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생강 뿌리 추출물이 함유된 생분해성 젤라틴 메타크릴로일의 항균 효과

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Antibacterial effect of biodegradable gelatin methacryloyl loaded with ginger rhizome extract

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천연 세포외 기질을 모방한 Gelatin methacryloyl (GelMA) 3D 메쉬는 유망한 약물 전달 시스템으로 사용될 수 있으나, 불충분한 기계적 및 열화 특성은 의료적 적용에 가장 큰 장애물로 남아 있다. 본 연구에서는 항균 효과를 향상시키기 위해 천연 향신료로 사용되는 생강 추출물을 함유하는 하이드로겔을 개발하였다. 생강 추출물을 함유한 GelMA 하이드로겔을 제작하여 푸리에 변환기 적외선 분광기와 주사전자현미경으로 화학적, 형태학적 특성을 분석하였고, 압축 시험과 표면 젖음성 분석을 통해 구조적 특성을 평가하였다. *S. mutans, S. aureus* 및 *P. gingivalis*를 사용하여 생강 추출물로 개질된 하이드로겔의 항균 효과를 확인하였다. 생강 추출물로 개질된 하이드로겔의 FT-IR 스펙트럼은 GelMA 스펙트럼과 비교하여 일부 피크에서 강도의 증가를 보였다. 생강 추출물로 개질된 하이드로겔은 기공 크기가 감소함에 따라 압축 탄성률은 감소하고, 표면의 내구성, 팽윤율 및 소수성은 증가하는 등 하이드로겔의 물리적 특성에 영향을 주었다. 고농도의 생강 추출물을 함유한 하이드로겔은 *S. mutans* 및 *S. aureus*에 대해 우수한 항균특성을 나타냈고, *P. gingivalis*는 모든 농도에서 더 높은 항균특성을 보였다. 따라서,
이 연구에서 생강 추출물로 개질된 GelMA 하이드로겔은 좋은 항균성, 내구성 및 팽창 안정성을 가진다는 것을 확인하였다

색인단어 : 젤라틴, 하이드로겔, GelMA, 생강 추출물, 항균력

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Introduction

Local drug delivery systems using natural matrices has been largely studied recently as a strategy to limit systemic toxicity (1, 2), including antibacterial drug loading systems (3). Hydrogel is a suitable strategy for drug delivery system, as they present several advantages as providing spatial and temporal control over drug release while protecting drugs from degradation. The tunability of hydrogel matrix controls it physicochemical properties and enables the control over release and degradation of the matrix by polymer-drug interactions (4). Gelatin, is a hydrolysis product of collagen with amino acid composition similar to that of collagen, it can form physical crosslinking at low temperatures, which restore the triple helical structure as collagen. Gelatin-based hydrogel including the delivery of natural essential oils can mimic the natural extracellular matrix and is being used as a promising tissue engineering material and drug delivery system (5, 6). However, gelatin film alone is water-soluble and at body temperature (37 ℃), hence application of gelatin film is limited due to it poor mechanical strength and rapid degradation (7).

Van Den Bulcke et al (8) developed a method to modify gelatin with methacrylic anhydride (MA) into gelatin methacryloyl (GelMA). This polymer mesh is produced by the substitution of the free amine groups of the gelatin with methacrylate anhydride it preserves the temporal crosslinking capability of gelatin at lower temperatures forming a triple helical structure (9), and can be cured by photo-crosslinking by exposure to UV light or visible light (10, 11), allowing the control over physicochemical properties of GelMA hydrogels. Three-dimensional (3D) GelMA hydrogels have been studied as drug delivery system (12, 13). The hydrogel polymeric chains and the entrapped molecules can interact influencing the chemical modification, hydrophobic interactions or physical properties of the hydrogel (2, 14, 15). Ultimately, this influence control the hydrogels stiffness which can be affected by altering other factors as the degree of functionalization, crosslinking conditions including photoinitiator concentration and curing time (11, 16, 17). The GelMA-based hydrogel mesh size and porosity constitution is correlated with the hydrogel stiffness and drug diffusion.

Essential oils, obtained from natural medicine and antimicrobial properties have been recently applied to improve antibacterial activity in polymeric drug delivery systems (18, 19). Ginger extract (Zingiber officinale), receive much attention as it used in cooking, as a spicy and seasoning, and used in natural medicine due to it pharmacological benefits, including antioxidant, analgesic, anticancer, and anti-inflammatory properties (20). It bioactive components as phenolics, flavonoids, gingerols, shogaols, and zingerone (21), had manifested antibacterial activity (22, 23). This natural phytochemical had been previously studied to improve antibacterial effect of polymeric hydrogels (5, 24) and gelatin films (6).

Periodontal disease is a highly prevalent degenerative inflammatory process characterized by progressive destruction of supporting structures as gingiva, periodontal ligament, alveolar bone degeneration and, dental cementum (25, 26). Periodontitis is caused by the accumulation of bacteria residing in deep of the periodontal pockets and the current therapies to treat this disease include nonsurgical treatment as cleaning and scaling, however remaining of bacteria in the deep of the pocket after debridement kept local inflammation active (26). Therefore, more strategies had been study as treatment against periodontitis like local drug administration to the affected site (27), which can prolong the drug action and various polymers had been use to control release of drugs, including the study of GelMA hydrogels antibacterial effect against *P. gingivalis* and *S. mutans* (28, 29).

In this study, ginger extract which is a natural antimicrobial spicy was loaded into 3D GelMA hydrogels

to provide an improvement of its antibacterial activity. Then, the chemical interaction, morphology, compressive modulus, swelling ratio, degradation ratio, and antibacterial effect of the GelMA hydrogels and ginger-modified hydrogels were evaluated to compare its effect and potential as drug delivery system against common oral bacteria strains.

Materials and Methods

1. Materials

Gelatin (Type A, from porcine skin, 300 bloom), methacrylic anhydride (MA), Triethanolamine (TEA), N-vinylcaprolactam (VC), Eosin Y disodium salt and dialysis tubes, high retention seamless cellulose tubing (12-14 kDA MWCO, 40 mm flat width) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ginger extract was provided by the National Institute of Agricultural Sciences in Rural Development Administration in Korea.

2. Synthesis of Gelatin Methacryloyl

Gelatin methacryloyl macromer was synthetized according to previously reported methods (8), using gelatin type A. Briefly, a total of 10% (w/v) of gelatin was completely dissolved in Dulbecco's phosphate-buffered saline (DPBS, Sigma-Aldrich, St. Louis, MO, USA) at 60 ℃ for 1h. Then, MA (0.8 mL MA to 1g gelatin ratio) was added to the gelatin solution at a rate of 0.5 mL/min and stirred at 50 ℃ for 3h. The reaction was terminated by adding 5 times pre-warmed DPBS. The solution was dialyzed against distilled water using 12-14 kDa dialysis tubing at 37 ℃ for 7 days. Dialyzed GelMA solution was freeze-dried for 7 days, and the foam-like GelMA was stored at -20 ℃ freezer until further use.

3. Preparation of GelMA Hydrogel Modified with Ginger Extract

Hydrogel were formed by first dissolving gelatin methacryloyl lyophilized macromer (10% w/v) in the photoinitiator solution containing TEA (1.875% w/v) and VC (1.25% w/v) in distilled water at 37 ℃. Eosin Y was separately dissolved in distilled water at a concentration of 1 mM. To prepare 10% GelMA hydrogel, 800 μL of GelMA/photoinitiator solution was mixed with 100 μL of Eosin Y solution and 100 μL of distilled water.

To prepare the GelMA hydrogel modified with ginger extract samples: First, stock solution of 100% (w/v) of ginger extract dissolved in Dimethyl sulfoxide (DMSO, Duchefa Biochemie, BH Haarlem, The Netherlands) was prepared, then this was diluted in distilled water (DW) to obtain five working solutions with 10%, 20%, 30%, 40% and 50% ginger extract concentrations. Then GelMA hydrogels modified with ginger extract were prepared by mixing 800 μL of GelMA/photoinitiator solution with 100 μL of Eosin Y solution and 100 μL ginger extract working solution.

The final concentration of chemicals after forming the hydrogel was 10% (w/v) GelMA with 1.5% (w/v) TEA, 1% (w/v) VC, and 0.1 mM Eosin Y for the 10% GelMA hydrogel (Control (0)), the GelMA ginger-modified groups keep the final concentrations of the chemicals mentioned above with the variation of the addition of ginger extract to the final formula, resulting in five modified groups with ginger extract concentrations: 1%, 2%, 3%, 4% and 5% ginger extract/10% GelMA (Table 1).

To form the hydrogels, 150 μL of the precursor solution was pipetted into polydimethylsiloxane (PDMS) square molds (width: 10 mm; height: 1 mm) and square molds (width: 10 mm; height: 2 mm) for compressive test. Finally, the solutions were photo-crosslinked by exposure to visible light (440-480 nm) for 60 s using a CO_2 Curing Light dental light unit ($C0_2$, Premium Plus, UK, England).

Table 1. Composition of hydrogels and groups labelling.

Name	Group label	GelMA (% w/v)	Ginger (% w/v)
10% GelMA	Control(0)	10	0
10% GelMA/1% Ginger extract	1 GE	10	1
10% GelMA/2% Ginger extract	2 GE	10	2
10% GelMA/3% Ginger extract	3 GE	10	3
10% GelMA/4% Ginger extract	4 GE	10	4
10% GelMA/5% Ginger extract	5 GE	10	5

4. Nuclear Magnetic Resonance (^{1}H NMR) Spectra

The degree of functionalization (DoF) of GelMA was evaluated using ^{1}H NMR spectroscopy according to the previous studies (30, 31). ^{1}H NMR spectra were obtained using a 600 MHz Fourier transform-NMR spectrometer (JNM-ECZ600R, JEOL, Japan) installed in the Center for University-Wide Research Facilities (CURF) at Jeonbuk National University. For ^{1}H NMR analysis, GelMA samples (50 mg/mL) and gelatin samples (50 mg/mL) were prepared in deuterium oxide. The DoF was calculated using the following equation:

$$DoF(\%)=1-\left(\frac{\text{Lysine methylene proton of GelMA}}{\text{Lysine methylene proton of Gelatin}}\right)\times 100$$

5. Fourier-Transform Infrared Spectroscopy (FT-IR)

The cured hydrogels were previously freeze-dried overnight, ginger extract (10 μL) was coated on platinum foil and, analyzed by Fourier transform infrared (FT-IR) spectrometer (Perkin Elmer Frontier, Waltham, MA, USA) with KBr pellet in the range of 400~4000 cm^{-1}, using the attenuated total reflctance (ATR) method, to confirm their functional groups and changes in chemical structures.

6. FE-SEM Characterization

The morphology of hydrogels was studied by field emission scanning electron microscopy (FE-SEM, JSM-5900, JEOL, Japan). First, samples were immersed in PBS (37 ℃ overnight), lyophilized overnight and, then sputter platinum coating. ImageJ software (FE-SEM; Gemini500, Carl Zeiss Co., Oberkochen, Germany) was used to analyze the mean pore size and distribution through SEM images.

7. Compressive Mechanical Properties

The samples were hydrated in deionized water overnight previous to compressive test. The mechanical test of hydrogel samples (width: 10 mm; height: 2 mm) was performed using a universal tester (GB 4201, Instron, UK) with 50 N load cell, at a speed 0.5 mm/min rate until fracture. The measured data was obtained using Bluehill 2 software, and the compressive modulus was calculated as the slope of the linear region (0~20%) of the stress-strain curve.

8. Surface Wettability

The water contact angles of hydrogel samples were measured with a SEO contact angle analyzer (Surface

Electro Optics Co. Ltd, Phoenix-300, South Korea) using the touch-drop method. First, the square shape samples (width: 10 mm; height: 1 mm) were attached to a glass coverslip. Then, a droplet of deionized water (5 μL) was automatically dispersed onto the sample surface and was recorded with a video camera attached to the equipment. The water contact angles along time were automatically calculated by the equipment software. The contact angles of four independent samples of the same group were analyzed with the software Surfaceware 8 and averaged.

9. Swelling Ratio

For the evaluation of swelling ability, the initial weight (Wi) of hydrogels was measured after freeze-drying overnight. Then samples were incubated in PBS (pH 7.4) at 37 ℃. The hydrogel samples were pulled out of PBS after 24 hours and residual solution on the surface was removed using Kim wipes. The weights of the samples in the swollen status (Ws) were recorded. The swelling ratio was calculated using the formula:

$$\text{Swelling ratio}(\%)=(W_s - W_i)/W_i \times 100$$

10. Enzymatic Degradation

The evaluation for the degradation rate of the hydrogel samples was performed by an enzymatic degradation process, according to the previously reported method (32) with a slight modification. First, the initial weight (W_i) of hydrogels was measured after freeze-drying overnight. Then, 4 U/mL of collagenase (Type II, pH:7.4) was prepared in PBS, 1 mL was added to the hydrogel samples and incubated at 37 ℃. The samples were removed at several time points (2, 4, 6, 24 and 48 hours), lyophilized and weighted (W_t). The degradation rate was calculated by the following equation:

$$\text{Mass loss}(\%)=(W_i - W_t)/W_i \times 100$$

11. Antibacterial Effect

P. gingivalis, S. aureus and S. mutans were acquired from Korean Collection for Type Culture (KCTC, Jeollabuk-do, Korea), and were used to evaluate the antimicrobial properties for samples of 10% GelMA and GelMA modified with ginger extract.

The strains were first incubated in a sheep blood agar plate (BAP, Baudio Co, Gyeonggi-do, Korea) at 37 ℃ in 5% CO_2 atmospheres for 1 day, except for *P. gingivalis* which was incubated under anaerobic conditions for 7 days. Then, Brain Heart Infusion (BHI, Difco Laboratories, Le Pont de Claix, France) was inoculated with one colony of fresh pre-cultured dish and optical density was measure using DensiCheck plus (Biomérieux, SA) until lecture reached 0.5, then 1 ml of this suspension was diluted in BHI (1:10 ratio) and this inoculum was used for the antibacterial activity test (1.5×10^7 CFU/ml). For antimicrobial tests, 1 mL of a 10^7 CFU/mL bacteria solution was seeded on square shape hydrogels in 24-well plates. After 24 h in 5% CO_2 atmospheres incubation for *S. aureus* and *S. mutans,* and after 7 days of anaerobic incubation for *P. gingivalis,* the media was collected and the absorbance was measured at a wavelength of 600 nm by a microplate reader (Emax Precision microplate reader, Molecular devices, USA). The lowest concentration which inhibited the growth of the bacteria was considered as the MIC.

12. Statistical Analysis

All assays were performed in triplicate, and each group was tested at least three times. Quantitative data were expressed as the mean±standard deviation (SD). One-way Analysis of variance (ANOVA) and Tukey's post hoc test were used to determine the level of significance in comparison to control group. Statistical analysis was performed using SPSS 24.0 program (IBM, Chicago, IL, USA). A value of $p<0.05$ was considered to be significant.

Results

1. Preparation of GelMA hydrogels modified with ginger extract

The GelMA macromer synthesis mechanism is shown in Figure 1. The resultant GelMA was obtained and characterized by ^{1}H NMR (Figure 2(a)), the increase of signal at δ = 5.4 and 5.7 ppm of the proton methacrylate vinyl groups of MA, and the decrease of signal δ = 2.9 ppm of methylene lysine groups confirmed the modification of gelatin by the addition of MA. The proton signal of aromatic amino acids in gelatin and GelMA remained constant, so its intensity was used to normalized the proton signal of methylene lysine groups in Gelatin and GelMA macromere. The DoF of GelMA macromer was 73.81%.

GelMA-ginger hydrogels were crafted to enhance antibacterial properties. As shown in Figure 2(b), it is observed after visible light photocuring for 60 seconds square and thin film shaped hydrogels were obtained, it was observed that the color of 10% GelMA hydrogel turned into red-ish due to the addition of Eosin Y, while the color of ginger-modified hydrogels changed to a lighter tone as the ginger extract content increased and its transparency also increased. After freeze-dry, the 10% GelMA shape and length remained similar to the shape before freeze-dry, however, in case of the ginger-modified samples the shape changed, it shrinkage and the border became less defined, the samples also became thinner as the ginger concentration increased. This change could be influenced by Eosin Y cured time, as well the samples with higher ginger extract content (4% to 5% ginger) possessed a softer texture which could not preserve the square shape and defined border.

2. GelMA Morphology Assessment

The morphology of the hydrogels was observed by FE-SEM (Figure 3). All hydrogel groups showed a non-uniform porous morphology that resemble an irregular spider web and the detailed information of pore size is presented in Figure 3b. The pore size of the 10% GelMA group was significantly greater than the samples modified with ginger extract. The average of pore size

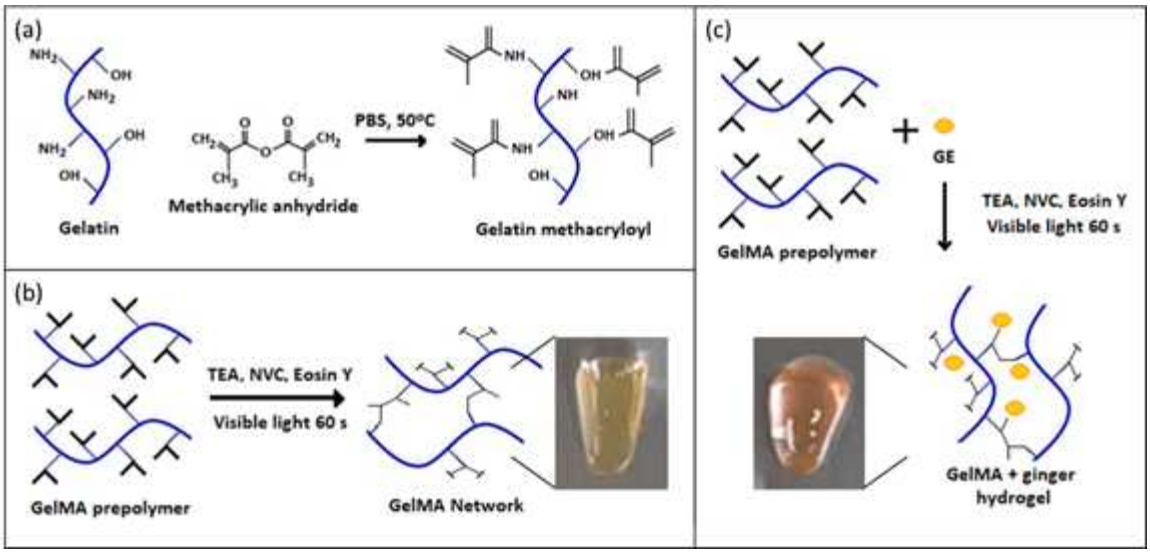

Figure 1. Schematic representation of the structure formation on (a) Gelatin methacryloyl (GelMA) macromer, (b) GelMA hydrogel by photo-induced activation of Eosin Y (10% GelMA, yellow color), and (c) GelMA modified by addition of ginger extract before photo-activation of Eosin Y (GelMA modified with 5% ginger extract, red color).

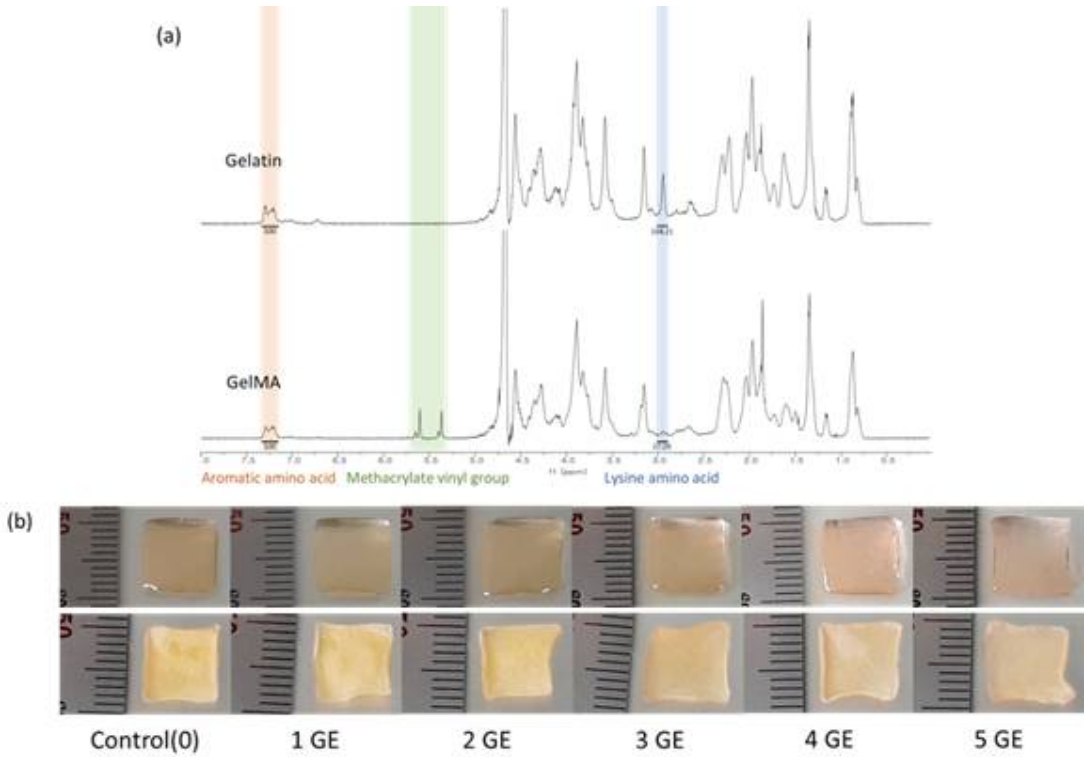

Figure 2. (a)^{1}H NMR spectra of Gelatin and GelMA macromere, (b) morphology of 10% GelMA [Control(0)], and ginger-modified hydrogels (1% to 5% ginger extract), on top row photocured samples and bottom row samples after freeze-dry.

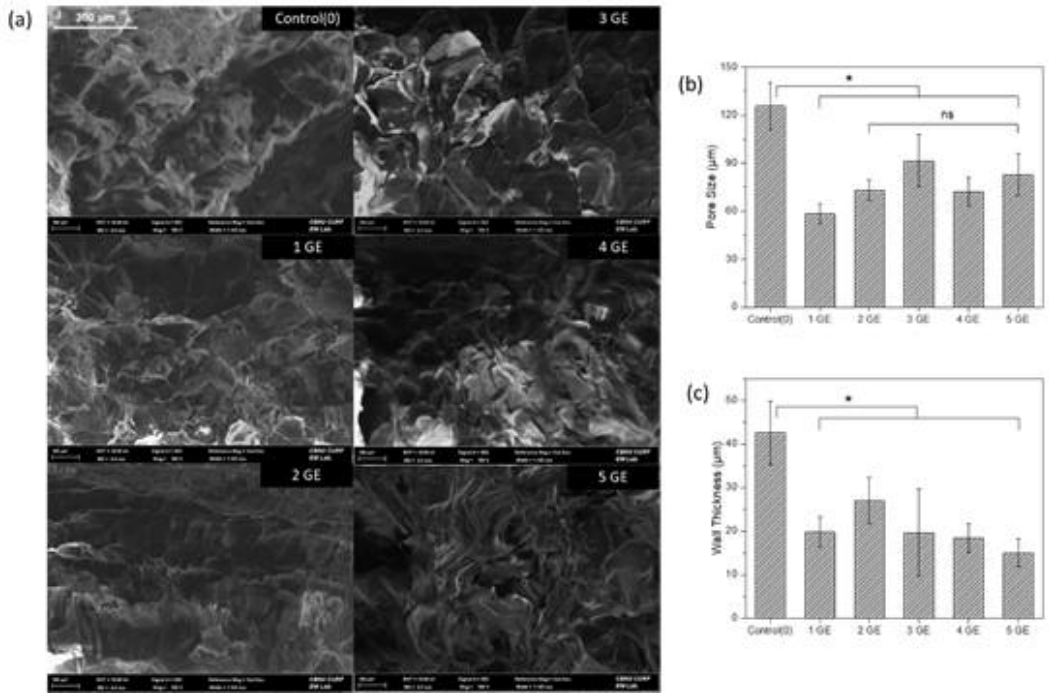

Figure 3. (a) FE-SEM images of 10% GelMA hydrogel and ginger-modified hydrogels (1% to 5% ginger extract), (b) average pore size and (c) average wall thickness on FE-SEM images (*p<0.05).

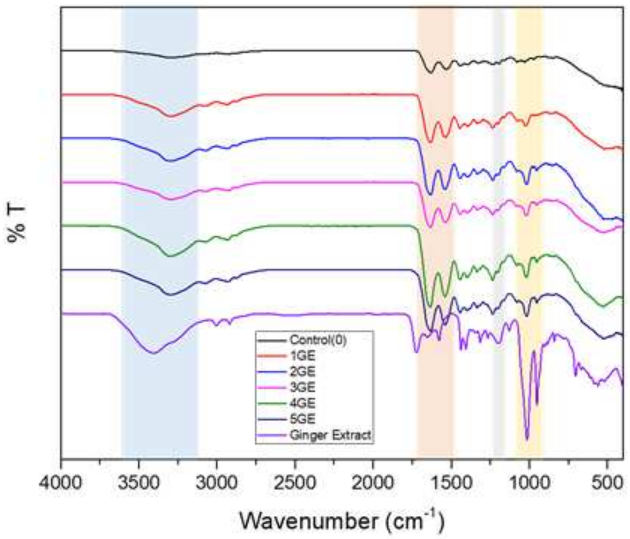

Figure 4. FT-IR spectra of 10% GelMA (Control(0)) hydrogel and ginger-modified hydrogels (1% to 5% ginger extract).

for 10% GelMA group was 125.6±14.63 μm. The pore size of the ginger-modified groups decreased significantly, with the exception of GelMA modified sample with 3% ginger extract. The wall thickness of the 10% GelMA group presented the higher mean of 42.61±7.24 μm in comparison with the samples modified with ginger extract. It is observed that the pore structure became unorganized wavy structure at highest concentrations (〉3%) of ginger extract.

3. FT-IR Spectra Analysis

To evaluate the influence of Ginger extract content to the chemical bonds, the 10% GelMA and each of the hydrogels modified with ginger extract were analyzed using FT-IR, as a comparison parameter ginger extract alone was also analyzed. As shown in Figure 4, the 10% GelMA spectra showed a band from 3200 to 3400 cm^{-1} which represents the presence of the peptide bonds (N-H stretching) from amide A, peaks at 1640, 1541 cm^{-1}, and 1240 cm^{-1} attributed to the C=O stretching (amide I), N-H bending (amide II), and C-N stretching plus N-H bending (amide III), as well as stretching at 2930 cm^{-1} related to the symmetric and asymmetric stretching in the CH_2 groups of alkyl chains.

Ginger extract alone presented a stretching vibration O-H band between 3200 to 3400 cm^{-1}, with a higher intensity than the N-H stretching at the same wavenumber on 10% GelMA, the presence of aromatic stretching (C-H in plane) near 1512 cm^{-1} was observed, another stretching peaks are observed between 1230 to 1380 cm^{-1} related to C-N stretching, finally C-C bending is observed between 900 to 1200 cm^{-1}.

The introduction of ginger extract to the GelMA hydrogels presented modification of the intensities of the peaks in comparison with the spectra in 10% GelMA, this intensity on the peaks became marked as the increase of ginger extract content increased on the sample. The higher changes were observed in the band from 3200 to 3400 cm^{-1}, peaks at 1640 and 1541, and the appearance of C-C bending peak between 900 to 1200 cm^{-1} on the GelMA modified with ginger samples. This FT-IR results indicated the introduction of ginger extract to GelMA at different concentrations.

4. Mechanical Properties of Hydrogels

As shown in Figure 5, the compressive stress-strain curve indicated a negative correlation between the ginger extract content ratio and the compressive modulus. The compressive modulus (49.33 kPa) of 10% GelMA hydrogel group was significantly higher than that of the groups modified with ginger extract. The compressive modulus of groups modified with ginger extract was 1 GE: 31.28 kPa, 2 GE: 30.74 kPa, 3 GE: 22.13 kPa, 4 GE: 4.37 kPa and 5 GE: 6.61 kPa respectively.

5. Swelling and Degradation Ratio

The biodegradation of 10% GelMA and samples modified with ginger extract was stimulated by collagenase. As shown in Figure 6(a), the 10% GelMA

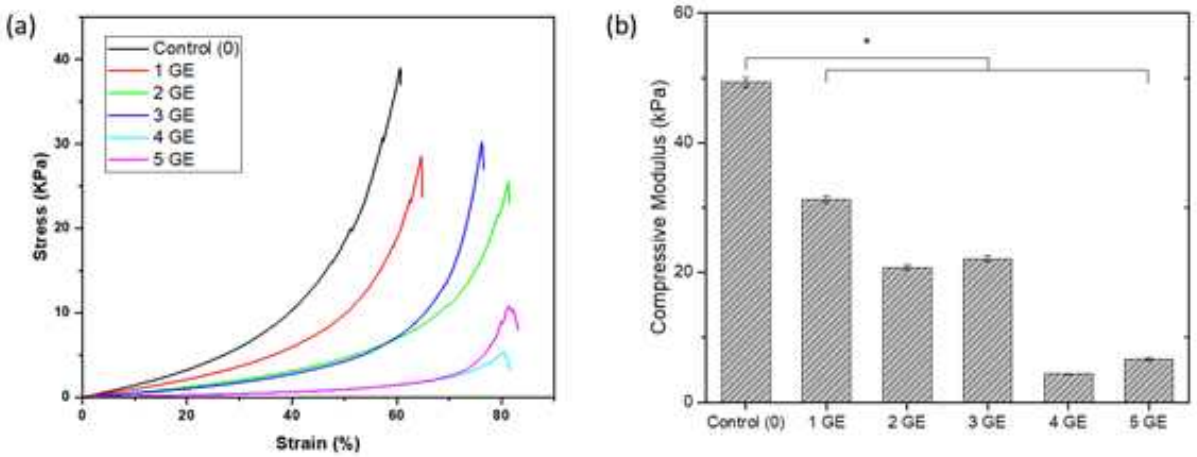

Figure 5. Mechanical properties of 10% GelMA hydrogels (Control(0) as Pristine 10% GelMA), and hydrogels modified with ginger extract (1% to 5% ginger extract inclusion), the compression was performed up to 90%, (a) Stress-strain curve for al hydrogels and, (b) Compressive modulus (*p〈0.05).

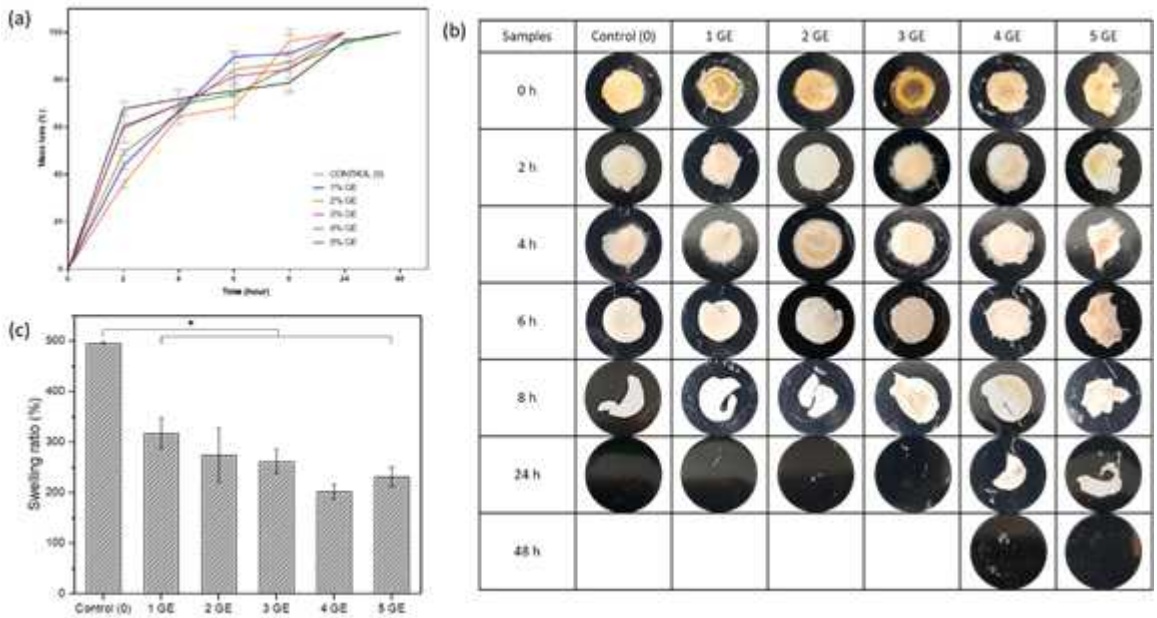

Figure 6. (a) Mass loss of 10% GelMA hydrogels (Control(0) as Pristine 10% GelMA), and hydrogels modified with 1% to 5% ginger extract by treatment time with collagenase type II (4 U/ml), (b) representation image of macroscopic changes during biodegradation test (freeze-dried) and (c) swelling ratio.

hydrogel presented low degradation after 2 hours immersion meanwhile the modified groups showed degradation ratios from 40% to around 65% as the content of ginger increased in the sample. Similar behavior was observed up to 6 hours immersion in which the lowest degradation ratio was presented by 10% GelMA, however at this period of time, higher degradation of the groups with lowest ginger content (〈3% ginger extract) showed over 80% mass loss, while the higher content of ginger hydrogels presented less than 75% mass loss. A significant

mass loss (96.24%) was observed by 10% GelMA after 8 hours immersion while mass loss of groups modified with ginger extract remained similar to the 6 hours behavior. After 24 hours immersion on collagenase, four groups with the lowest content of ginger from 10% GelMA to 3% ginger extract presented total degradation in comparison with the 4% and 5% ginger content samples with ≥5% of initial mass remaining. The surface changes of dry hydrogels were observed after different periods of immersion time and the degradation behavior of the samples affected the borders first (Figure 6(b)).

The effect of different concentrations of ginger extract on the swelling ratio of GelMA hydrogels samples was investigated. As shown in Figure 6(c), the swelling ratio of the samples significantly decreased in comparison with 10% GelMA hydrogel as the concentration of ginger extract increased. The highest swelling ratio is observed at 10% GelMA group (496% of the initial weight), meanwhile the samples modified with ginger presented swelling range between 317% to 201% after 24 hours immersion in PBS.

6. Wettability of Hydrogels

Surface wettability of the hydrogels samples was evaluated by contact angle sessile drop. As presented in Figure 7, contact angle values on the surface of the samples modified with ginger extract were higher than the 10% GelMA with a contact angle of 39.28±3°. Most of the samples with lower ginger content showed hydrophilic states (θ a〈90°), while 4 GE and 5 GE surface with a contact angle of 91.27±5° and 99.22±1° respectively, showed hydrophobic states (θ a〉90°) (33).

7. Antibacterial Properties

The antibacterial effect of ginger extract alone at different dilution concentrations before the addition on the GelMA hydrogels samples was evaluated against *S. mutans, S. aureus* and *P. gingivalis* (Figure 8(a)). The

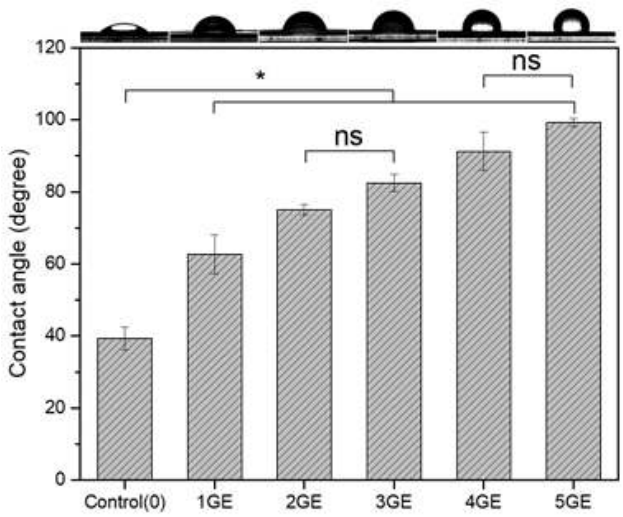

Figure 7. Contact angle of 10% GelMA hydrogels (Control(0) as 10% GelMA), and ginger-modified hydrogels (1% to 5% ginger extract inclusion) (*p〈0.05)(ns = no statistical difference).

growth of *S. aureus* was significantly decreased at concentrations higher than 1% ginger extract. Meanwhile, *S. mutans* presented a higher sensitivity against ginger extract at the same concentration (〉1% ginger extract) with an inhibition of more than 50% bacterial growth. On the other hand, *P. gingivalis* presented a higher sensitivity than the rest of the bacteria strain tested even at lowest concentrations (〉0.25% ginger extract).

The addition of ginger extract into the GelMA hydrogels deceased it antibacterial effect against *S. mutans* and *S. aureus* in comparison with the growth against the dilution of ginger extract on media. The bacteria strain with less sensitivity against the GelMA samples modified ginger extract was *S. aureus*, with a statistically significant inhibition growth against the samples modified with higher ginger content (4% and 5% ginger extract). *S. mutans* growth was affected against samples modified with concentrations higher than 3% ginger extract. GelMA hydrogel sample with 5% ginger extract was the one that presented a growth inhibition up to 50% against *S. mutans* and *S. aureus*. It is important to notice, *P. gingivalis* presented the highest sensitivity after incubation on the

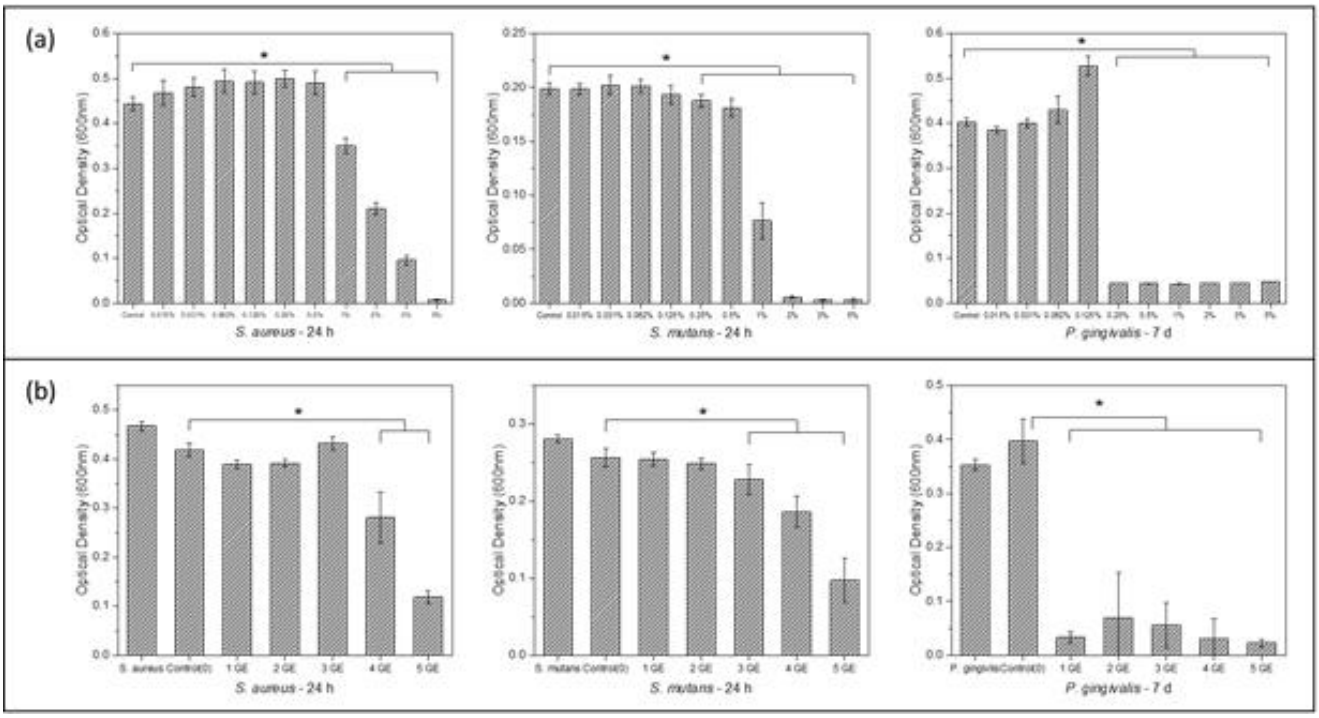

Figure 8. Bacterial growth of *S. aureus, S. mutans* and *P. gingivalis* (a) after incubation in media with different concentrations of ginger extract (BHI media without ginger extract was used as Control), and (b) after incubation on 10% GelMA hydrogels [Control(0)], and ginger-modified hydrogels (including 1% to 5% ginger extract) (*p<0.05).

GelMA samples modified with all concentrations of ginger extract addition, which was similar pattern as in the case against incubation of ginger extract/media dilution.

Discussion

GelMA macromer, in which methacrylic anhydride reacted with the free amine and hydroxyl groups on the gelatin chain, were prepared by the method reported by Van Bulcke et al (8). The degree of functionalization of GelMA can be calculated by ^{1}H NMR, by comparing the lysine methylene proton signal at 2.9 ppm of unmodified and modified gelatin, and the appearance of typical methacrylate proton signal between 5.4 and 5.7 ppm (30). In this case, the increase of methacrylate proton signal and the decrease of lysine methylene proton signal confirmed the formation of gelatin methacryloyl with a DoF of 73.81%, ranking between medium to high methacrylation degree.

The resulting GelMA precursor can be crosslink to form hydrogels through water-soluble photo-crosslinking systems to initiate the polymerization, one of these systems constituted the bimolecular photo-initiation mechanism which include at least two components: a photosensitizer (Eosin Y) and an initiator (TEA) (11, 34). The addition of Eosin Y can influence the final color after light exposure of the hydrogel, which can change color from red to yellow after the formation of the activated Eosin Y (16). In the visual analysis of the macroscopic structure of hydrogels, it can be confirmed that the addition of ginger extract and the increase of concentration in the GelMA hydrogels samples lead to a formation of hydrogels network not fully crosslinked at the higher concentrations

(4% and 5%) of ginger at similar light exposition (60 seconds). An alteration on the crosslinking conditions can influence the properties of the hydrogels.

The microstructures of GelMA hydrogels can be furthermore modified by the degree of methacrylation and the addition of drug into the structure, since the higher degrees of methacrylation decreased the pores size and result in stiffer and more durable hydrogels with small pores as reported by Chen et al (35). In comparison, on this study was observed the pore size was modified by the addition of ginger extract on the samples resulting in smaller pore diameters and wall thickness than 10% GelMA. This could be related with the hydrophobic interactions that could cause a rapid loss of water from the interconnected pores and a collapse of the microporous structure on the samples with higher ginger content, which could be the reason of the irregular wavy microporous structure with small size and low stiffness (36). These collapsed pores presented a wavy structure in 4 GE and 5 GE giving the impression of smaller pore size in comparison with 3 GE, however there is no statistical difference between each of the samples pore size. A similar irregularity in porous structure of the GelMA was observed by Darvishi et al (37), by the addition of reduced graphene oxide (RGO) nanoparticles into gelatin methacryloyl hydrogels, related to the polymeric chain in GelMA containing hydrophilic and hydrophobic segments which reacted through hydrophobic-hydrophobic interactions to the RGO surface. However, 10% GelMA hydrogel revealed the pore size of 125.6± 14.63 μm, which is similar to the GelMA hydrogel developed by Rizwan et al (17) and Sadeghian et al (38), with a pore size average of 135 μm, and 105 μm respectively.

It is known GelMA is a polypeptide polymer with repeating amide units. The characteristic bands from 3200 to 4300 cm^{-1} represent the peptide bond (N-H stretching) from amide A, as well as amide B located at the stretching vibration of C-H bond at 3080 cm^{-1}. The localization of amide I, amide II and amide III at 1640, 1541, and 1240 cm^{-1} was also recognized by previous studies (10, 39). This amide groups were observed on this study confirming the chemical structure of amide units in the GelMA hydrogels. On the case of ginger extract alone a band in similar location at 3200 to 3400 cm^{-1} corresponding to O-H vibration is identified, with the appearance of methylene C-H asymmetric stretching at 2950 to 3020 cm^{-1}, in conjunction with aromatic stretching and other functional groups typical from ginger extract spectra were found (40). In this study, the spectrum of 10% GelMA hydrogel derived from the spectra of the hydrogels modified with ginger extract, which presented an increasing of the intensity of certain peaks showing an interaction between GelMA and ginger extract, mainly related with a shift between amine N-H from 10% GelMA hydrogel and O-H bonding from ginger extract. The intensity of GelMA spectra peaks can be modified by the addition of drugs during the hydrogel formation (10), as in the case of the interaction between Tannic Acid and GelMA hydrogel, Tannic acid is a natural plant source of polyphenolics and when is include into the GelMA hydrogel the resulting spectra shown a typical wave between 3100 to 3600 cm^{-1}, related to the formation of hydrogen bonds due to a shift of the amine N-H of GelMA and H-bonding from Tannic Acid (41).

Alteration of the crosslinking conditions can affect the properties of the hydrogels. Successful polymerization reaction on gelatin methacryloyl hydrogels requires the generation of radical species in optimal concentrations and light exposure time, if the reaction contain too many free radicals close to each other's, it can lead to smaller molecular weight chains observed as small pores (42). To study the effect between the crosslinking conditions and GelMA hydrogel microstructure Noshadi et al (16), varied the TEA (0.5 to 1.5% w/v), VC (0.5 to 1.5% w/v) and EY (0.1 Mm) concentration finding a direct correlation

between VC and TEA content to the properties of the hydrogels, affecting the porosity and compressive modulus, related with the influence of crosslinking sites numbers in higher concentrations of VC an TEA resulting in an increase on density and hydrogel stiffness, decreasing pore sizes and swelling ratio. Similarly, in this study the compressive modulus of 10% GelMA hydrogel (49.33 kPa) is in range of results reported by Noshadi and UV cross-linked hydrogels (43), with a fracture at 60% strain (38). The compressive modulus of ginger-modified hydrogels differed from the tendency, in which is proclaimed small pore size are related to high stiffness (15), in this study the higher concentrations of ginger influenced negatively the compressive modulus even though the pore size were smaller than the 10% GelMA hydrogel with similar crosslinking conditions, this could be related to a rapid loss of water from the interconnected pores of the ginger-modified samples which decreased the stiffness of the hydrogels independent of the mesh density (36). Swelling and degradation ratio of hydrogels determine the suitability of this polymer for different tissue engineering applications. The physical and morphological characteristic on GelMA hydrogels can be manipulated during it synthesis and process varying the degree of methacrylate substitution and crosslinking conditions (35, 42, 43). In this study, the methacrylation degree and crosslinking conditions were kept constant in all groups, only changing the ginger extract loading concentrations on each group, the different concentrations influenced the pore size of the ginger-modified hydrogels polymer network, which influenced the degree of swelling in comparison with the 10% GelMA. This influence is related to the closeness on the polymer chains as new bonds form after crosslinking, making the mesh denser (small pores size) with higher retraction forces (44). The effect on drug loading to GelMA hydrogel in the swelling behavior was similarly observed by Huang et al (14), which incorporated different concentrations of Red Jujube powder, a natural extract, as drug loading solution into the hydrogel formulation before crosslinking, finding a correlation between high drug concentration with a decrease in swelling ratio. The degree of degradation is also influenced by the crosslinking degree, degradation on GelMA hydrogels decreased with an increase of crosslinking degree and the presence of less unreacted components (44). To simulate *in vivo* environments enzymatic degradation of GelMA is studied using collagenase, the concentration of collagenase as varied in different studies from 2.0 to 28 U/mL (45-47). Zheng et al, investigated the degradation ratio on bioactive glass modified GelMA hydrogel in which the degradation ratio was reduced in samples with lower swelling ratio and small pores, the denser mesh structure reduced the penetration of collagenase. Is this study, the hydrophobic interactions and small pore size shown in 4 GE and 5 GE prevented the penetration of collagenase into the mesh, reducing the degradation rate in comparison with the rest of the samples. Surface wettability that is evaluated by studying hydrogels contact angle is also an important characteristic property. It has been previously reported that the surface of GelMA hydrogel presented a hydrophilic character which can be modified by the photoinitiator concentration modification (39, 48). Similarly, as the influence on swelling and degradation ratio an increment in crosslinking bond formations result in a compacted structure, a denser mesh affected the wettability of the hydrogel surface. According to Cha et al (33), a surface presented a hydrophilic state when the droplet-surface degree is $\langle 90°$ and hydrophobic state at degree $\rangle 90°$. The results in contact angle increasing in samples with smaller pore size were similarly to other studies (2, 39, 48), with hydrophobic state as ginger concentration increased to 4% and 5%.

Hydrogels can serve as a platform for delivering different molecules as a drug delivering system through polymer-drug interactions, including antimicrobial agents' delivery

(49, 50). GelMA hydrogel alone as being reported as non-effective to reduce bacterial colonization (13, 51). The phenolic compound in ginger extract has been employed as an anti-bacterial agent (22, 52). Previously, gelatin alone was modified with ginger extract by Li et al (6) to improve the antibacterial effect of the resulting films, finding a high antimicrobial activity against gram-positive bacteria at starting 0.5% ginger extract content, involving the destruction of bacteria walls by the active compounds (52, 53). In comparison with this study, low concentrations of ginger extract incorporated into GelMA hydrogel exhibited non-antibacterial effect while high concentrations (over 3%) presented inhibition against *S. aureus* and *S. mutans* growth in comparison with the control group, this could be related with the higher integrity of GelMA hydrogel than gelatin film alone. However, it is remarkable to mention than *P. gingivalis* presented a high sensitivity against all GelMA modified with the concentrations of ginger extract, which could be applied as antibacterial delivery therapy against periodontal disease (50, 54, 55). *S. mutans* is a pivotal species during early stages of dental plaque colonization and also in the pathogenesis of dental caries due to its ability to produce glucans from sucrose and tolerate acid (56). *P. gingivalis* is a model pathogen persistence in the subgingival region and during host late stage colonization it alter immune responses and oral microbiota leading to inflammatory process and bone loss (25). Therefore, GelMA modified samples with ginger extract can contribute to drug release therapy during the treatment of periodontitis and dental caries prevention by bacterial growth inhibition.

In summary, a hybrid hydrogel GelMA modified with ginger extract with antibacterial and relatively acceptable swelling and degradation ratio was constructed, which can be promising as drug delivery polymeric systems against oral infections. This GelMA modified hydrogel can be applied for treatment of oral infections as periodontitis. However, further studies are needed to investigate more beneficial applications of this materials and it compatibility with cellular environment in oral tissues, overall bone structures, as more modifications to improve compressive modulus and durability of the modified samples.

Conclusions

GelMA hydrogels modified with different concentrations of ginger extract were prepared. The main difference between pristine GelMA hydrogel and ginger-modified hydrogels was observed in the mechanical properties, in which ginger extract could affect the compressive modulus resulting in softer hydrogels. The swelling ratio and degradation ratio decreased with increasing ginger-concentrations in the hydrogels, which resulted in better durability over time than the 10% GelMA hydrogel. Also, the surface turned into hydrophobic by increasing ginger-concentrations in the hydrogels, which could be related to a denser mesh with small pore size. Finally, the enhanced antibacterial property was identified on the hydrogels modified with higher concentrations of ginger extract (especially against *P. gingivalis*). GelMA hydrogels modified by the addition of natural extracts is considered as promising biomaterials, but further studies are required to investigate a clinical side effects in order to use as drug delivery systems against infections in the oral cavity.

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Original Article

Antibacterial effect of biodegradable gelatin methacryloyl loaded with ginger rhizome extract

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Gelatin methacryloyl 3d mesh mimics the natural extracellular matrix which allow loading as promising drug delivery systems. However, insufficient mechanical and degradation properties remain the biggest obstacle for this material application. In this study, a modified hydrogel with natural phytochemical was developed to improve the antibacterial effect by the addition of ginger extract, a natural spicy used in traditional medicine. GelMA hydrogels with ginger extract were fabricated and their chemical and morphological characteristics were analyzed by Fourier transformer infrared spectroscopy and scanning electron microscopy, structural characteristic were evaluated by compressive test and surface wettability analysis. *S. mutans, S. aureus* and *P. gingivalis* were used to confirm the antibacterial effect of the modified hydrogels. The FT-IR spectra of the hydrogels modified with ginger presented an increase in intensity of some peaks in comparison with the 10% GelMA hydrogel. The pores of ginger-modified hydrogels decreased it size which affected the hydrogels physical properties, decreasing the compressive modulus and increasing the durability, swelling ratio and, hydrophobicity of the surface. The ginger-modified hydrogels exhibited excellent antibacterial properties against *S. mutans* and *S. aureus* at high concentrations of ginger extract, while *P. gingivalis* presented a higher sensitivity at all tested concentrations. Hence, this study concludes that ginger-modified GelMA hydrogels presented better antibacterial effect, durability over time and, swelling stability.

Keywords : Gelatin, Hydrogel, GelMA, Ginger extract, Antibacterial activity.