https://doi.org/10.17952/37EPS.2024.P1144

Development of a HPMC-patch containing RGD and KTTKS peptide sequences for application in tissue healing

Naiara Maia Pereira, Reinaldo Marchetto

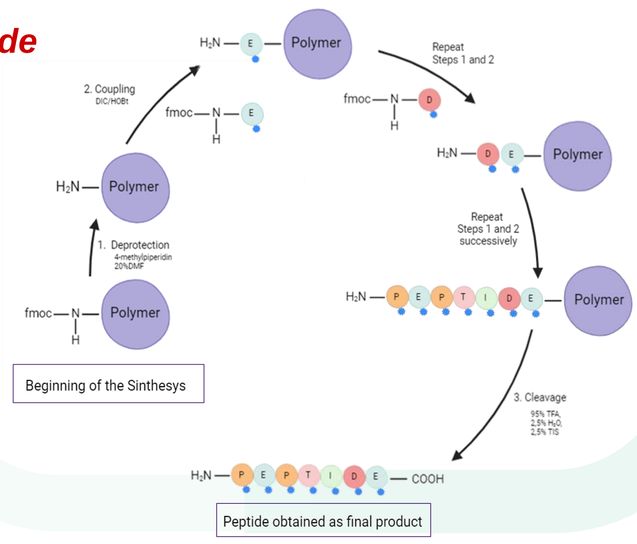
UNESP – Institute of Chemistry – Department of Biochemistry and Organic Chemistry, Araraquara, São Paulo, Brazil

INTRODUCTION

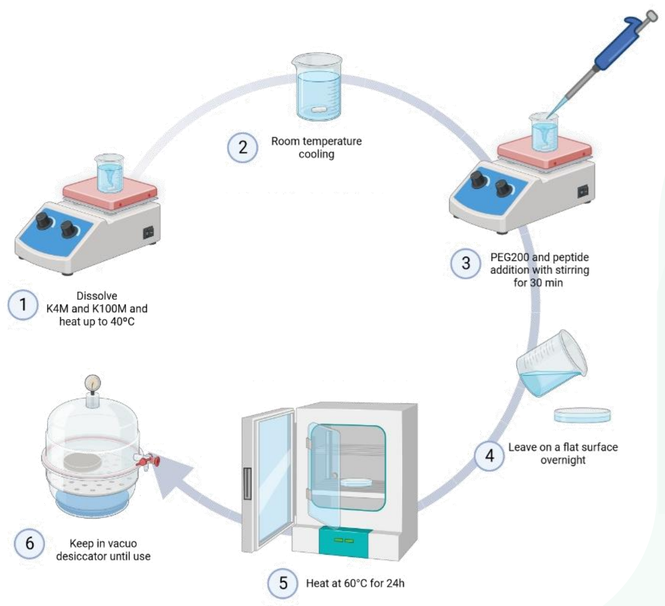
The tissue healing is a complex process involving biological and molecular cascades events including hemostasis, inflammation, cell proliferation and remodeling phases. Studies indicates that RGD and KTTKS peptides are able to stimulate the Extracellular Matrix to increase an and sustains these events, due to the ability of the RGD sequence to stimulate cell adhesion in fibronectins [1] and KTTKS sequence to trigger cellular processes to induce Extracellular Matrix synthesis, collagen and fibronectin expression [2]. Therefore, peptide based on these sequences can be used in compounds meant for tissue recovery and healing, such as transdermal films. Thus, the peptide named NMC was designed, synthesized and loaded in patches prepared using different hydroxypropyl methylcellulose (HPMC) types and concentrations. The peptide was incorporated during the gel preparation process. The patches were cut as disks of 5 mm diameter and characterized by scanning electron microscopy (SEM), x-ray diffractometry (XRD) and Fourier transform infrared spectroscopy (FT-IR). The cytotoxic effect was investigated by MTT assay in HaCaT cells (BCRJ 0341). The antimicrobial activity was evaluated by the minimum inhibitory concentration (MIC) assay in Staphylococcus aureus (ATCC 14458).

METHODOLOGY AND RESULTS

Peptide



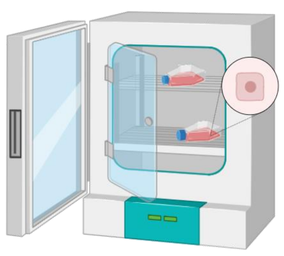
Film



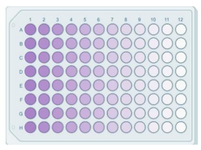
Films preparation

The solvent evaporation technique was used in the films preparation. Various formulations were tested using different types and concentrations of HPMC, combined with another polymers and solvents as previous described [3,4,5,6]. The best formulation (V30) was obtained by dissolving of HPMC K4M and K100M (3:1) at 40ºC, followed by cooling to RT. Then, PEG200 and the peptide (for V30P), when necessary, were added and stirred for more 30 minutes. The obtained gel was left on a flat surface overnight until air bubbles disappeared. Afterwards, the set was taken to an oven at 60ºC for 24 hours. The formed film was then left in a vacuo desiccator until use.

In vitro assays

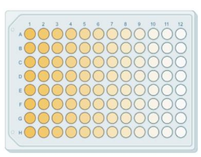


Cellular culture



Citotoxicity and proliferation assays

Bacterial cultivation and preparation of inoculum



Antimicrobial assay

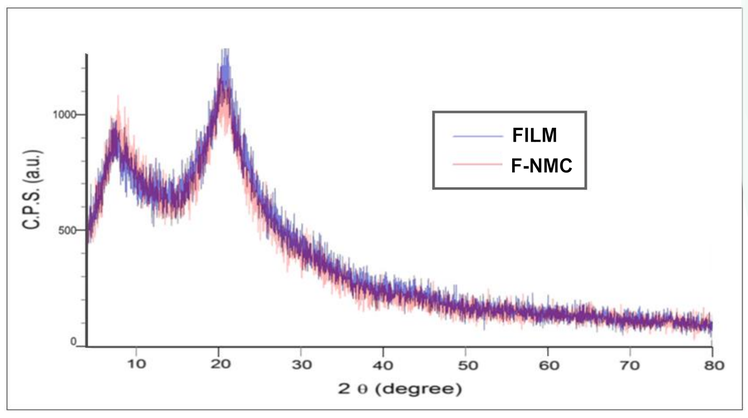
Synthesis, purification and characterization of peptide

The peptide NMC, WTGRGDSP-Ahx-KTTKS, was synthesized by solid-phase methodology, using Fmoc/tBu Chemistry with a RinK amide MBHA resin (0.55 mmol.g^{-1}) and DIC/HOBt activation. After deprotection and cleavage with TFA/water/EDT/TIS (94.5:2.5:2.5:0.5) for 2h at RT, the peptide was precipitated, solubilized and dried under vacuum. Analysis and purification was performed by RP-HPLC and the identity confirmed by ESI-MS.

Material Characterization

SEM images were obtained with a JSM-7500 scanning electron microscope. The samples were sputter coated with a 6 nm thick carbon layer. The morphology was observed at an accelerating voltage of 2 kV. XRD patterns were performed on a SmartLab SE Rigaku Diffractometer with a Kβ filter and Cu Kα radiation from 4° to 80°.
FTIR spectra were obtained with dried samples on a Bruker Hyperion 2000 Fourier transform infrared spectrophotometer, with ATR detector.

X Ray Difraction



The pure HPMC film had broad diffraction peaks at around 7.72º and 20.48º attributable to the (101) and (002) crystalline planes and indicates its semi crystalline polymer nature (FILM). No significative variation was showed after peptide incorporation (F-NMC).

Cytotoxicity and proliferation assays

Cytotoxicity analysis was performed using the MTT colorimetric method for 72 h, using HaCaT cells, immortalized human keratinocytes (BCRJ 0341). To perform the MTT assay, the films were removed, the supernatants were discarded and 100 µl of sterile PBS buffer was added to the wells to remove dead cells and other metabolic residues present. Next, 180 µL of DMEM and PBS (1:4) solution were added, followed by the addition of 20 µL of MTT solution at 1.0 mg.mL^{-1}. The plates were kept in incubation for 4 hours at 37°C and 5% CO_2. Finally, the supernatants were discarded and 100 µL of a mixture 1:1 of isopropyl alcohol and dimethyl sulfoxide was added to each well to dissolve the formed crystals, keeping the set protected from light and under agitation for 1 hour. The absorbance was read using a microplate reader spectrophotometer at 540 nm.

Antimicrobial activity

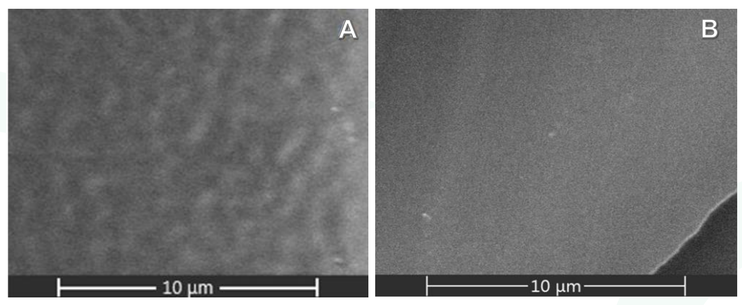
Antimicrobial activity was performed using the plate microdilution method with modifications described by the Clinical and Laboratory Standards Institute (CLSI – M7-A10, 2015 and M27-A3, 2017). After initial growth, a cellular suspension was adjusted to McFarland scale 0.5 (10^8 CFU/mL) and diluted to 10^7 CFU/mL. For peptide analysis, 80 µL of media and 100 µL of peptide solution were added to each well, followed by 20 µL of microbial suspension, and incubated for 24 hours at 37°C. Similarly, 5mm discs were used in place of peptide solution under the same conditions. Positive controls were prepared similarly, using microorganisms and media as growth controls. Negative controls included media-only for peptide plates and media with a pure film disc for film analysis. After the incubation period, bacterial growth was assessed by measuring the optical density at 595 nm using a microplate reader spectrophotometer.

Peptide Structure and Characterization

WTG**RGD**SP-Ahx-**KTTKS-**NH_2

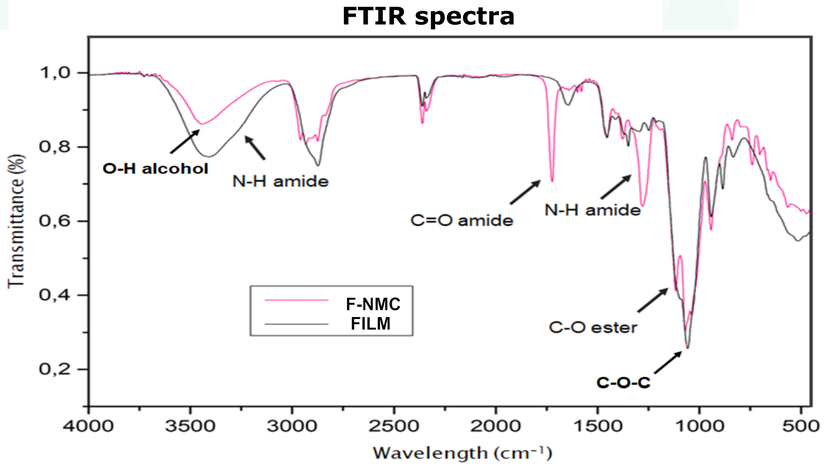
Calculated MW (g.mol^{-1})	ES+	ES m/z (g.mol^{-1})	Obtained MW (g.mol^{-1})
1.533,68	+1	1535,69	1534,67
	+2	767,90	
	+3	512,33	
	+4	384,80	

Scanning Electron Microscopy

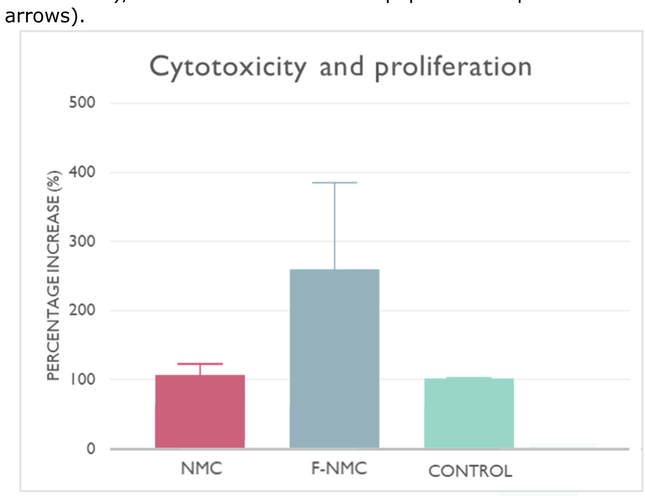


SEM images shows that the films exhibited smooth and homogeneous morphology with no sign of phase separation, which indicated the complete miscibility between HPMC and PEG200 (A). In addition, the film blended with peptide displayed smoother and more homogeneous topography (B).

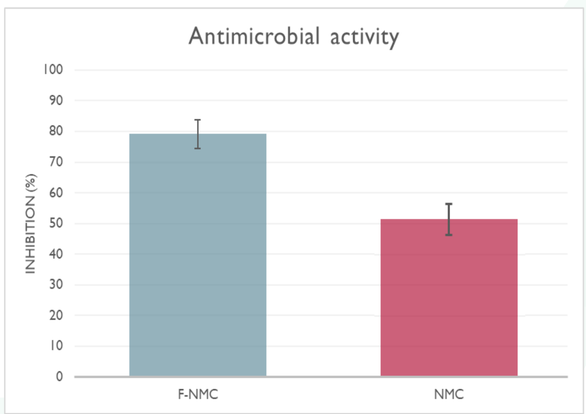
FTIR spectra



Characteristic vibrational frequencies assigned to HPMC and PEG200 were observed at 3500–3200 cm^{-1} (OH stretching), 2909 cm^{-1} (CH stretching of CH_2 and CH_3 groups), 1645 cm^{-1} (water OH bending), 1425 cm^{-1} (CH_2 symmetric bending), 1370 cm^{-1} (CH bending), 1160 cm^{-1} (antisymmetric bridge C–O–C stretching). Spectroscopy analysis showed bands assigned to amide I (3300-3180 cm^{-1}), C=O amide (1700 cm^{-1}) and amide II (~1300 cm^{-1}), which confirms the peptide incorporation on the films (indicated by arrows).



Each film sample was loaded with peptide in order to maintain equivalent amount to the free peptide assayed (10 µmol.L^{-1}). The MTT assay showed that peptide-loaded film (F-NMC) had low cytotoxicity and higher proliferation capacity of queratinocytes compared to the free peptide (NMC).



The Antimicrobial assay showed that F-NMC was more effective than the NMC to promote the control of the bacterial load against S. aureus with an inhibition rate of 79,18%±6,95 and 51,43%±5,33, respectively, for the same concentration.

CONCLUSIONS

The peptide-loaded film were successfully obtained using a modified peptide from natural human fibronectin containing the RGD and KTTKS sequences. The addition of the peptide to didn't show significantly changes on film chemical or physical properties but increased positively the cytotoxicity, proliferation and antimicrobial activity in comparison to the behavior of the free peptide. In general, the peptide-loaded film is very promising for use in biomedical applications, especially in cutaneous tissue healing as a transdermal patch.

Support

CAPES · CNPq Conselho Nacional de Desenvolvimento Científico e Tecnológico · FAPESP

REFERENCES

[1] HERSEL, U.; DAHMEN, C.; KESSLER, H. *Biomaterials*, 24(24), p. 4385-4415, 2003

[2] ABU SAMAH, N.H.; HEARD, C.M. *Int. J. Cosmetic Sci*., 33(6), p. 483-490, 2011.

[3] KHAN, S.; et al.; *AAPS PharmSciTech.,* 16(4), 800–810, 2015.

[4] KATHE, K.; HATHPALIA, H. *Asian J. Pharm. Sci*., 12(6), 487–97, 2017.

[5] PATEL D.P.; et al.; *AAPS PharmSciTech.*, 10(2), 437–42, 2009.

[6] VIJAYA, R.; et al. *Int. J. Pharm. Chem. Biol. Sci*., 5(3), 548–56, 2015.