International Journal of Biological Macromolecules 279 (2024) 135187

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Mangostanin hyaluronic acid hydrogel as an effective biocompatible alternative to chlorhexidine

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ARTICLE INFO

Keywords:
Mangostanin
9-hydroxycalabaxanthone
Chlorhexidine
3D engineered gingival tissue
Antibacterial oral gel

ABSTRACT

Periodontal disease (PD) prevention and treatment products typically demonstrate excellent antibacterial activity, but recent studies have raised concerns about their toxicity on oral tissues. Therefore, finding a biocompatible alternative that retains antimicrobial properties is imperative. In this study, a chemically modified hyaluronic acid (HA) hydrogel containing mangostanin (MGTN) was developed. Native HA was chemically modified, incorporating amino and aldehyde groups in different batches of HA, allowing spontaneous cross-linking and gelation when combined at room temperature. MGTN at different concentrations was incorporated before gelation. The structure, swelling characteristics MGTN release, rheological parameters, and in vitro degradation performance of the loaded hydrogel were first evaluated in the study. Then, antimicrobial properties were tested on *Porphyromonas gingivalis* and its biocompatibility in 3D-engineered human gingiva. HA hydrogel was very stable and showed a sustained release for MGTN for at least 7 days. MGTN-loaded HA hydrogel showed equivalent antimicrobial activity compared to a commercial gel of HA containing 0.2 % chlorhexidine (CHX). In contrast, while MGTN HA hydrogel was biocompatible, CHX gel showed high cytotoxicity, causing cell death and tissue damage. Modified HA hydrogel allows controlled release of MGTN, resulting in a highly biocompatible hydrogel with antibacterial properties. This hydrogel is a suitable alternative therapy to prevent and treat PD.

1. Introduction

Periodontal disease (PD) is a chronic inflammatory disease characterised by the destruction of gingival connective tissue and the resorption of alveolar bone, resulting in eventual tooth loss [1]. This disease is initiated by certain periodontopathogenic bacteria in the subgingival plaque, such as *Porphyromonas gingivalis (P. gingivalis)* [2]. These bacteria provoke a host immune response, releasing cytokines and inflammatory mediators in the periodontal tissues, which contributes to periodontal breakdown [3].

Currently, PD is treated using mechanical and/or chemical therapies. Mechanical treatments like scaling and root planning remove plaque, calculi, and necrotic tissue [4]. However, it often does not produce effective therapeutic results, requiring complementary therapies such as antimicrobial agents [5]. Despite that, they do not entirely regenerate periodontal tissue [3].

Gels and mouthwashes containing antiseptics are the most marketed treatment options for subgingival irrigation in order to control microbial biofilms in PD [6]. Chlorhexidine (CHX) is one of the most commonly used antiseptics due to its exceptional antimicrobial properties. However, several studies have raised concerns regarding cytotoxicity associated with commercial periodontal gels containing CHX or other antibacterial agents [7]. Additionally, prolonged use of CHX has been associated with adverse effects, including oral mucosa ulceration or erosion and increased levels of the potentially carcinogenic compound p-chloroaniline in saliva [8,9].

Alternatively, various antibiotics have been used to treat PD, but they are restricted and used only in cases of severe periodontitis. In the case of patients with PD being prescribed antibiotics, antimicrobial susceptibility testing patterns should be encouraged for a more targeted

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https://doi.org/10.1016/j.ijbiomac.2024.135187
Received 27 September 2023; Received in revised form 16 August 2024; Accepted 28 August 2024
Available online 29 August 2024
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approach to prevent bacterial resistance due to inappropriate systemic use [10]. It starkly contrasts with the current level of investment in developing novel antimicrobials to treat infections resulting from the global spread of multidrug-resistant bacterial pathogens, especially in the field of natural-product-derived and synthetic small molecules [10,11].

In traditional medicine, natural products isolated from plants are considered good alternatives to synthetic chemicals like antibiotics and antiseptics [10,11]. *Garcinia mangostana Linn,* commonly known as mangosteen,is a fruit tree widely cultivated in Southeast Asia [12]. Its pericarp has been used in traditional medicine for treating a wide range of diseases due to the presence of abundant quantities of xanthones, a class of polyphenolic compounds with a wide range of biological properties [13–18]. Experimental studies has shown that xanthones possess antioxidant, anti-inflammatory, anti-allergic, antibacterial, antitumor, antifungal, antimalarial, cytotoxic, and HIV-1 inhibitory effects [13,14,17–20].

Based on all the effects of xanthones, we focused on mangostanin (MGTN) [21], also known as 9-hydroxycalabaxanthone (Fig. 1), a less studied xanthone with demonstrated cytotoxicity against cancer cells and with antioxidant, antibacterial and antimalarial activities [13,19,22–24]. Structurally, the important parts responsible for the antibacterial activity of the MGTN have been reported to be the H-10, 9-OH, and C-7 prenyl group of ring A, together with the dimethylchromene ring attaching to ring B (Fig. 1) [25].

Among various biopolymers, hyaluronic acid (HA) has been extensively studied for periodontal regeneration [26]. HA, a major component of the extracellular matrix (ECM) in all connective tissues, has diverse biological functions. These include direct receptor-mediated effects on cell adhesion, growth, and migration, and it acts as a signaling molecule in processes such as cell death, inflammation, and wound healing [27–32]. Therefore, HA-based biomaterials combined with MGTN could be a promising option for oral antibacterial and regenerative treatments. However, most gel formulations using unmodified HA exhibit poor mechanical properties and rapid degradation, making a controlled release unviable. To overcome these drawbacks, HA chains can be crosslinked by directly adding a cross-linker to form a three-dimensional network or by pre-modifying the HA chains with functional groups amenable to crosslinking. [33]. The latter approach generates active moieties that add new functionalities to the hydrogel [34]. Among the various possible derivatives, in this study we chose to carry out a chemical crosslinking using HA derivatives. Specifically, a derivative with amino groups was synthesised by incorporating hydrazide (ADH) into the carboxylic acid groups and a derivative with aldehyde groups was synthesised by oxidation of HA with sodium periodate

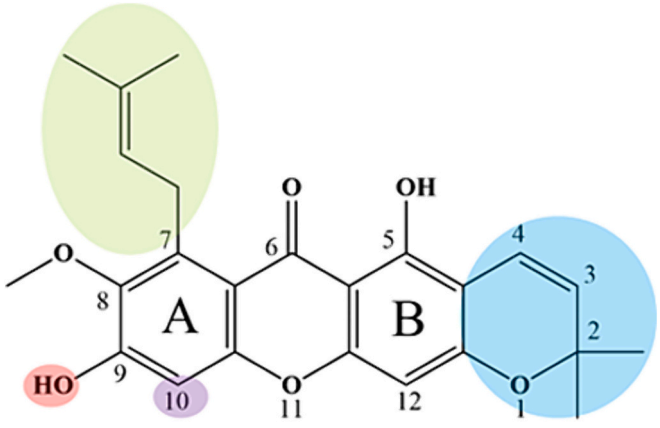

Fig. 1. Structure of MGTN (9-Hydroxycalabaxanthone). The groups responsible for the antibacterial activity appear shadowed, H-10 (coloured in purple), 9-OH (coloured in red), C-7 prenyl group of ring A (coloured in green) and dimethylchromene ring attached to ring B (coloured in blue).

[35]. Thus, the reactivity between the amino groups of the hydrazide and the aldehyde groups via the Schiff reaction allows spontaneous gel formation, generating stable 3D gel matrices. This HA-ADH/HA-CHO crosslinking system also offers other advantages, such as being non-toxic and metabolisable by the human body [36]. Additionally, the gel precursor solutions can be applied as a liquid to form a gel in situ, allowing for the easy incorporation of a molecule such as MGTN, provided it can be dissolved or suspended in aqueous or water-miscible solutions.

Therefore, here we aimed to develop a chemically modified HA hydrogel combined with MGTN to target *P. gingivalis* and to evaluate its biocompatibility using a 3D cell culture model of human tissue equivalents of gingiva under inflammatory conditions.

2. Materials and methods

2.1. Biomolecule of MGTN

MGTN IUPAC name 5,9-dihydroxy-8-methoxy-2,2-dimethyl-7-(3-methylbut-2-enyl) pyrano[3,2-b] xanthen-6-one (9-hydroxycalabaxanthone) (BioCrick Biotech, Sichuan, PRC and Cymit Química S.L., Barcelona, Spain) was used. It was stored at a concentration of 2 % in dimethyl sulfoxide (DMSO)(Sigma-Aldrich, St. Louis, MO, USA) and aliquoted at −70 °C and diluted with H_2O milliQ to obtain the desired concentration and eliminate the DMSO toxicity risk. This biomolecule was originally isolated from the petrol extract of the fruit hulls of *Garcinia mangostana Linn (family Clusiaceae)* [21].

2.2. Synthesis and characterisation of HA hydrogel and loading with MGTN

2.2.1. Synthesis of derivative HA-amino (HA-ADH)

In a typical reaction 500 mg (1.32 mmol; Mw = 1.2×10^6 g/mol) of HA (F002103, Bioibérica, Barcelona, Spain) were dissolved in H_2O milliQ at a concentration of 3 mg/mL at room temperature. Then, a 30-fold molar excess of Adipic acid dihydrazide (ADH) (97 %) (6887.9 mg; 39.54 mmol) (Sigma-Aldrich) was added, and when it was completely dissolved, the pH of the reaction mixture was adjusted to 6.8 with [sodium hydroxide (NaOH)] = 0.1 M and [hydrochloric acid (HCl)] = 0.1 M solutions. Next, 1012.2 mg (5.28 mmol) of EDC (Sigma-Aldrich)/ 735.5 mg (5.28 mmol) of 1-Hydroxybenzotriazole hydrate (HOBt) (Sigma-Aldrich) dissolved in 5 mL of DMSO:H_2O 1:1 were added to the solution. The reaction was carried out overnight in the dark at room temperature at a pH of 6.8. After the time reaction, the pH was adjusted to 7.0, and the product was dialysed at 37 °C for 7 days. During dialysis, the H_2O milliQ was changed twice daily. Then, Sodium chloride (NaCl) (Sigma-Aldrich) was added to the solution until reaching 5 % (*W/V*) and modified HA was precipitated using three equivalent volumes of ethanol. Then, the precipitate was recovered, re-dissolved in H_2O milliQ at a concentration of 5 mg/mL, and lyophilised for 7 days. Finally, HA-ADH was stored at −80 °C under a N_2 atmosphere. The degree of substitution by ADH was assessed by proton nuclear magnetic resonance (^{1}H NMR) (Bruker Avance 300).

2.2.2. Synthesis of derivative HA-aldehyde (HA-CHO)

In a typical reaction 1000 mg (2.64 mmol; Mw = 1.2×10^6 g/mol) of HA (F002103, Bioibérica) was dissolved in H_2O milliQ in a concentration of 3 mg/mL at room temperature. Then, 21 mL of a solution with 563.9 mg (2.64 mmol) of Sodium periodate (NaIO4) (99.8 %) (Sigma-Aldrich) in milliQ H_2O was added dropwise. The reaction was carried out for 2 h at room temperature in the dark. Then, 671 μL (12 mmol) of ethylene glycol (Sigma-Aldrich) was added to inactivat any unreacted periodate. The solution was dialysed for 7 days and lyophilised for another week. During dialysis, the H2O milliQ was changed twice daily. HA-CHO was stored at −70 °C under a N_2 atmosphere. The degree of substitution by CHO was assessed by ^{1}H NMR spectroscopy (Bruker

Avance 300).

2.2.3. Synthesis of HA hydrogels with MGTN

Two solutions of [MGTN] = 0.025 % and 0.05 % were prepared in H_2O milliQ. Then, HA-ADH and HA-CHO were dissolved separately in those solutions at a concentration of 7 mg/mL. Finally, the hydrogels with MGTN were formed by mixing solutions 1:1.

2.2.4. Release studies of MGTN from HA hydrogels

50 μL of HA hydrogel containing 0.05 % of MGTN was incubated in 950 μL of distilled water at 37 °C for 7 days. At prefixed time points (0.4, 1, 2, 3, 6, and 7 days), samples were centrifuged at 16.000 ×*g* for 15 min. The supernatants were collected and analysed by UV–vis spectrophotometer (PowerWave Ht) at a wavelength of 338 nm to determine the amount MGTN released. The experiment was performed in triplicate, with two replicates at each condition ($n = 6$).

2.2.5. Equilibrium swelling ratio determination and scanning electron microscopy (SEM)

The equilibrium swelling ratio (ESR) was evaluated as previously described [37]. Briefly, the ESR of the hydrogels was evaluated as follows: 1 mL of the hydrogels wereincubated in distilled water at 37C. At predetermined time points (0.5, 1, 3 and 24 h), each sample was centrifuged at 16.000 x*g* for 15 min. The supernatants were discarded, and the wet gels were immediately weighed (Ws). The hydrogels were then frozen at —80C and lyophilised during at least 72 h. The products were reweighed (Wd) to determine ESR values according to the formula showed below. Assays were performed in triplicate for each condition ($n = 3$). The swelling ratios of the resulting gels were determined using the following equation: ESR = (Ws - Wd)/Wd.

Qualitative determination of the hydrogel structure was carried out by SEM (SEM, Hitachi S-3400 N, Hitachi High-Technologies Europe GmbH, Krefeld, Germany) to evaluate the changes in the hydrogel network and the effects of MGTN on the structure of the network (pore size and pore distribution). The hydrogels were frozen at −80 °C and freeze-dried for 72 h. Samples were then frozen in liquid N_2 to allow an accurate transversal section using a sharp scalpel and observed at 15 kV, 40 Pa, and 1009 magnification.

2.2.6. Rheological measurements

Rheological measurements of the HA hydrogels were conducted using an MCR 702e Multidrive modular compact rheometer (Anton Paar, Germany), equipped with parallel plate geometry, a 25 mm plate diameter, 1.0 mm gap, and Peltier temperature control. Measurements were conducted at 37 °C. Amplitude sweep tests were carried out at 37 °C and a frequency of 1.59 Hz over a strain range of 0.01–100 %. Storage modulus (G') and loss modulus (G''), within the linear viscoelastic range (LVR) were determined. The assays were carried out in triplicate at each condition ($n = 3$).

2.2.7. In vitro degradation test of HA hydrogels

An in vitro degradation test of HA hydrogels and HA 0.05 % MGTN was performed by placing 100 μL of each hydrogel sample into 2 mL vials. Then, 1.45 mL of PBS was added to each vial. Subsequently, 0.45 mL of PBS containing 100 U/mL of hyaluronidase (HAse) from sheep testes (Sigma-Aldrich) was added to each solution, which was then incubated at 37 °C for the indicated times (0–48 h). At predetermined sampling times, 200 μL of supernatant was collected from each solution, and 200 μL of fresh PBS was added back to each solution to maintain the total volume. The collected supernatant samples were placed in a boiling water bath (100 °C) for 3 min to stop the enzymatic activity. The degradation of HA hydrogels was analysed by measuring the amount of HA degradation products in the supernatant released from the HA hydrogel samples using the carbazole assay [38]. The assays were carried out in triplicate at each condition ($n = 3$).

2.3. Experimental groups used in the in vitro assays

The present study used two different concentrations of modified HA containing MGTN (0.025 % and 0.05 %). Also, two different controls were added, modified HA without biomolecule (HA) and commercial Periokin Hyaluronic 1 % gel (Laboratorios Kin, Barcelona, Spain) that contains CHX at 0.2 % (HA 0.2 % CHX) with unmodified HA, which served as the commercial gold standard. For the studies on bacteria, gels were used at 5 % (*v*/v) concentration, as described in Section 2.4. For the study on 3D-engineered human gingival tissue equivalents, gels were placed directly onto the tissues as described in Section 2.5.

2.4. Testing of antimicrobial activity of the different HA gels on *P. gingivalis*

2.4.1. Culture and proliferation assay of *P. gingivalis*

As previously described [7], the strain of *P. gingivalis* (ATCC 33277TM, Manassas, VA, USA) was cultured on Brain Heart Infusion (BHI) (Scharlab, Barcelona, Spain) medium under anaerobic conditions (10 % H_2, 10 % CO_2, and 80 % N_2) for 24-72 h at 37 °C using an Oxoid Anaerogen™ sachet (Thermo Fisher Scientific, Waltham, MA, USA) . After overnight incubation, 1 mL of bacterial suspensions ($\approx 3 \cdot 10^8$ bacteria/mL) were incubated under anaerobic conditions for 10 h with the different HA gels.

Bacterial viability was determined using the LIVE/DEAD BacLight bacterial viability kit (L7012, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions using the fluorescence spectroscopy protocol. Three independent experiments were conducted with two replicates per condition ($n = 6$).

2.4.2. Gingipain activity of *P. gingivalis*

P. gingivalis secretes gingipains, which are cysteine proteases that play a key role in tissue destruction and are related to its virulence [39]. As described previously [7], to determine the Arg-gingipain proteolytic activity, 10 μL of bacterial suspension with the different treatments, after 10 h of incubation, were mixed with 50 μL of assay buffer (10 mM cysteine-HCl, 1 M HEPES (Biowest), pH 7.5) in microtiter plates. Next, 100 μL of substrate solution (0.5 mM benzoyl-arginine p-nitroanilide (Sigma-Aldrich), 10 mM cysteine-HCl, 50 mM Tris-HCl, pH 7.5) were added to each well. Following a 16-h incubation at 37 °C, the absorbance at 405 nm was measured using a spectrophotometer (PowerWave HT). A total of three independent experiments were performed, with two replicates per condition ($n = 6$).

2.5. Testing of HA gels on a 3D model of gingival tissue equivalents (GTE)

2.5.1. Cell culture

[7] Immortalized Human Gingival Fibroblasts-hTERT (iHGF) (Applied Biological Materials Inc., Richmond, BC, Canada) were grown at 37 °C in an atmosphere of 5 % CO_2 using fibroblast medium that consists in Dulbecco's modified Eagle's medium (DMEM) low glucose (Biowest)/Ham's F12 (3/1) (Biowest), supplemented with 10 % (*v*/v) fetal bovin serum embryonic stem cells tested (FBS) (Biowest), 100 μg/mL penicillin and 100 μg/mL streptomycin (Biowest).

Immortalized Human Gingival Keratinocytes (iHGK)(Gie-No3B11, abbreviated as iHGK) (Applied Biological Materials Inc) were grown on tissue culture flask for sensitive adherent cells (Sarstedt, Numbrecht, Germany) at 37 °C in an atmosphere of 5 % CO_2 using keratinocyte medium that consist in DMEM without magnesium and calcium (Gibco, Grand Island, NY, US)/Ham's F12 (3/1) (Biowest), supplemented with 0.01 mg/mL of insulin (Sigma-Aldrich), 0.4 ng/mL of hydrocortisone (Sigma-Aldrich), 6.7 ng/mL of selenium (Sigma-Aldrich), 0.01 μg/mL of human epithelial growth factor (ThermoFisher Scientific), 1 M HEPES-buffer (Biowest), 5.5 μg/mL of transferrin (Sigma-Aldrich), 10^{-10} M of cholera toxin (Sigma-Aldrich), 2 mM of L-glutamine (Sigma-Aldrich), 5 % (*v*/v) of FBS embryonic stem cells tested (Biowest) and 100 μg/mL

penicillin and 100 μg/mL streptomycin (Biowest).

The GTE was constructed using cultures of iHGK and iHGF with 70–80 % confluency, as described in the next section.

2.5.2. Engineering 3D model of GTE

The GTE was created using Dongari Bagtzoglou and Kashleva's technique [40] with some modifications published in a previous paper [7]. In short, a rat tail type I collagen solution (ThermoFisher Scientific) (2.2 mg/mL) was mixed with iHGF (1×10^5 cells/well) and pipetted into a 24-well transwell insert (Sarstedt) with 0.4 μm pores. The fibroblast-embedded collagen was cultured at 37 °C, 5 % CO_2 for 7 days submerged in fibroblast medium. iHGK (2.5×10^5 cells/well) were then seeded on top and GTE were cultured submerged in keratinocyte medium for 3 days at 37 °C, 5 % CO_2. GTE were then lifted to the air–liquid interface and incubated cultures at 37 °C, 5 % CO_2 for 15–17 days in airlift culture medium (AL) consisting of DMEM low glucose (Biowest)/Ham's F12 (3/1) (Biowest), supplemented with 5 μg/mL of insulin (Sigma-Aldrich), 0.4 μg/mL of hydrocortisone (Sigma-Aldrich), 2×10^{-11} M of 3,3′, 5-triiodo-L-thyronine (T3) (Sigma-Aldrich), 1.8×10^{-4} M of adenine (Sigma-Aldrich), 5 μg/mL of transferrin (Sigma-Aldrich), 10^{-10} M of cholera toxin (Sigma-Aldrich, St. Louis, MO, USA), 2 mM of L-glutamine (Sigma-Aldrich), 5 % (*v*/v) of FBS embryonic stem cells tested (Biowest) and 100 μg/mL of penicillin and 100 μg/mL of streptomycin (Biowest). The AL medium was refreshed every other day. After 25 days GTEs were treated as explained in Section 2.5.3.

2.5.3. GTE treatment with different gels

Tissues were treated on top with 50 μL of 1 μg / mL *P. gingivalis* Lipopolysaccharide (LPS) (InvivoGen, San Diego, CA, USA) for 24 h. Following that, 30 μL of different gels or PBS (Biowest, Nuaille, France) for the negative control (C-) and sodium dodecyl sulfate (SDS) at 5 % for the positive control were applied on top of the tissue for 48 h. Three independent experiments were performed, with two replicates at each condition ($n = 6$).

2.5.4. MTT test

Once the tissues had been incubated with the HA gels, they were rinsed with PBS before being placed on 400 μL of 0.5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)(Thermo Fisher Scientific), as previously described [7]. Each condition was replicated twice in three independent experiments ($n = 6$).

2.5.5. Prostaglandin E2 (PGE2) levels

PGE2 was detected from GTE culture media after 48 h of treatment using an enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen) according to the manufacturer's instructions. Three independent experiments were conducted, with two replicates per condition (n = 6).

2.5.6. Histology

Once the tissues were incubated with HA gels on top of the GTE for 48 h, they were rinsed with PBS. The GTE was then fixed in paraformaldehyde (Scharlab) at 4 % and then embedded in paraffin. Histological examination was performed on paraffin sections (6 μm) stained with hematoxylin and eosin (H&E) ($n = 3$). In order to acquire the images, Zeiss Axioskop 2 microscope (Carl Zeiss, S.A., Barcelona, Spain) was equipped with AxioCam ICC3 digital camera and AxioVision 40 V 4.6.3.0 Software.

2.6. Statistical analysis

The data are presented as mean values ± standard error of the mean (SEM). Shapiro-Wilk tests were used to determine whether normality tests assumed parametric or non-parametric distributions. Based on the normal distribution of the groups, Kruskal-Wallis or one-way ANOVA with Bonferroni as post-hoc or T-student were used to analyse the differences between groups. SPSS® program (SPSS Inc., Chicago, IL, USA) for Windows, version 17.0, and GraphPad Prism (La Jolla, CA, USA) (version 9.0.2) were used. Results were considered statistically significant at p-values <0.05.

3. Results

3.1. HA chemical modification

3.1.1. Synthesis of HA-ADH and HA-CHO derivatives

The HA was modified by adding ADH and periodate in two routes to provide amine and aldehyde groups to allow HA hydrogel crosslinking. The complete synthesis of HA derivatives is shown in Fig. 2A.

From the ^{1}H NMR of HA-ADH, the bars show the characteristic signals of the functional groups present in the HA before and after chemical modification with ADH [41] (Fig. 2B). HA is a polymer with very high molecular weight (Mw = 1.2×10^6 g/mol); therefore, a broad multiplet appears between approximately d = 3.0–3.9 ppm, (d - > δ), corresponding to the signal of the protons in the sugar rings. They are all superimposed, which makes it impossible to assign each proton individually. The signal at d = 1.89 ppm highlighted by the green bar (letter a) correspond to the *N*-acetyl methyl functional group and was used as an internal standard to calculate the degree modification (DM) of HA because this signal rarely is modified during the synthesis of HA derivatives [41]. In this case, the DM was 45 %, a good result considering the high molecular weight of our polymer. The signals at d = 1.53 ppm, d = 2.14 ppm, and d = 2.27 ppm, highlighted by the red, orange, and purple bars, respectively, correspond to the methylene groups of ADH and confirmed the successful chemical modification of HA by ADH.

From the ^{1}H NMR of HA-CHO, the green bar shows only the characteristic signal of the *N*-acetyl methyl functional group at d = 1.91 ppm. This signal remains unmodified during the chemical reaction with NaIO4. The difference between the HA before and after oxidation only shows some changes in the region of the broad multiplet between approximately d = 3.0–3.9 ppm, corresponding to the signal of the protons in the sugar rings (Fig. 2B). Therefore, the ^{1}H NMR did not clearly reveal the presence of aldehyde groups due to the hydrogen exchange with D_2O or a trace of water in it. Therefore, these changes or signals do not permit the calculation of the degree of oxidation.

Nevertheless, the small changes in the region of the protons for the sugar rings allowed to confirm a new chemical structure in HA. Furthermore, the viscosity of the reaction mixture visibly decreased over the course of the reaction, indicating the oxidation of HA and a slight loss of molecular weight, typical in these processes [42]. The combination of HA-ADH and HA-CHO resulted in the crosslinked hydrogel.

3.1.2. Results of characterisation HA combined with MGTN

The MGTN release profile from HA 0.05 % MGTN hydrogel is depicted in Fig. 3A. A controlled release of MGTN into the aqueous medium occurred, maintaining a moderately sustained release of 15 % of the hydrogel after 10 h and up to 7 days. The quantification of released MGTN shows that the concentration released and accumulated in one-week was 80 % of the HA 0.05 % MGTN hydrogel.

Quantitative ESR results of HA and HA 0.05 % MGTN hydrogels are shown in Fig. 3B. No differences in ERS were observed when comparing HA hydrogel at the different time points tested. However, when we compared the HA 0.05 % MGTN hydrogel at different times, there was a significant decrease in ESR at 0.5 h, 1 h, and 3 h compared to 24 h. Moreover, when comparing differences in ESR capacity between both hydrogels, the ESR capacity was significantly higher in HA hydrogel compared to HA 0.05 % MGTN hydrogel at 0.5 h, 1 h, and 3 h, until equilibrium was reached after 24 h in both samples.

The images of the hydrogels after lyophilization for 72 h (Fig. 3C) and SEM images (Fig. 3D and E) show that the HA 0.05 % MGTN hydrogel had a more reticulated and compact structure. In addition, HA 0.05 % MGTN hydrogel also showed a smaller and more compacted pore size.

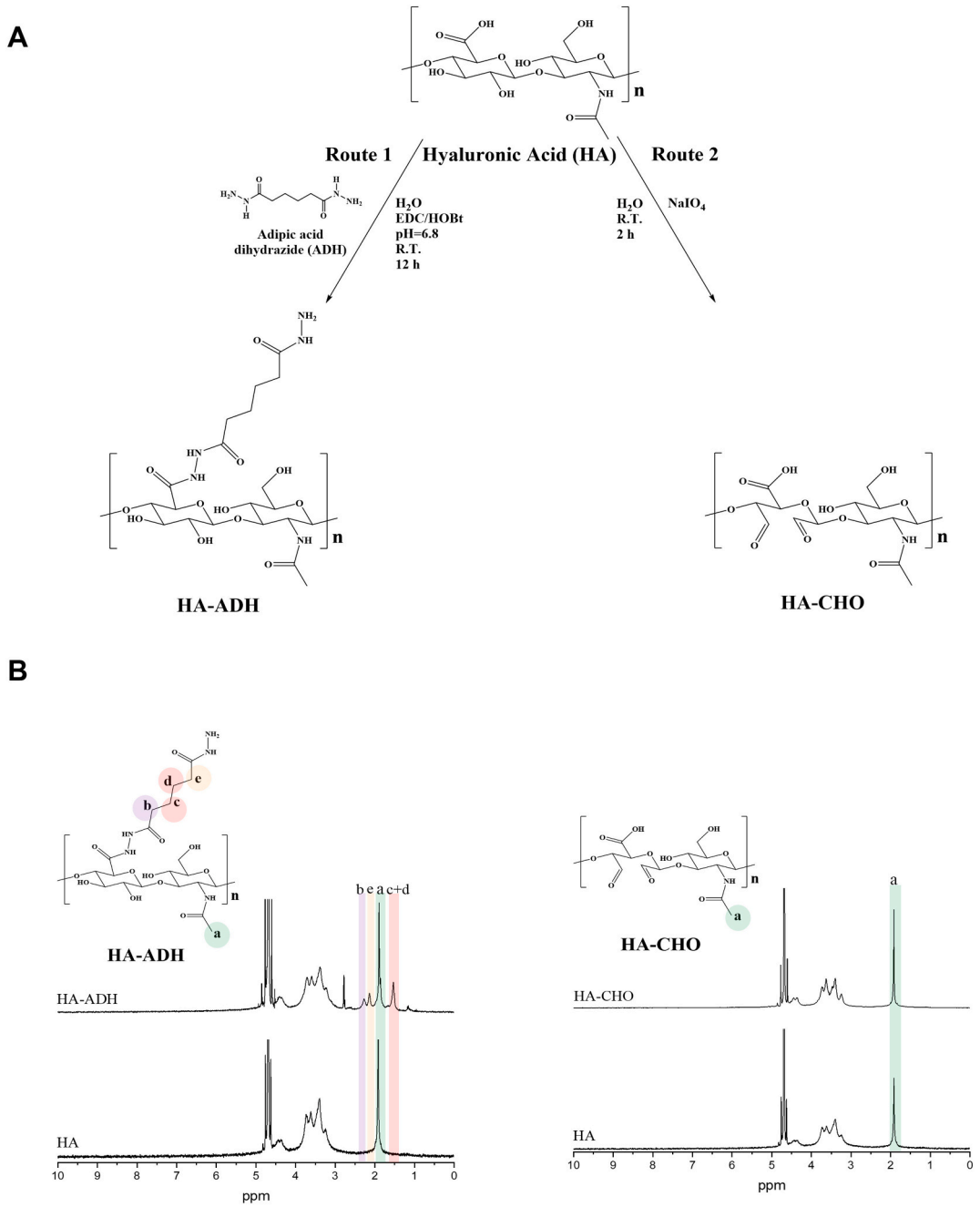

Fig. 2. HA-ADH and HA-CHO derivatives synthesis and characterisation. (A) Route 1: HA-ADH synthesised by ADH addition to HA at room temperature, in H_2O, pH = 6.8 and protected from light. Route 2: HA-CHO synthesised by oxidation of HA by NaIO4 at room temperature, in H_2O, and protected from light. (B) Route 1: ^1H NMR characterisation of HA and HA-ADH derivative to confirm the modification of HA. The assignation of signals was as follows: the signal at d = 1.53 ppm was assigned to the methylene group (coloured in red, letters c + d), the signal at d = 1.89 ppm was assigned to the *N*-acetyl methyl group (coloured in green, letter a), the signal at d = 2.14 ppm was assigned to methylene group (coloured in orange, letter e) and signal at d = 2.27 ppm was assigned to methylene group (coloured in purple, letter b). Route 2: ^1H NMR characterisation of HA and HA-CHO derivative to confirm the modification of HA. In this case, the signal at d = 1.89 ppm was assigned to the *N*-acetyl methyl group (coloured in green, letter a). Chemical structures from ChemDraw software and graph spectrum processed with Origin software.

As indicated by the rheometric parameters in Fig. 3F and G, the HA 0.05 % MGTN hydrogel displayed a higher G' and G" than the HA hydrogel, indicating that the HA 0.05 % MGTN hydrogel possesses greater rigidity or capacity to store elastic energy during deformation and greater capacity to dissipate energy during deformation, typically associated with increased viscosity or damping ability. To mimic in vivo conditions, the degradation of HA hydrogels by Hase was examined (Fig. 3H). According to the carbazole assay of HA degradation products in the supernatant, no significant degradation was observed between the two hydrogels, the HA hydrogel and the HA 0.05 % MGTN hydrogel.

3.2. Testing of antimicrobial activity of the different HA gels on *P. gingivalis*

For testing the antimicrobial activity of newly formulated HA gel containing MGTN, this was compared to HA alone and to a commercial gold standard gel containing CHX. Fig. 2A shows that the HA 0.2 % CHX, HA 0.025 % MGTN, and HA 0.05 % MGTN hydrogels produced a

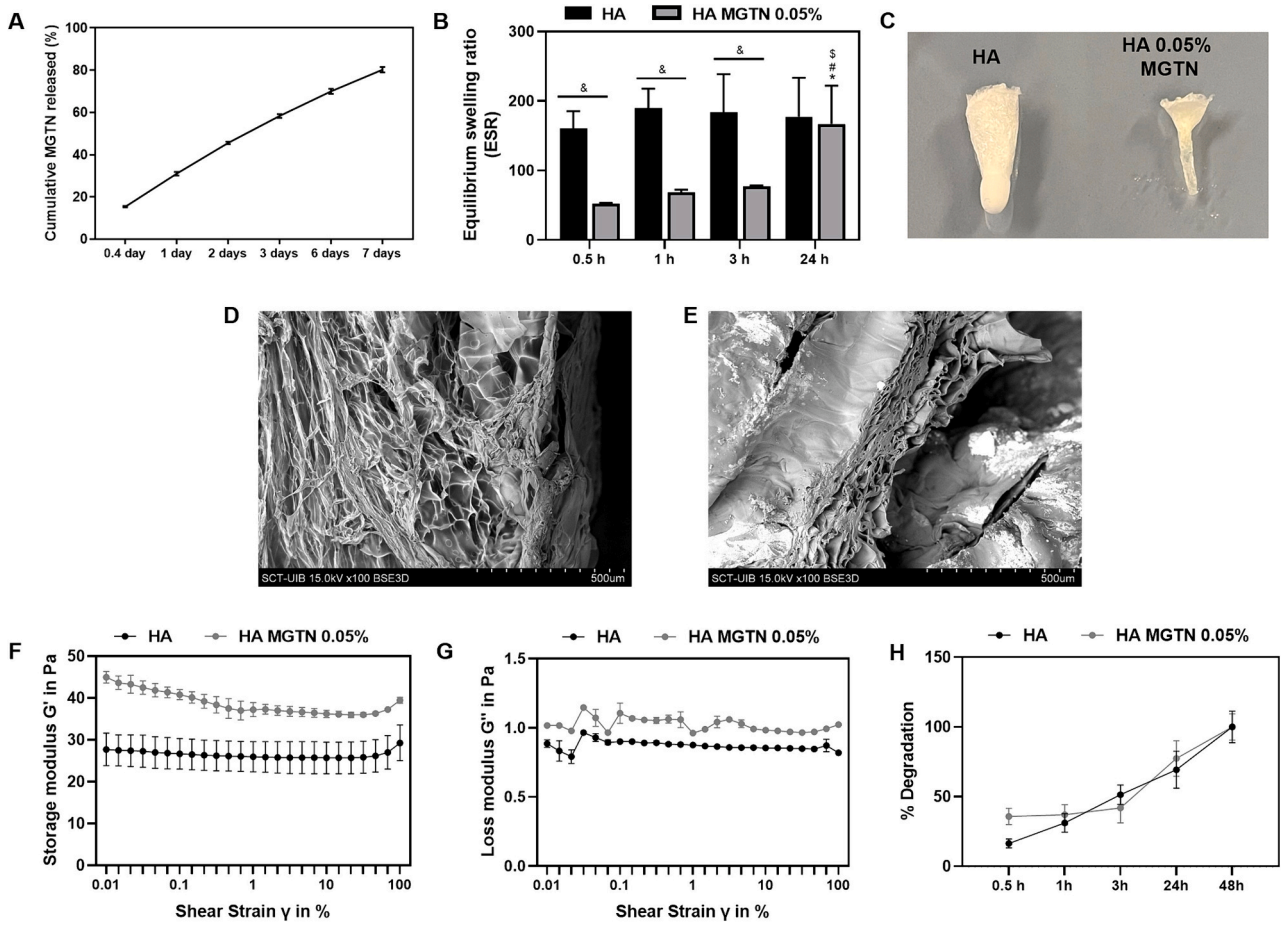

Fig. 3. Hydrogels characterisation. (A) Cumulative MGTN release in % from HA 0.05 % MGTN hydrogel at 0.4, 1, 2, 3, 6, and 7 days ($n = 6$). (B) ESR at 0.5, 1,3, and 24 h for HA hydrogel and HA 0.05 % MGTN hydrogel formulation ($n = 3$). (C) Images of hydrogels after freeze-drying for 72 h. (D, E) These images show the microscopic structure of HA (D) and HA 0.05 % MGTN (E) hydrogels. SEM observations were done at 15 kV, 40 Pa, and 9100 of magnification. Rheological parameters of HA hydrogel and HA 0.05 % hydrogel: (F) Storage modulus and (G) Loss modulus, measured at a frequency of 1.59 Hz at 37 °C (within the LVR). (H) In vitro degradation of HA hydrogel and HA 0.05 % hydrogel by hyaluronidase at a concentration of 100 U/mL. Values represent the mean ± SEM. Results were statistically compared by ANOVA using Bonferroni as a post-doc; *& $p < 0.05$ HA* vs. *HA 0.05 % MGTN; *p < 0.05 HA 0.05 % MGTN 24 h* vs. *HA 0.05 % MGTN 0.5 h; # p < 0.05 HA 0.05 % MGTN 24 h* vs. *HA 0.05 % MGTN 1 h; $ p < 0.05 HA 0.05 % MGTN 24 h* vs. *HA 0.05 % MGTN 3 h.*

significantly high inhibition of bacterial growth rate concerning the negative control, HA. Furthermore, HA 0.025 % MGTN hydrogel produced a significantly lower inhibition of bacterial growth with respect to the HA 0.2 % CHX and HA 0.05 % gels.

P. gingivalis Live/Dead ratio after 10 h with different hydrogels was analysed to test their effect on bacterial survival. Fig. 4B shows a significantly lower Live/Dead ratio on HA 0.2 % CHX, HA 0.025 % MGTN, and HA 0.05 % MGTN gels concerning HA, which indicates that they decreased bacterial survival. Moreover, HA 0.025 % MGTN hydrogel showed a significantly higher Live / Dead ratio than the HA 0.2 % CHX and HA 0.05 % MGTN gels.

After treatment with the different gels, we also evaluated the gingipain activity as the major virulence factor of *P. gingivalis* (Fig. 4C). All gels showed significantly lower gingipain activity compared to HA.

3.3. Testing of HA gels on a GTE

The GTE treated for 48 h showed that hydrogels containing MGTN (0.025 % and 0.05 %) presented very good biocompatibility, similar to HA hydrogel, and to untreated gingival control tissue (C-), as measured by an MTT assay (Fig. 5A). These results contrasted to the groups treated with SDS 5 % or with the commercial HA 0.2 % CHX gel, which presented significantly lower viability.

Histology was done to confirm the morphology and viability of GTE tissues, as shown in Fig. 5B. The images for HA, HA 0.025 % MGTN, and HA 0.05 % MGTN confirmed the good multilayer gingival structure, where the fibroblasts embedded in the collagen matrix showed a good morphology and a good layer of keratinocytes was present on the tissue surface. Images for HA 0.2 % CHX showed a smaller and destroyed layer of keratinocytes on the tissue surface compared with the other treatments, and the fibroblasts embedded in the collagen matrix show a round-shaped morphology, indicative of cell death.

In addition, the hydrogels containing MGTN (0.025 % and 0.05 %) presented significantly higher levels of PGE2 at 48 h of treatment compared to the negative control and the commercial HA 0.2 % CHX gel (Fig. 5C).

4. Discussion

In this study, we demonstrate the antimicrobial effect of a chemically modified HA periodontal hydrogel containing different concentrations of MGTN on *P. gingivalis* bacteria and its excellent biocompatibility over a commercial CHX gel on a 3D-engineered model of human gingiva under inflammatory conditions to simulate the in vivo situation.

HA is one of the most widely utilized hydrogels in periodontal drug delivery systems due its exceptional properties. HA is a natural occurring polysaccharide present in connective tissues such as gums, periodontal ligaments, and various other tissues [26]. HA hydrogels are

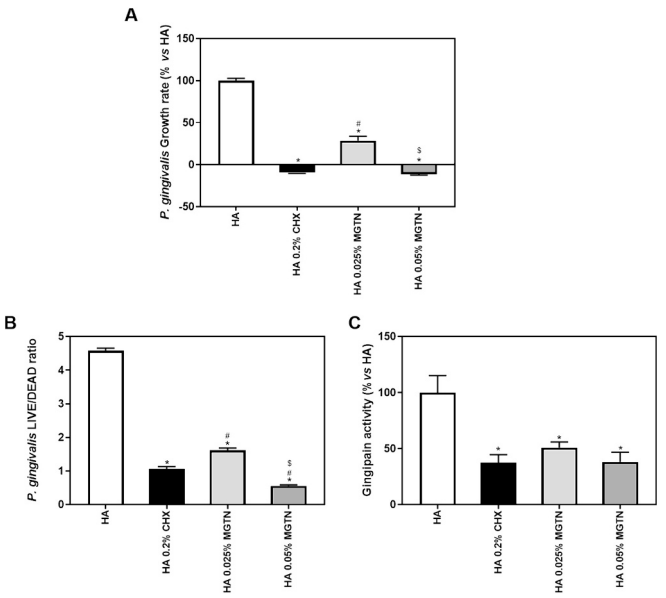

Fig. 4. Antimicrobial activity of different gels. (A) *P. gingivalis* growth rate cultured with different gels. (B) *P. gingivalis* Live/Dead Ratio cultured for 10 h with different gels. (C) In vitro gingipain activity from *P. gingivalis* after 10 h of treatment. Results are expressed as % vs Negative control (HA) that was set to 100 %. Data represent the mean ± SEM (n = 6). Negative control (HA) was bacterial suspension with HA treatment, and positive control (C+) was bacterial suspension with commercial Periokin gel (HA 0.2 % CHX). Results were statistically compared by Kruskal-Wallis for *P. gingivalis* growth rate and *P. gingivalis* Live/Dead ratio and by ANOVA and Bonferroni as post hoc for gingipain activity: *$p < 0.05$ treatment* vs *HA; # $p < 0.05$ treatment* vs *HA 0.2 % CHX; $ $p < 0.05$ treatment* vs *HA 0.025 % MGTN.*

highly valued for their biocompatibility, biodegradability, and excellent water-retaining capacity. These hydrogels can be engineered to release therapeutic agents in a controlled manner, ensuring sustained therapeutic effects at the target side [43]. The advantages of HA hydrogels extend to their ability to facilitate periodontal wound healing and tissue regeneration. Research has shown that HA hydrogels promote the migration and proliferation of periodontal cells, enhance the formation of new blood vessels, and modulate the inflammatory response, which is crucial for effective tissue repair [27–32]. Additionally, HA exhibits bacteriostatic properties, which help control periodontal pathogens and contribute to the management of periodontal diseases [44–46]. Moreover, HA is a highly porous and hydrophilic biomaterial that allows a proper interaction with the physiological environment [47]. The HA hydrogel was produced by mixing solutions of HA-ADH and HA-CHO, which crosslink at room temperature and result in a stable hydrazone bond. Compared to natural hydrogels, a general advantage of these bioconjugate hydrogels is their versatility in optimizing their characteristics, such as gelation time and mechanical strength, according to their intended use. In this case, it is important to say that MGTN is partially insoluble in water, although the HA hydrogel's complex matrix helped to stabilize and solubilize the biomolecule and carried out a well-controlled release of MGTN. Furthermore, it is well known that due to the high-water content, the HA hydrogels are usually poor at delaying the release of low molecular weight drugs [48], but they can retain precipitates of hydrophobic drugs relatively well [49]. So, our strategy to chemically modify HA to allow its crosslinking resulted in a stable hydrogel in physiological conditions and enabled to carry out a controlled release of MGTN. Curiously, loading the HA hydrogel with MGTN led to a decrease in ESR capacity at early time points. Thus, the presence of MGTN in the hydrogel structure caused a decrease in water absorption in the first hours, which could be associated with a significant increase in the viscosity of the reaction mixture, as shown by the results of the analysed rheometric parameters [50]. Furthermore, the addition of xanthone increased both the stiffness of the sample and its ability to store energy in the form of elastic energy. This indicates that xanthone contributes to the mechanical reinforcement of the hydrogel, making it more robust and capable of withstanding deformation. Another possible explanation could be that MGTN increased the crosslinking density of the hydrogel, resulting in a more rigid, elastic, compact structure with a smaller mesh size in the network. This structure might hinder the diffusion of water inside the hydrogel [50,51]. Thus, the design and synthesis of innovative HA derivatives, such as the one we show here, may offer some benefits over native HA, such as optimum MGTN concentration maintenance, improved efficiency of treatment with less MGTN, very low or insignificant toxicity and prolonged in vivo release rates for the treatment of periodontal disease.

In this study, modified HA combined with 0.025 % and 0.05 % of MGTN exhibited significant high inhibition of *P. gingivalis* growth rate. MGTN has demonstrated antimicrobial activity against various bacteria: *Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Vibrio rotiferianus* and *Vibrio campbellii* [23,52,53]. Moreover, previous studies have shown that mangosteen peel extract effectively inhibits *Streptococcus mutans* and *P. gingivalis* biofilms [54]. Therefore, based on our results, modified HA combined with 0.05 % of MGTN is highly effective at blocking bacteria growth.

In vitro studies to test periodontal gels on gingival tissues have been carried out mainly in monolayer cultures. However, these cultures have limitations in their ability to simulate the in vivo situation [55]. To overcome these limitations, 3D models have been developed in recent years, providing a higher degree of complexity similar to natural tissues [55]. In this study, the 3D model of GTE results in a good multilayer epithelial structure, with layers organized similarly to the cells in the native oral mucosa. We tested different hydrogels of modified HA combined with MGTN, which did not cause a deleterious effect on the GTE. In line with these results, a recent study showed that MGTN was not cytotoxic to human epidermal keratinocytes in vitro, and it was proposed to protect the skin from the action of free radicals, thus preventing skin aging [22]. In addition, the HA gels containing MGTN showed high PGE2 levels [56]. PGE2 acts as an inflammatory mediator and a fibroblast modulator, and it has been proposed as a promising agent for tissue repair and regeneration agent. Therefore, HA combined with MGTN treatment would be very suitable for treating periodontal diseases, given its high biocompatibility with oral tissue and allowing tissue regeneration.

Comparing the effects of the modified HA combined with 0.05 % MGTN to the commercial HA gel containing 0.2 % CHX with unmodified HA, we observe that both gels were very effective at inhibiting bacteria growth. However, MGTN was more efficient, as it was used at a lower concentration. Moreover, the commercial gel (HA 0.2 % CHX) exhibited higher cytotoxicity in the 3D model of the oral mucosa, resulting in keratinocyte monolayer destruction and cell death. This is consistent with the findings of CHX, the gold standard in periodontology, which demonstrates strong antimicrobial effects, cytotoxic properties, and altered cell morphology [7]. The gel containing 0.2 % CHX also showed significantly lower levels of PGE2, potentially related to inhibited tissue regeneration and excessive wound scar [56]. In addition, the prolonged use of CHX has reported to cause side effects such as impairment taste, teeth staining, and peeling of oral mucosa, which limit its use [57].

The mechanisms of action of CHX and xanthones differ significantly, shedding light on their distinct effects on bacteria and tissues. CHX, a synthetic antiseptic, primarily disrupts bacterial cell membranes, increasing permeability and causing cell death by leaking essential components and interfering with DNA and proteins [58]. In contrast, xanthones, such as MGTN, use a multifaceted approach: they inhibit bacterial enzymes, hinder DNA replication, and disrupt biofilm formation. Xanthones penetrate bacterial cells, damage the cytoplasmic membrane, and target intracellular components, including biofilm matrix proteins [59–62]. This mechanism, which involves the inhibition of

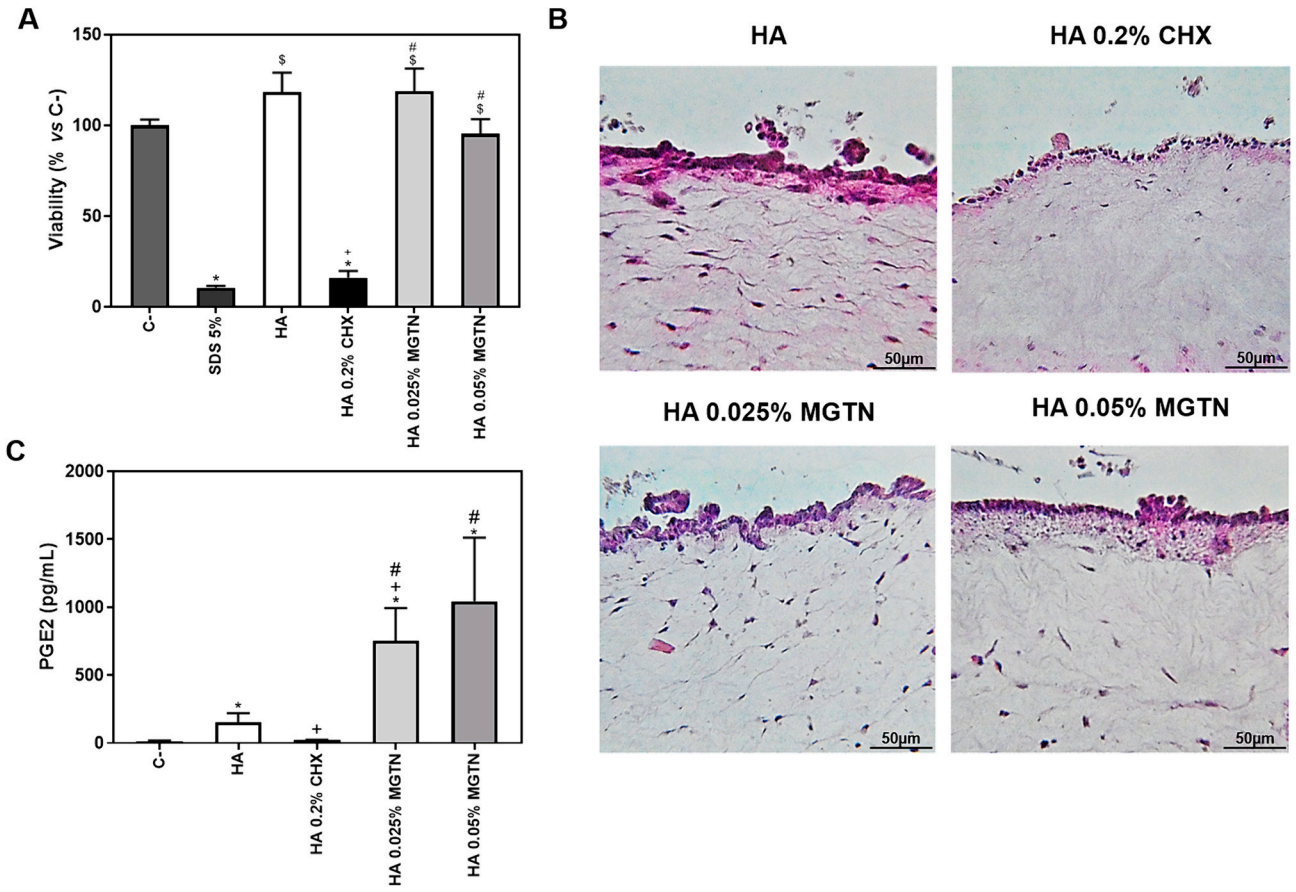

Fig. 5. Assay of biocompatibility of different gels. (A) MTT assay on GTE after applying hydrogels and LPS for 48 h. Negative control (C-) was obtained GTE treated with PBS that was set at 100 %. Positive control (SDS 5 %) was obtained from GTE treated with 10 % SDS diluted in PBS (1:1) (n = 6). (B) Histologic characterisation of GTE. Representative images were present for each treatment—detail of H&E staining of GTE at 400×. Values represent the mean ± SEM (n = 3). (C) Effect of different hydrogels on PGE2 release by GTE 48 h after treatment and LPS stimulation. Negative control (C−) was obtained from culture media of GTE treated with PBS. Results were statistically compared by ANOVA and Bonferroni as post hoc for MMT assay and by Kruskal-Wallis for PGE2 levels: *$p < 0.05$ *treatment* vs *C-; $ $p < 0.05$ treatment* vs *SDSS 5 %; +$p < 0.05$ treatment* vs *HA; # $p < 0.05$ treatment* vs *HA 0.2 % CHX.*

bacterial enzymes, may explain why xanthones are effective against bacteria without being toxic to human tissues [63].

The next step will be validating the modified HA gel containing MGTN in an in vivo inflammatory animal model of periodontitis, to verify its biocompatibility, antibacterial, anti-inflammatory, and regenerative properties. As for future experiments, it would be interesting to further investigate the interaction of MGTN with the hydrogel structure. Due to the complex nature of the hydrogel, the interaction between the hydrogel and the MGTN molecule could be explored using UV/Vis spectroscopy. UV/Vis can provide valuable insights into the molecular interaction between the hydrogel and MGTN, as modifications in the MGTN structure may alter the UV/Vis absorption signal or the extinction coefficient. Additionally, a high-resolution magic angle spinning (HR-MAS) NMR approach could be employed. The use of pulse field gradient spin-echo (PGSE) NMR in HRMAS-NMR has the potential to provide direct, highly accurate, robust, and precise information regarding the molecular mechanisms of drug diffusion [64]. Furthermore, this method is suitable for small molecules with a molecular weight of <20 kDa.

In conclusion, the gel composed of modified HA allows the controlled release of MGTN, resulting in a biocompatible hydrogel with high antibacterial activity. Therefore, this hydrogel can be an alternative therapy to prevent and treat periodontal diseases to products currently on the market with antibacterial activity but with a lack of long-term tissue biocompatibility and side effects.

CRediT authorship contribution statement

Marta Munar-Bestard: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Nelson Vargas-Alfredo:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Joana Maria Ramis:** Writing – review & editing, Validation, Resources, Project administration, Funding acquisition. **Marta Monjo:** Writing – review & editing, Validation, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors are inventors of a pending patent application based on some aspects of this work (WO2023180564A1).

Acknowledgments

This research was funded by the Fundación Universidad-Empresa de las Islas Baleares (Proof of concept Call 2019), the FOLIUM postdoctoral program within the FUTURMed, financed at 50 % by the sustainable tourism tax of the Balearic Islands and at 50 % by the European Social Fund (contract to N.V.A.), the Instituto de Salud Carlos III, the Ministerio de Economía y Competividad, co-funded with ESF European Social Fund (contract to M.M.B; FI18/00104) and Mecanisme de Recuperació i Resiliència, intended to execute research projects of «Noves polítiques

públiques per a un mercat de treball dinàmic, resilient i inclusiu», collected in Pla de Recuperació, Transformació i Resiliència, financed by European Union-Next Generation EU and driven by SOIB and Conselleria de Fons Europeus, Universitat i Cultura i la Conselleria de Model Econòmic, Turisme i Treball.

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