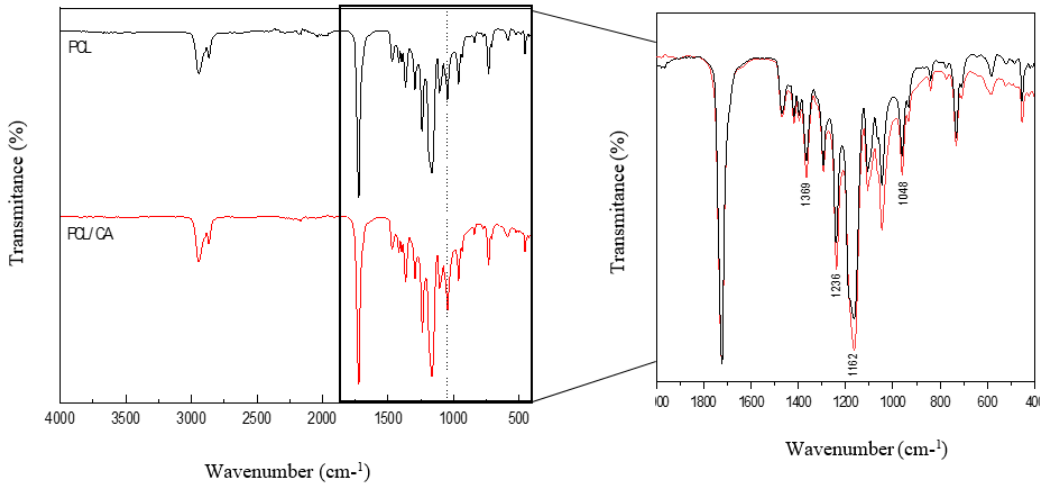

Figure 2. ATR-FTIR spectrum obtained for the two polymeric mats of PCL and PCL/CA

The chemical composition of the fibers formed by the PCL polymer and the PCL/CA mixture was evaluated by ATR-FTIR spectra (Figure 2). In the literature, the structure of PCL is characterized by typical absorption bands at 2948 and 2868 cm^{-1} corresponding to the chemical group CH_2, at 1725 cm^{-1} of the group C= O and at 1246 and 1166 cm^{-1} of the C–O–C group. By comparison, the two spectra obtained appear to be the same only with a % transmittance more intense in some characteristic peaks. These peaks are at 1369 cm^{-1} and 1236 cm^{-1} of the CH_3 group, 1167 cm^{-1} corresponding to the symmetrical vibrations of C–O–C stretching (higher presence in the PCL) and at 1048 cm^{-1} of the C–O functional group. –C (greater presence in the CA). The peak 1728 cm^{-1} characteristic of the C=O group (present in both polymers) does not show any difference in intensity. This may be due to the establishment of bonds between the polymers by the carbonyl group of PCL and the hydroxyl group of CA.

By analyzing the thermal stability of the polymeric films by the TGA technique (Figure 3), as already verified with previous techniques, the results obtained for the polymeric mixture approached the results obtained for the PCL control, being this in a higher % in the mixture. However, the presence of CA was confirmed, and it is clear that the sample has a greater level of degradation and that it starts at lower temperatures (290ºC) than when we have only PCL. This is explained by the properties of PCL, which have a higher thermal resistance compared to CA and in the presence of a mixture of the two polymers, this characteristic disappears.

Hydration and porosity tests were performed to evaluate the absorbent and mechanical potential of polymeric mats for application in chronic wounds. The degree of hydration of the mats was determined by measuring the weight variations after 7 days of immersion in simulated body fluid (SBF) at 37ºC. It was found that in the presence of CA, the degree of hydration is reduced compared to the PCL control. This was not expected, as this polymer should have a greater fluid retention effect as it makes polymeric mats less hydrophobic. However, as noted, it is likely that many of its hydroxyl groups were conditioned in combinations with PCL or protected by such a polymer. In this way, PCL and CA groups were lost, reducing the possible bonds with the water molecules in the medium. With regard to porosity, the determination of this value was carried out indirectly through the immersion of the mats in ethanol. It was concluded that the addition of CA compromised the mechanical and structural strength of the film, making it less compact and more susceptible to rupture, with a higher percentage of porosity.

To evaluate the wettability of the polymeric films, contact angles measurements were made and subsequent determination of the value of surface free energy, for the solvents water, benzene and polyethylene glycol.

The PCL polymeric mat, in contact with water, generated high contact angles (150º), characteristic of its hydrophobic nature. At the same time, the PCL/CA mat in the same solvent also produced even higher contact angles (165º), confirming that despite the presence of CA, the polymeric mat maintained its hydrophobic characteristics.

The contact angle values in PCL/CA for the nonpolar solvents showed that the surface presents a hydrophobic behavior, so the addition of CA did not contribute to a better hydrophilicity of the polymeric surface. A total surface free energy of 53.0 mN/m was obtained for the PCL mat and 31.4 mN/m for the PCL/CA. Contrary to the literature, the surface with the highest surface energy value (PCL) was the one with the highest contact angles, except in the presence of water. The addition of CA caused the surface energy value to decrease, which should mean that the contact angles would be larger as the chemical interactions would be weaker. This was not proved, considering that even with the benzene solvent (nonpolar), the contact angles were the lowest (21º). With this perspective, it is concluded that there will be a lack of uniformity of the PCL and CA polymer in the polymeric mat, where sometimes we can find the PCL more on the surface, making the film much more hydrophobic and at another time, the CA may be more on the surface, only slightly increasing the hydrophilicity of the polymeric mat.

The immobilization of the peptide on the surface of the polymeric films was evaluated using an antimicrobial microbial growth kinetics test. This test was performed on immobilized and non-immobilized films with the peptide and evaluated by the number of viable bacteria at specific incubation periods, 1, 2, 4, 6 and 24 h, as well as the associated log reduction (Figure 4). The data reported an improved antimicrobial action on films immobilized with the peptide only in the first hour of culture.

Films were modified with Tiger 17C peptide, at 2xMIC value, for 72 h. However, due to the highly hydrophobic nature of PCL-based mats, repulsion of the peptide was expected even after such a long contact period (the films remained in the peptide incubation bath). These results demonstrate that the amount of peptide bound was small and that it was released very quickly. It is likely that the surface-bound peptide interacted with the polymers via weak bonds. Of the two polymer mats, PCL/CA appeared to have the preferable bond to Tiger 17C. This is a very interesting result, considering that the hydrophobicity of PCL/CA films was even more important than that of PCL. It is likely that due to the prolonged exposure of the mats to the peptide, some molecules have moved towards the free hydroxyl groups of the CA, forming more interactions. After 2h of incubation, a continuous reduction in cell number is observed for all surfaces. This is promoted by nanostructured polymeric mats, which retain many bacterial cells. Also, between 6h and 24h of culture, the importance of the film structure is nullified by the continuous growth of the bacteria. Without the antimicrobial peptide to inhibit their growth, the bacteria continued to evolve, overcoming the initial reduction introduced by either the peptide or the polymeric mat.

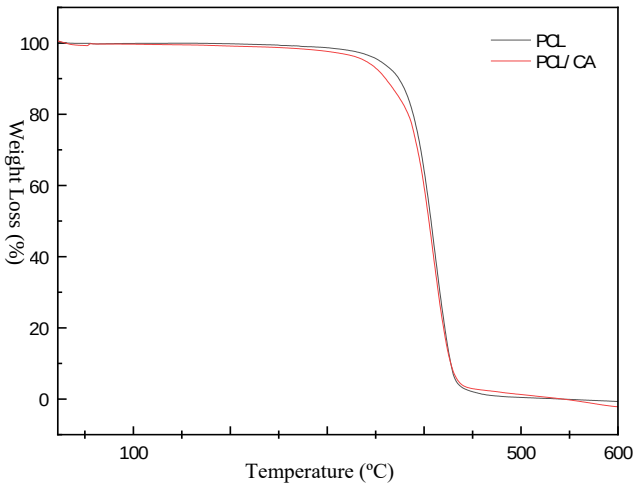

Figure 3. a) TG graph of % loss of mass and temperature (ºC) of the two polymeric mats PCL and PCL/CA.

4. CONCLUSIONS

The antimicrobial activity of the synthesized Tiger 17 peptide is a small explored area, and it was of interest to test whether this peptide could have antimicrobial activity against the most prevalent bacteria in skin wounds, namely *S. aureus*, since previous studies had already demonstrated its high healing capacity in injured tissues. As it is a peptide never before synthesized in an academic laboratory context, several optimization processes were carried out, and it was concluded that the synthesized peptide was impure and without conditions for microbial tests to be carried out.

As a way of establishing its characteristic antimicrobial activity, the commercially obtained Tiger 17 peptide was subjected to MIC microbial tests and microbial growth kinetics where it was shown that this peptide has antimicrobial activity that combined with its ability to regenerate tissues and heal chronic wounds, it becomes an extremely promising alternative in tissue engineering and biomedical applications.

With a polymeric mixture of PCL/CA and a control solution of PCL, nanofibrous polymeric mats were obtained. Both PCL and PCL/CA films are considered to be hydrophobic and temperature resistant and the addition of CA did not allow an effective increase in advantageous characteristics for the use of these fibers in bioactive dressings. It is possible that the degree of miscibility between the two polymers was not sufficient, which justifies that the peptide has poor immobilization on the polymeric surface and it is necessary to achieve better solutions so that the peptides have a more controlled action and release when applied on the nanofibrous mats.

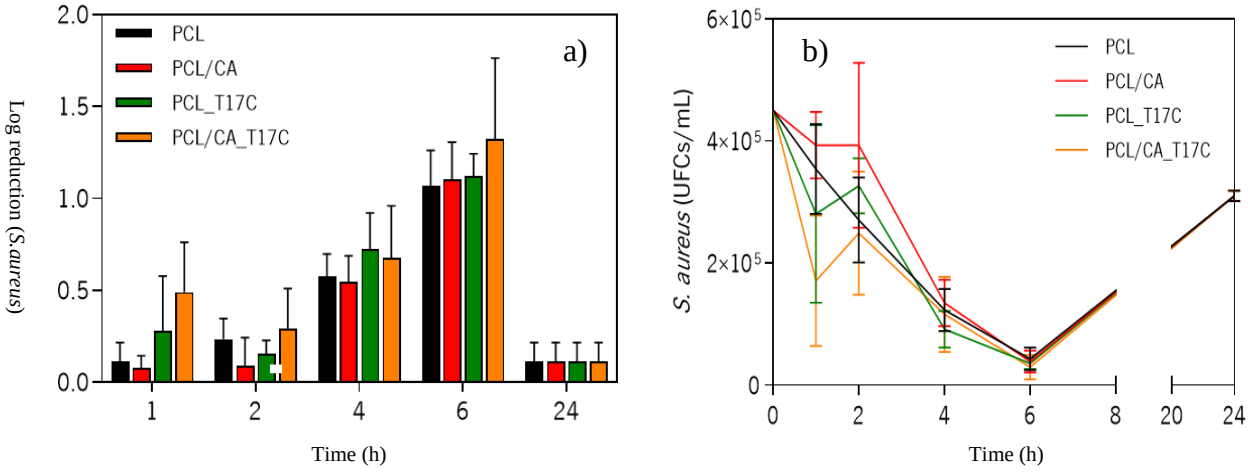

Figure 4. a) Evolution of the number of S. aureus bacteria over time (up to 24h of incubation), in contact with Tiger 17C (T17C). The positive control for this bacterium reached ≈ $8.1x10^5$ colony forming unit (CFU)/mL. b) Reduction of the S. aureus log in relation to time 0h.

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