Iranian Journal of Basic Medical Sciences
ijbms.mums.ac.ir

Therapeutic potential of a pullulan polysaccharide-based hydrogel with antibacterial peptide for skin wound healing

Maziar Malekzadeh [1], Mehrdad Moosazadeh Moghaddam [1], Masoud Ghorbani [1]*

[1] Tissue Engineering and Regenerative Medicine Research Center, New Health Technologies Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran

ARTICLE INFO

Article type:
Original

Article history:
Received: Jul 6, 2025
Accepted: Oct 25, 2025

Keywords:
Carboxymethyl chitosan
Hydrogel
Oxidized pullulan
Schiff base reaction
Skin regeneration

ABSTRACT

Objective(s): Eliminating skin defects using scaffolds, especially hydrogels, has been a promising method to accelerate regeneration. The utilization of hydrogels composed of carboxymethyl chitosan and pullulan oxide, synthesized via Schiff base reaction, represents an effective approach for skin repair.

Materials and Methods: Carboxymethyl chitosan was reacted with oxidized pullulan (CMCS/OPL), and the hydrogel was formed. In addition, an antibacterial peptide was incorporated into a hydrogel. In *in vitro* conditions, the samples were evaluated by a characterization test. *In vivo*, H&E and real-time PCR tests were taken from the removed samples. Experiments confirmed the formation of the Schiff base reaction.

Results: Two groups of hydrogels exhibited the appropriate viscosity, porosity, and degradation. The rate of cell proliferation on the 7th day in the hydrogel with the peptide was the same as that of the control group. An *in vivo* test showed that the peptide-containing hydrogel significantly enhanced the regeneration process ($P \leq 0.05$). Tissue examination of the samples showed that the rate of angiogenesis and skin repair increased in the hydrogel with the peptide group. The use of peptides strengthened the repair processes in the skin area.

Conclusion: These hydrogels represent a promising strategy to promote skin regeneration and provide a significant avenue for future clinical applications in regenerative medicine.

▶ Please cite this article as:
Malekzadeh M, Moosazadeh Moghaddam M, Ghorbani M. Therapeutic potential of a pullulan polysaccharide-based hydrogel with antibacterial peptide for skin wound healing. Iran J Basic Med Sci 2026; 29: 412-422. doi: https://dx.doi.org/10.22038/ijbms.2026.90215.19450

Introduction

Wound healing is a complex biological process that requires a specific microenvironment to proceed optimally. While minor wounds may heal spontaneously through the body's innate regenerative mechanisms, more extensive injuries often necessitate medical intervention (1).

When a burn injury occurs, the skin cells along with the underlying dermal layers are destroyed. This damage is often extensive, and the wound may become desiccated. At this stage, the application of an appropriate dressing is essential to promote optimal wound healing and accelerate tissue regeneration. One of the main goals of wound healing is to reduce healing time and minimize side effects (2). Currently, various dressings are used for wound treatment, such as fiber, sponge, hydrogel, foam, and hydrocolloid dressings. Hydrogels can provide mechanical support and a moist environment for wounds and are widely used in the biomedical field (3).

Hydrogels prepared from polymers are increasingly popular in tissue engineering and reconstruction due to their ability to enhance and support cell migration and proliferation, as well as their high biodegradability (4). Characteristics of an ideal scaffold for skin tissue engineering include mechanical strength, biocompatibility, biodegradability, and the ability to control the release of biological substances. Hydrogel scaffolds are one of the main external agents in the healing process (5). Hydrogels are considered the most viable therapeutic approach for chronic wounds. It is suspected that such products will soon go mainstream (6).

Hydrogels can create a moist microenvironment at the wound site, facilitate gas exchange, and absorb excess exudates. Hydrogels possess a three-dimensional network structure that enables substantial water retention and creates an optimal environment conducive to the wound regeneration process (7). In addition, hydrogels need to have a structure resembling natural extracellular matrix substrates to promote cell growth and facilitate nutrient exchange during the wound-healing process (8, 9).

Skin scaffolds should completely cover the wound surface and protect the wound from external influences and factors. In the majority of biological applications, hydrogels are derived from non-toxic, biocompatible, and biodegradable natural polymers due to their similarity to native tissue's physical properties (10). Polysaccharide backbones offer diverse molecular weights and multiple functional groups that enable customization of functions, substrates, and properties such as water solubility, bioadhesion, and bio

***Corresponding author:** Masoud Ghorbani. Tissue Engineering and Regenerative Medicine Research Center, New Health Technologies Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran. Email: Dr.ghorbani62@yahoo.com

recognition (7). In this study, two polymers, pullulan and carboxymethyl chitosan, were used to make a hydrogel. Pullulan is a polysaccharide recognized as a suitable polymer for tissue engineering due to its unique characteristics. It is a linear, neutral, and non-ionic glucose homopolysaccharide made up of maltotriose units linked together by both α-1,6 and α-1,4 glycosidic bonds. Its chemical formula is $(C_6H_{10}O_5)$ n. Pullulan exhibits biocompatibility and biodegradability, low toxicity, compatibility with hemocompatible materials, good adhesiveness, elasticity, thermal stability, and excellent barrier properties against oxygen and carbon dioxide, making it valuable in various applications. The proper chemical structure of this polymer makes it soluble in various types of solvents. This capability enables widespread use of this polymer in skin repair (11, 12). Carboxymethyl chitosan is one of the derivatives of chitosan that dissolves in water. To enhance the structural strength of the hydrogel, this polymer has been used (13).

Use of this compound in various tissue-engineering applications has demonstrated proper cell growth, differentiation, and repair of damaged tissue (14).Various methods for making hydrogels exist. A hydrogel based on the Schiff-base reaction is based on the bond between the aldehyde and amine group (15). Crosslinks, by themselves, can introduce toxicity into their composition, but the formation of a Schiff base between two functional groups does not introduce these disadvantages. The addition of a cross-linker can cause toxicity. Hence, the Schiff-base reaction strategy is performed without cross-linking, which is nontoxic (3).

One way to treat infections is to use antibiotics. However, excessive antibiotic use can lead to bacterial resistance. The researchers showed that using an antibacterial peptide along with a scaffold minimizes these problems (8, 10). Also, peptides play an essential role in cell stimulation and proliferation. In addition to its role in cell proliferation, an antibacterial peptide plays a vital role in destroying microbes and other small microorganisms. Eliminating microbes in the area of a wound or skin burn will be a factor in repairing the damaged tissue (16, 17).

There are different methods of making hydrogel. The Schiff base method is used to create a hydrogel with the desired strength without a crosslinker. Also, the combination of carboxymethyl chitosan and pullulan oxide yields a transparent hydrogel, allowing the wound to heal clearly within 14 days. In this study, a hydrogel prepared via a Schiff base reaction with an antibacterial peptide was used to reduce infection in burn wounds. CM11 peptide has been used as an antibacterial peptide in various experiments, drug delivery, and tissue engineering to reduce the burden of infectious agents. Due to the favorable properties of hydrogels and the peptide's antibacterial properties, this research has been used for wound healing (18). Hydrogel with the combination of pullulan and carboxymethyl chitosan, along with antibacterial peptide, can play an important role in improving burn wound healing, which we have investigated in this study.

Materials and Methods

Materials

Materials used in this research were Pullulan (1 g, Sinopharm, China), sodium periodate (1 g, 99% w/w, Sinopharm, China), Carboxymethyl chitosan (1 g, medium molecular weight, degree of deacetylation - 75–85 %, viscosity 200–800 cps, Merck, Germany), ethylene glycol (99% v/v, Merck, Germany) and CM11 peptide (32 µg /ml, Produced in Baqiyatallah University of Medical Sciences, Molecular Weight: 1179.46; M. Wt, 1179.46; Formula, C58H82N8O14S).

Oxidation of pullulan

In this study, 1 g of pullulan and 1g of sodium periodate were used for oxidation. Oxidation reaction was carried out under the following conditions: reaction temperature: 25 °C, pH 3.5, weight ratio of sodium periodate (Merck, Germany) to pullulan: 1/1 (reaction time: 4 hr). The quantity of aldehyde produced by the FTIR technique was determined at a reaction temperature of 35 °C (4 hr, pH 3.5), with a sodium periodate-to-pullulan weight ratio of 1:1. The details of the oxidation reaction were as follows, 1 g of pullulan was added to 100 ml of distilled water. It was stirred at room temperature until the solution became clear. Then, sodium periodate and pullulan were added to the container with a weight ratio of 1/1. Following that, 1 g of sodium periodate was added to the solution. The solution was adjusted to around 3.5, and half-molar sulfuric acid was used to increase the pH. The oxidation reaction was carried out at 35 °C, and the reaction vessel was wrapped in several layers of aluminum foil to prevent spontaneous oxidation from exposure to light. The oxidation reaction lasted for 4 hr. Then, 100 µl of ethylene glycol were added to the prepared solution, and the mixture was stirred for 30 min. After oxidation, oxidized pullulan was precipitated with isopropanol to obtain a solid form. Finally, to dry, the samples were placed in a freeze-drying device (duration: 48 hr, temperature:50 °C, vacuum pressure: 0.1 mbar) (15).

Hydrogel fabrication with and without the antibacterial peptide CM11

This process involves the composition of two essential components: Aldehydes and Amines. The reaction between these functional groups forms imine groups. Upon generating the aldehyde compound within pullulan through oxidation, 0.1 g of the oxidized pullulan powder was immersed in 2 ml of purified water to present aldehyde groups to subsequent reactions, which are available for reaction with amine groups. After complete dissolution of pullulan oxide in distilled water, 0.1 g of carboxymethyl chitosan (Merck, Germany) was transferred for hydrogel formation. This hydrogel was made without CM11. However, to make a hydrogel with an antibacterial peptide, we added 32 µg /ml of CM11 peptide (Produced in Baqiyatallah University of Medical Sciences, Molecular Weight: 1179.46; M.Wt, 1179.46; Formula, C58H82N8O14S) to the solution (19, 20). The resulting ingredients are put into a round-bottom container to take the shape of the container. The gelation time was measured and recorded. Hydrogel *in vitro* test included two groups: one that included hydrogel with and without CM11.

Fourier transform infrared spectroscopic (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) is a technique employed to acquire the infrared absorption or emission profile of various substances, including solids, liquids, and gases. FTIR spectrometers (Huazheng, China) can simultaneously acquire high-spectral-precision data across an extensive wavelength range. This feature provides

a notable advantage over dispersive spectrometers, which can only measure intensity within a limited wavelength range at a time. Wavelengths were measured in the range of 500–4000 cm^{-1}.

Scanning electron microscopy (SEM)

To evaluate the microscopic structure of the hydrogel and the relationship of its porosity, an assessment was done with SEM (XL30, Philips, Netherlands). Since the samples were non-conductive, they were coated with gold. The porosity dimensions were assessed using ImageJ.

Rheometry test

Texture Analysis stress-controlled AR-G2 rheometer (ARR 150 ex, Germany) was utilized to conduct small-scale oscillating shear experiments. To carry out stress and frequency scans, the gels were relocated to the Peltier platform and adjusted to fit a 40mm parallel plate configuration. Prior to frequency sweeps, amplitude sweeps were conducted to maintain the experiments within the linear viscoelastic range. A stress sweep test was performed by linearly increasing the shear stress from zero to a value well above the yield stress. The storage and loss moduli were measured by conducting dynamic frequency scans at 25 °C, with a stress amplitude of 0.1% and a frequency range of 0.1–100 rad/s (16).

Degradation rate of hydrogels

The prepared hydrogel's degradation rate was assessed by soaking the samples in PBS (CinnaGen, Iran) at 37 °C. The percentage of weight loss was measured at different time points, and the sample weight was reported as a percentage of the initial sample weight (N=3). Percentage of degradation is obtained based on the following relationship: (W0= initial weight, Wt= Secondary weight)

$$\text{Degradation rate (W\%)} = ((W0 - Wt) \div W0) \times 100\%$$

Hydrogel water absorption

To evaluate the swelling, samples were immersed in distilled water at room temperature for 24 hr. Then, the sample was weighed in the wet state (Ww) and allowed to dry and reach a constant weight (Wd). The inflation ratio was calculated by equation (N=3):

$$\text{Swelling ratio (\%)} = ((Ww - Wd) \div Wd) \times 100\%$$

***In vitro* cytotoxicity**

The MTT (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide) test was used to evaluate the toxicity of the hydrogel. Cells (mouse bone marrow mesenchyme stem cells, Pasteur Institute cell bank, Iran) were cultured in DMEM/F12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, Merck, Germany) medium supplemented with 10% FBS (Sigma-Aldrich, Germany) and 1% penicillin-streptomycin. Third passage cells were used for this research. To investigate the cytotoxicity of hydrogels using the MTT method, 96-well plates were used (N=3). For sterilization, hydrogels were sterilized under UV light for 20 min (one side of the scaffold every 10 min). After incubating the scaffolds in the culture medium used, DMEM/F12 containing 10% FBS and 1% penicillin-streptomycin, the culture medium was replenished to prevent contamination. Cells previously cultured in the flask were separated using trypsin. 10^4 cells were added to each well. After the specified period, MTT was removed, and DMSO (Dimethyl sulfoxide, Sigma-Aldrich, Germany) was added to each well to dissolve the formazan sediment. The solutions were then transferred to 96-well plates. The absorbance of the samples was measured using an ELISA reader at 595 nm. The toxicity of hydrogels was assessed on days 1, 3, and 7.

Antibacterial activity of hydrogels

Disc diffusion method used to evaluate the antibacterial properties of hydrogels. A piece of hydrogels containing CM11 peptide was placed on the surface of solid agar plates already seeded by Staphylococcus aureus (ATCC 25923) and Pseudomonas aeruginosa (ATCC 27853). Two controls were included, positive control (ciprofloxacin, 5 µg) and negative control (blank disc), and incubated for 24 hours at 37 0C. The proliferation rate of S. aureus and P. aeruginosa around the hydrogels was checked and photographed with a digital camera to measure the antibacterial activity.

Creating a burn wound and applying hydrogel

A total of 18 Male mice (NMRI mice) aged 6–8 weeks and weighing between 25–35 g were obtained from Iran University of Medical Sciences. All conditions applied to mice were performed under the National Research Council's Guide for the Care and Use of Laboratory Animals. (IR.BMSU.AEC.1402.018). All animals were anesthetized by intraperitoneal injection of xylazine (10 mg/kg; Alpasan, Netherlands) and ketamine (100 mg/kg; Alpasan, Netherlands). The animals' backs were shaved with a 10% povidone-iodine disinfectant solution. A cylindrical rod measuring 1 cm^2 was placed on the shaved skin of the mice's backs and held for 20 sec (the burn model temperature was 105 ℃). The control group was treated without hydrogel application, while the experimental groups consisted of mice with burn injuries that received hydrogel treatments containing CM11 or none. On the 14^{th} day, alterations in the defect region were photographed (Figure 1). The amount of repair in all images was evaluated using ImageJ size analysis software.

Histology evaluation

At the 7th and 14th days, the restored tissue was taken out, and the specimen was preserved in formalin (10% v/v) for 2 days. Following that, the samples were dehydrated with ethanol and subsequently cleaned in xylene. After embedding in paraffin, the sample was cut into 5 µm sections. The Hematoxylin & eosin (H&E) staining (Merck, Germany) was assessed by examining the stained areas under a light microscope.

Extraction and cDNA synthesis

As per the manufacturer's guidelines (Invitrogen, San Diego, CA), mouse tissue samples were used to extract RNA. This action was performed using the Micro-Fast Track mRNA extraction kit from Merck (Germany). After being placed in 10 µl of distilled water, the RNA's concentration was determined by directly using light absorption at 260 nm. Subsequently, reverse transcriptase was used to convert a portion of the mRNA into cDNA, which was then amplified using specific primers.

Real-time RT-PCR

The NCBI Primer-BLAST (COL1: NC_000017.10, VEGF: NC_000006.12, GAPDH: NC_000012.11) tool was used to

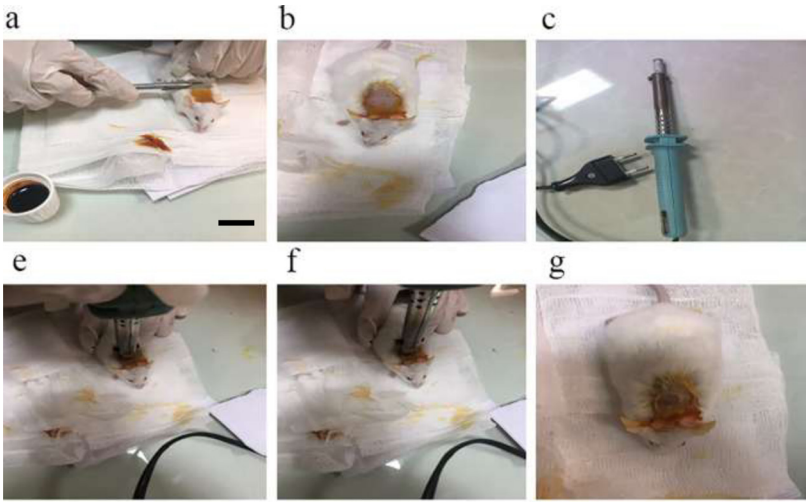

Figure 1. (a-g) The researchers created a burn model on the backs of mice by shaving and disinfecting them with a solution
A 1 cm-diameter rod was placed on the shaved skin, and hot water was poured into it, allowing it to sit for 20 sec. The area was then treated with a hydrogel dressing and covered. The changes in the burned area were photographed 14 days later (Scale bar: 3 cm)

Table 1. Primer sequence of mouse genes (21, 22)

Gene	Primer sequence (5 '→3 ')
COL1	F' TCCGACCTCTCTCCTCTGAA
	R' GAGTGGGGTTATGGAGGGAT
VEGF	F' AGCGGAGAAAGCATTTGTTTG
	R' AACGCGAGTCTGTGTTTTTGC
GAPDH	F' TGACATCAAGAAGGTGGTGAAGC
	R' CCCTGTTGCTGTAGCCGTATTC

develop gene-targeted primers, and we chose GAPDH as a housekeeping gene (Table 1). We assessed primers using Oligo Analyzer and conducted real-time RT-PCR with a QuantiTect SYBR Green RT-PCR kit (Qiagen, France) on an ABI Step One instrument (Foster, USA). In the real-time RT-PCR process, the $2^{-\Delta\Delta Ct}$ method was employed to assess the outcomes (N=3), as it is a convenient way to analyze relative changes in gene expression from real-time quantitative PCR experiments.

Statistical analysis

Graphpad Prism (8.0.1) was used to assess differences between samples. A value of $P \leq 0.05$ was considered statistically significant (* $P<0.05$, ** $P<0.01$, and *** $P<0.001$)

Results

Gelation time

The gelation time of hydrogels was controlled at 25 ℃. For both hydrogels, gelation time occurred within 9±2 sec. Hydrogels are formed based on the Schiff base reaction in all groups. In this case, the amine group of carboxymethyl chitosan is combined with the aldehyde group of pullulan oxide. This combination led to the formation of an *in situ* hydrogel. The viscosity of the hydrogels was suitable, and the formed consistency was gel-like. Interestingly, the gelation time was similar in both cases, indicating that CM11 addition did not affect the process. The Schiff base reaction, which is responsible for the formation of gelation, occurred efficiently in both types of samples (Figure 2).

Fourier transform infrared spectroscopic analysis (FTIR)

FTIR results of carboxymethyl chitosan, pullulan oxide, and CMCS/OPL hydrogels are shown in Figure 3. Carboxymethyl chitosan displays three peaks at 3420, 1610, and 1426 cm^{-1} in its spectrum, which correspond to N-H stretching vibrations and asymmetric and symmetric vibrations, respectively. O-H and N-H stretching vibrations contributed to the broad peak in carboxymethyl chitosan at 3200–3400 cm^{-1}, and C-H stretching vibrations caused the

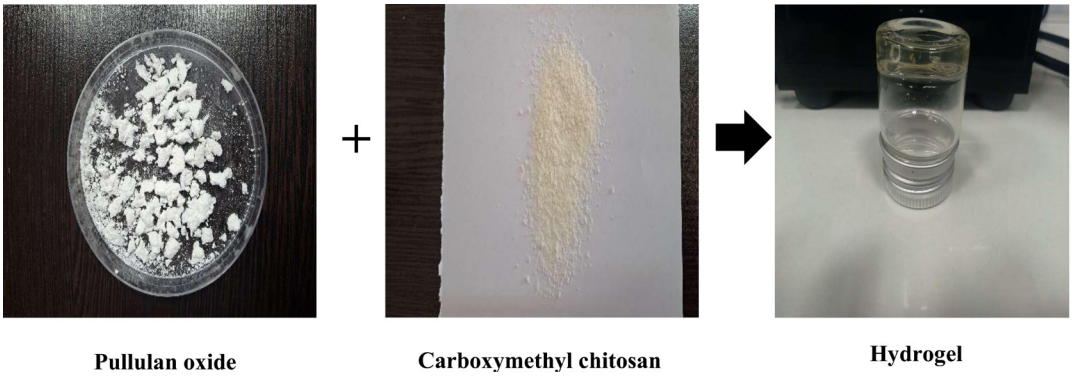

Figure 2. Created a hydrogel by combining two polymers and allowing them to react *in situ*, forming a Schiff base
Interestingly, the time to gelation was similar whether or not CM11 was present, suggesting that the peptide didn't influence the gelation process.

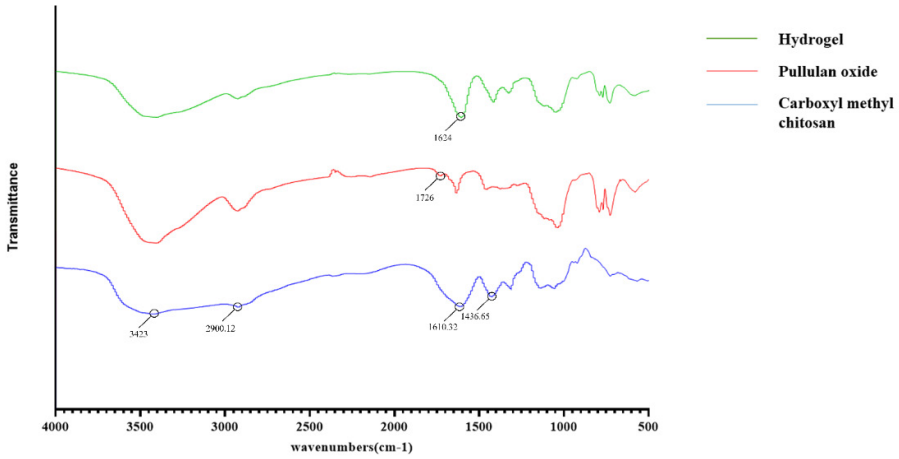

Figure 3. FTIR result of carboxymethyl chitosan, pullulan oxide, and CMCS/OPL hydrogels
The peak at 1730 cm^{-1} indicated the oxidation of pullulan, attributed to the aldehyde groups of OPL. Next, a main Schiff base structural band (C=N) appeared at about 1600 cm^{-1} in the hydrogel spectrum. Wavelengths were measured in the range of 500-4000 cm^{-1}. CMCS/OPL: Carboxymethyl chitosan/pullulan

broad peak at 2900 cm^{-1}. Also, the peak at 1726 cm^{-1} appears after the oxidation of pullulan, due to the C--O stretching vibration of the aldehyde groups of OPL. Next, a main open Schiff structural band (C=N) appeared at about 1600 cm^{-1} in the hydrogel spectrum.

Scanning electron microscopy (SEM)

After examining the desired photos under an electron microscope, pores were observed. The hydrogels were porous, with interconnected pores. The pore size was suitable for cell proliferation. Hydrogel porosity is essential for cell proliferation, differentiation, and angiogenesis. The pore size in the samples was approximately 100 µm (Figure 4).

Degradation rate of hydrogels

After data analysis, no significant difference was observed between the study groups. Also, in both groups, a suitable percentage of damage was observed in the skin tissue on the 14th day. Figure 5 shows the percentage of hydrogel degradation in the groups. On day 14, the hydrogel degradation rate in the group with and without peptide was

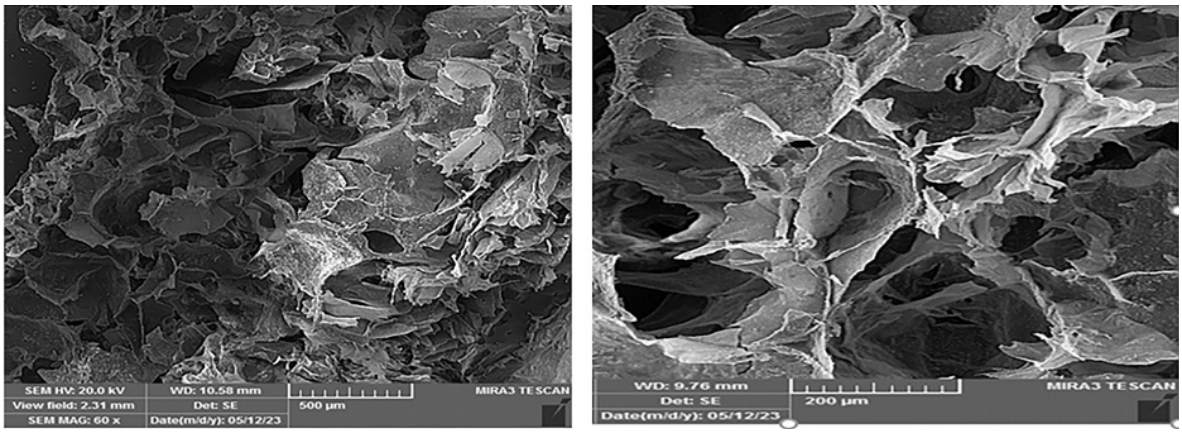

Figure 4. Examining the desired photos using an electron microscope
Hydrogel porosity images by SEM. The amount of hydrogel porosity is also suitable for cell proliferation and differentiation (Magnification: 500 & 200 µm)

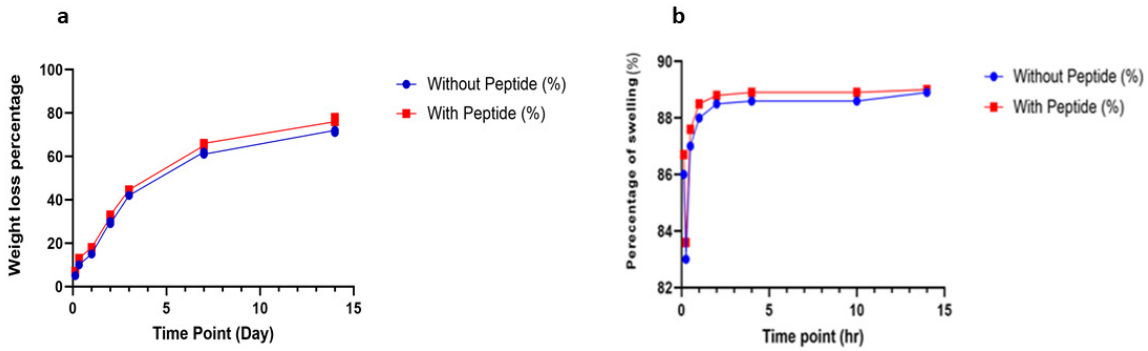

Figure 5. a. Investigating the degradation rate of hydrogels. No significant difference between the studied groups (P≥0.05, ANOVA test). b. Hydrogel water absorption test
Due to the hydrophilic functional groups in the materials, they show a high swelling ratio.

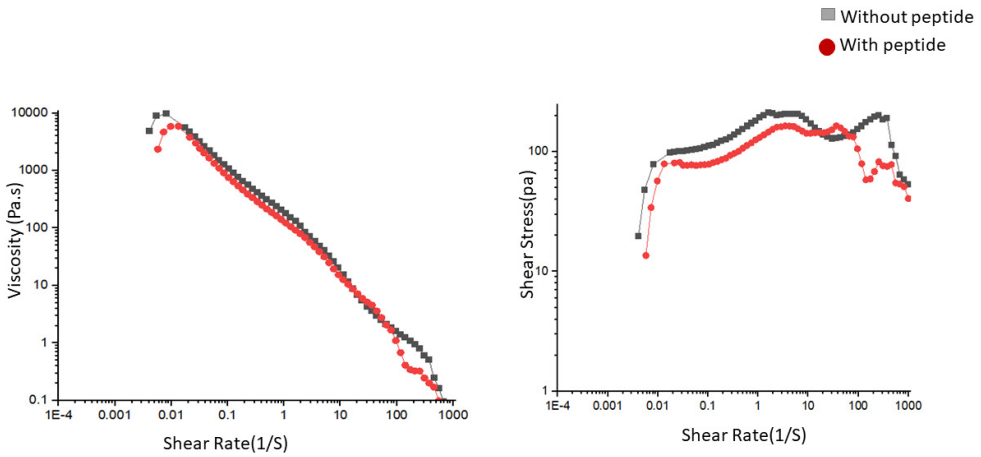

Figure 6. The hydrogel without CM11 exhibited slightly higher viscosity at low shear rates
Hydeogel with peptide, G'(storage modulus) = 103, G" (loss modulus) =102. Hydrogel without peptide G'= 102, G" =101 (≤ 0.1 s-1), but no significant difference between groups (P≥0.05, ANOVA test) was observed.

76% and 72%, respectively.

Hydrogel water absorption

Hydrogels synthesized via Schiff-base cross-linking between OPL and CMCS exhibit a high swelling ratio, attributable to the presence of hydrophilic functional groups within the constituent materials. In hydrogels, water absorption reached a steady state after the initial few hours, with no significant changes observed at subsequent time points. At the 14th hr, the water absorption percentage in both hydrogels was about 90%, a value observed when the hydrogels have suitable pores. Figure 5 shows this; both types of hydrogels had similar pore sizes, leading to the same swelling behavior.

Rheometry test

Rheometry examines the effect of peptide presence on the viscosity of hydrogels. The shear viscosity in relation to shear rate for OPL-CMCS hydrogel and hydrogel-peptide is demonstrated in Figure 6. As the shear rate rises, both viscosities decrease, indicating typical shear-thinning behavior, a sign of good injectability. The hydrogel without CM11 exhibited slightly higher viscosity at low shear rates (≤ 0.1 s^{-1}), but the differences were not significant. This might be because, in the hydrogel, all OCMCs were used for cross-linking with the -NH2 groups of CMCS, whereas in the hydrogel-peptide, some peptide -NH2 groups remained uncross-linked. This could lead to a weak, physically unstable assembly involving non-covalent interactions, thereby reducing viscosity.

Antibacterial activity of hydrogels

In this study, two Gram-positive and Gram-negative bacteria used. As seen in Figure 7, the zone formed around the hydrogel containing the antibacterial peptide is clearly visible for the two bacteria. Also, a clear zone was formed around the positive control (ciprofloxacin, 5 µg). This is while no inhibition zone was formed around the hydrogel without peptide and the negative control (blank disc).

In vitro cytotoxicity

After reviewing the data and analyzing the MTT test, the survival rate and cell proliferation have increased in all three groups on days 3 and 7. Cell proliferation and survival rate on day 7 in the hydrogel with the CM11 group were significantly different from those in the other groups (P≤0.05). On the 1st and 3rd days, there was no significant difference in cell viability among the groups (Figure 8).

Wound repair

To evaluate wound healing, the studied groups were treated using a hydrogel with and without CM11. In the control group, the hydrogel was not applied to the burn area

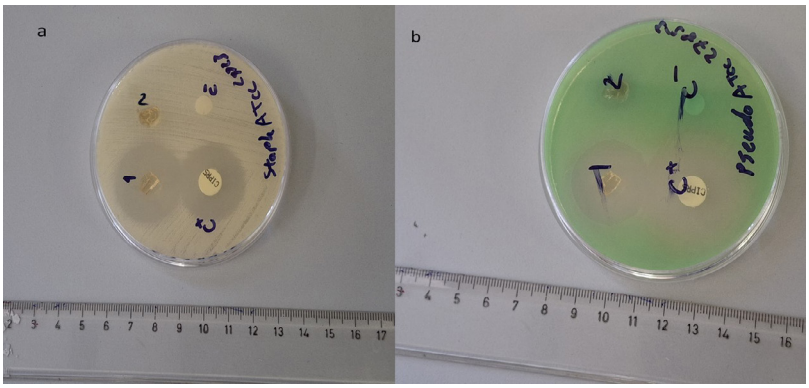

Figure 7. a). Staphylococcus aureus (ATCC 25923). b) Pseudomonas aeruginosa (ATCC 27853), antibacterial test
The antibacterial property of a hydrogel containing CM11 was demonstrated through a halo test using two types of bacterial. As shown in the accompanying Figure, the hydrogel with CM11 clearly exhibited a visible halo in 24 hr. C-= Negative control (blank disc), C+= Positive control (ciprofloxacin, 5µg), 1= Hydrogel with antibacterial peptide, 2= Hydrogel without antibacterial peptide.

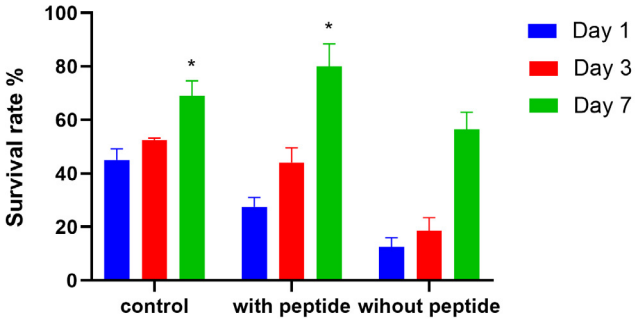

Figure 8. The survival rate (%) of cells increased in all three groups on days 3 and 7

The increased cell survival on day 7 in the hydrogel with the CM11 group was significant compared to other groups ($P \leq 0.05$). On days 1 and 3, the survival rates of cells across groups did not differ significantly.

after the burn. First, the mice were subjected to a second-degree burn at the injury site. After that, wounds were exposed to hydrogel (Figure 9). In each group, the number of hydrogel replacements on the wound was twice, until the 14th day. The rate of recovery varied between different groups. After 7 days of burn skin intervention, the wound-healing rate was 21±2.9 in the CM11 group, which was not significantly different from that in the control group. At day 7, the wound-healing percentage in the hydrogel group incorporating CM11 was 72±1.4%, which was substantially higher than in the other groups. On day 14, the rate of wound closure and healing rate in the control group was 24±8.4%. Meanwhile, the healing rate of the burn wound in the hydrogel without CM11 was 43.3%, which was significantly higher than that of the control group. In the CM11 group, the regeneration rate was 96±1.1%, which was considerably higher than in the other groups. In the control group, the wound was not closed morphologically, and improper coagulation took place. Scars were clearly visible in this group, indicating a lack of proper skin tissue regeneration. In the hydrogel group without peptide, the amount of scar formed and granulation was less than that of the group without hydrogel. In the hydrogel containing CM11 group, granulation and scars were not observed, and the skin was properly repaired.

Histology evaluation

To evaluate the therapeutic effect of the antibacterial CM11, pathological changes in the wound bed were assessed in each group at different time points (Figure 10). On day 7, the control and hydrogel groups without CM11 showed significant inflammation, with the epidermis and dermis not properly regenerated and no accessory glands in the dermis. Also, the number of inflammatory cells was

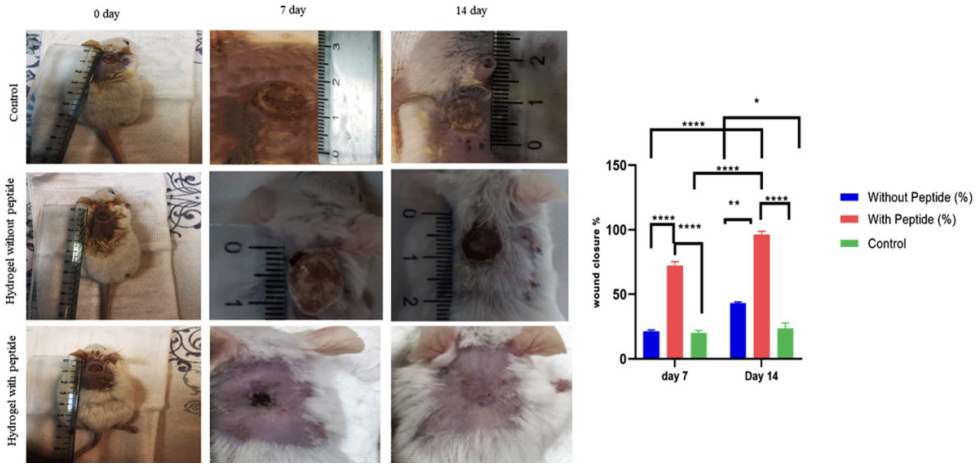

Figure 9. a) Images of wound healing after creating an animal model on days 7 and 14 in different groups using hydrogels with and without CM11. b) Wound healing percentage on days 7 and 14 and comparison of healing percentage in different groups.

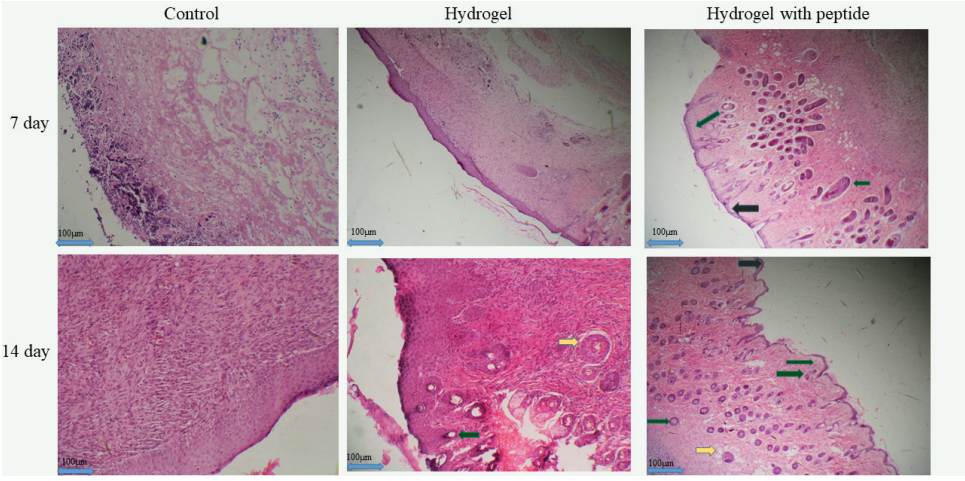

Figure 10. H&E staining 7 and 14 days after creating the animal model in different mouse groups

In a study comparing different hydrogel groups with an antibacterial peptide, the results showed that the group with the antibacterial peptide had a faster, more effective healing process than the other groups, particularly on days 7 and 14. The diagram features arrows indicating the different skin structures, sebaceous glands, glandular dermis, and epidermis, which are labeled as yellow, green, and black, respectively (scale bars: 100 µm)

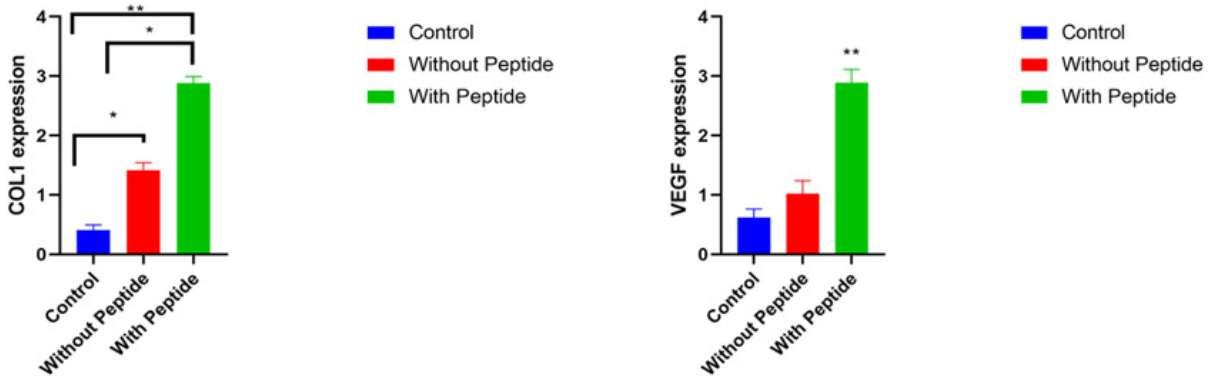

Figure 11. Converting a portion of mouse mRNA into cDNA
In the real-time RT-PCR process, the $2^{-\Delta\Delta Ct}$ method was employed to assess outcomes (N=3), as it is a convenient way to analyze the relative changes in gene expression from real-time quantitative PCR experiments. The expression level of VEGF and COL1 genes after 14 days of creating the burn model. In the group treated with the antibacterial peptide, the levels of gene repair and expression were significantly different from those in other groups ($P \leq 0.05$).

reduced in the hydrogel group containing the antibacterial peptide. In the hydrogel groups, re-epithelialization was observed 14 days after intervention, and epithelial thickness was normal compared with the control group. Additionally, in the control group, the newly formed epidermis appeared as granulation tissue. Compared with other groups, the hydrogel containing an antibacterial peptide showed skin tissue regeneration (Figure 10). Dermis and epidermis are completely defined with regular epidermal structure and hair follicles, which are normal skin appendages.

Real time – RT PCR

On the 14th day after creating the burn model, samples from the studied groups were collected to evaluate genes involved in skin development. The relative expression of the VEGF gene in the hydrogel treatment group with CM11 was significantly higher than in the other groups ($P \leq 0.05$). The expression level of this gene in the hydrogel treatment group without CM11 was not substantially different from that of the control group. The level of COL1 gene expression in the hydrogel with the CM11 group showed a significant difference compared with the treatment (without CM11) and control groups (Figure 11). Also, the expression level of this gene in the treatment group (without peptide) was significantly different from that in the control group ($P \leq 0.05$) (Gene expression measurements were obtained based on ΔCT data and the $2^{-\Delta\Delta Ct}$ formula).

Discussion

Various studies have been conducted to advance wound or burn regeneration. Recently, multiple studies have been conducted in the field of hydrogels, burn, and wound healing. Hydrogels have attracted the attention of researchers due to their properties of water retention and controlled drug release. The fabrication of biocompatible, transparent hydrogels from natural materials can effectively promote tissue regeneration at defect sites. Various methods are used to make hydrogels, including the Schiff base reaction (23). In this study, the Schiff base technique was used to create hydrogels. The Schiff base method involves the condensation reaction between aldehyde and amine compounds, resulting in the formation of an imine functional group. It is covalently bonded without the use of any toxic cross-linkers. However, to demonstrate the formation of the Schiff base, we used FTIR. In this method, the wavelength range was 4000-500 nm. The Schiff base reaction should give a strong peak in the 1600 nm range. At the same time, when this strong peak is formed, the aldehyde functional bond must also be disappearing after the formation of the Schiff base bond. These data, obtained from different studies, were consistent with the results of the present study. Sodium periodate is used to oxidize the PL, producing aldehyde groups. Amine groups can react with these aldehyde groups to form a covalent crosslink. This covalent cross-linking provides excellent stability, maintaining the hydrogel structure. FTIR analysis confirmed that aldehyde and amine groups were successfully incorporated into pullulan oxide and carboxymethyl chitosan, respectively (24, 25). Additionally, amine groups in carboxymethyl chitosan and aldehyde groups in oxidized pullulan were observed in the FTIR results. After confirming these results by FTIR, we began forming the hydrogel, which appears in the 1600 nm range, as mentioned. Also, Mehrabi *et al.* (26), who utilized this technique, observed Schiff base bonds in this range after preparing the hydrogel. They used it to treat wounds, and the effects were clearly observed in skin repair. This process was based on the Schiff base reaction. (26, 27).

In the present study, hydrogels produced with or without peptide showed equal water absorption. In addition to the swelling mechanism, water absorption in hydrogels results from the hydrophilic functional groups present on the surface and within the hydrogel, as well as the hydrogels' porosity, which traps water and causes them to swell (28). An essential characteristic of hydrogels in biomedical applications is their ability to absorb large quantities of water while preserving moisture and maintaining their structural integrity, which is necessary for tissue engineering and drug delivery systems. Chu *et al.* demonstrated that the application of these hydrogels can accelerate the healing process of tissue defects. When the hydrogel is placed in water, its hydrophilic nature causes water molecules to rapidly enter the structure and be absorbed by the functional groups (29). This has been proven in various studies. Lan *et al.* (30), after investigating the degradation rate of the produced hydrogel, concluded that the hydrogel degrades via hydrolysis upon exposure to an aqueous environment (30, 31). In this study, the hydrolysis speed was high until the 7th day. However, from days 7 to 14, since there were only a few breaks in the Schiff base bonds during degradation, they were unable to destroy the hydrogel matrix. Another cause was the degradation of parts dissolved from the hydrogel over time. By the 14th day, the bonds progressively degraded, and

the cleavage of the Schiff base reaction led to the complete disruption of the cross-linked network, disintegrating the hydrogel. (26, 32).

There was no significant difference in the degradation percentage of the two types of hydrogel. The present study showed that CM11 did not affect porosity, swelling, or degradation. Since this peptide neither functions as a crosslinker, a reinforcing agent, nor integrates into the hydrogel's chemical structure, no significant differences were observed between the groups (33). Various mechanical tests are used for biomaterials, but rheology tests are predominantly used to evaluate the mechanical properties of hydrogels. Evaluating the mechanical properties of hydrogels is crucial, as scaffolds must be durable enough to support cell growth. In fact, it can be said that the mechanical property of a scaffold affects its cell proliferation and differentiation process (34). Adding a substance or growth factor can alter a material's mechanical properties. Studies have demonstrated that adding a material can affect its mechanical strength, with the extent of the change depending on the material's role in the hydrogel composition. Rheological tests showed that the storage modulus was constant in both samples, with and without peptide. The rheology test results showed that adding the peptide to the hydrogel did not affect mechanical strength. This could be because this peptide was not used as a crosslinker or stabilizer. In fact, the peptide used has been utilized as a drug or as a restorative (35, 36).

In this study, it was demonstrated that both hydrogels exhibit favorable mechanical and viscoelastic properties for skin tissue regeneration. Results indicate that the inclusion of a CM11 did not alter the mechanical properties of the hydrogels used for the regeneration of skin defects. This is in contrast to earlier research on other antibacterial peptides and their impact on mechanical properties (37). These peptides can be used as reaction stabilizers in combination with other substances. The presence of amine and hydrophilic functional groups in the hydrogels facilitates the continuous and stable release of this peptide. Given this property, the appropriate degradability of the hydrogels, and the optimized amount loaded, the peptide is released. It is therefore essential to understand the role of a growth factor or peptide in the hydrogel structure (10, 18).

In this study, it was shown that the release of CM11 from the scaffold does not have cytotoxic effects on the cells (Figure 8). In addition, this antibacterial peptide was found to be a stimulator of cell proliferation. As a result, scaffolds containing this peptide showed the potential to stimulate cell proliferation. Although the cell proliferation rate was decreased in the CMCS/OPL group compared to the CMCS/OPL antibacterial peptide group, no cytotoxicity was observed. OPL and CMCS had no adverse effect on cell proliferation in either group. Zhao *et al* showed that the use of antibacterial peptides can play a role in accelerating the wound healing process. In fact, after loading the peptide into the hydrogel, its contents will come out of the formed pores and will play a role in accelerating the healing process by reducing the bacterial load around the wound (38).

In *in vivo* studies, the number of mice (n=18) was selected based on the feature and previous studies. In skin regenerative studies, this number of mice has been used to ensure tissue regeneration (3,26). *In vivo* studies have shown that burn wounds are prone to infection. The findings indicated that using an antibacterial peptide had beneficial effects on burn injury healing. According to this, it could be argued that using a potent antibacterial polymer creates a clean environment around the wound site and accelerates the regenerative and epithelialization processes. The H&E-stained results showed that this granular substrate creates a porous, moist environment, which accelerates tissue regeneration. In addition, the antibacterial peptide is designed to recognize integrin receptors on cells, providing mechanical and structural support for new tissue growth. (39). In contrast, the groups that were infected exhibited regeneration with an incomplete formation of the epidermal layer (40).

In Real Time - RT-PCR tests examining COL-1 and VEGF genes, results showed that CM11 improved wound healing by increasing gene expression. During remodeling, COL1 expression is increased in the skin. The increase in expression of this gene in the group, together with the peptide, indicates that skin repair is well underway and the repair process is taking shape (41, 42). In addition, this protein plays a role in improving angiogenesis and blood supply in damaged tissue by regulating angiogenesis through the TGF-β/VEGF pathway. CM11's modulation of the TGF-β/VEGF crosstalk can control new blood vessel formation. CM 11 could modify the extracellular matrix or cell adhesion molecules, influencing how TGF-β and VEGF signals are received and integrated. Thus, this protein plays a role in tissue regeneration by regulating angiogenesis. As a result, CM11 peptides not only reduce infection and inflammation at the wound site but also indirectly stimulate fibroblast and epithelial cell activity, thereby accelerating tissue regeneration (43).

Conclusion

In this study, a hydrogel was formed via a Schiff base reaction. In the characterization of this hydrogel, water absorption, destruction, and the appropriate degree of porosity were observed. No toxicity was detectable. Furthermore, CM11 showed antibacterial properties in both the hydrogel and in vitro. Hydrogels, along with antibacterial peptides, have been shown to accelerate wound healing. Healing rate of the burn wound on day 14 in the hydrogel group lacking CM11 was 43.3 ± 3. However, in the CM11 group, the regeneration rate was 96±1.1. This indicates that the hydrogel with CM11 contributed to burn wound healing. In addition to *in vivo*, tissue regeneration has been evident in H&E tests. In real-time RT-PCR, the group containing CM11 showed increased expression of COL1 and VEGF, indicating the effective role of the hydrogel combined with CM11 in skin regeneration. Finally, we concluded that a hydrogel based on the pullulan polysaccharide, combined with the aforementioned peptide, was non-toxic and had regenerative properties. To conclude, this study also had some limitations. Among these, the limited access to a large number of mice due to ethical considerations, the high cost of experimental tests (*in vivo* & *in vitro*), and the high cost of materials may be noted.

Acknowledgment

We would like to thank Baqiyatallah University of Medical Sciences, Tehran, Iran. We have received no funding for this research.

Funding

We did not receive any funding for this research.

Authors' Contributions

All authors contributed to the study conception and

design Data Processing, Collection. Perform experiment M M. Supervision, Funding Acquisition M MM, M Gh and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

There are no conflicts of interest or personal relationships among the authors that are known to have influenced or caused a conflict of interest in this work.

Declaration

We have not used any AI tools or technologies to prepare this manuscript.

References

1. Noori A, Afzal A, Masood R, Khaliq Z, Ahmad S, Ahmad F *et al.* Dressings for burn wound a review. J Mat Sci 2022; 12: 6536-6572.
2. Ladhani HA, Yowler CJ. Burn wound colonization infection and sepsis. Surg Infect (Larchmt) 2021, **22**: 44-48.
3. Karimi A, Peyravian N, Delattre C, Ghasemian M, Michaud P, Amini N, *et al.* Designing and synthesis of *in situ* hydrogel based on pullulan/carboxymethyl chitosan containing parathyroid hormone for bone tissue engineering. Materialia 2024; 33: 102026.
4. Kopecki Z. Development of next-generation antimicrobial hydrogel dressing to combat burn wound infection. Biosci Rep 2021; 41: 1-5.
5. Yao Y, Zhang A, Yuan C, Chen X, Liu Y. Recent trends on burn wound care: Hydrogel dressings and scaffolds. Biomater Sci 2021; 9: 4523-4540.
6. Yin B, Gosecka M, Bodaghi M, Crespy D, Youssef G, Dodda JM, *et al.* Engineering multifunctional dynamic hydrogel for biomedical and tissue regenerative applications. J Chem Eng 2024; 487: 150-403.
7. Yu Q, Sun H, Yue Z, Yu C, Jiang L, Dong X, *et al.* Zwitterionic polysaccharide-based hydrogel dressing as a stem cell carrier to accelerate burn wound healing. Adv Healthc Mater 2023; 12: 220-230.
8. Dong M, Mao Y, Zhao Z, Zhu L, Chen L, Cao L. Novel fabrication of antibiotic containing multifunctional silk fibroin injectable hydrogel dressing to enhance bactericidal action and wound healing efficiency on burn wound *in vitro* and *in vivo* evaluations. Int Wound J 2022; 19:679-691.
9. Fayyazbakhsh F, Khayat MJ, Leu MC. 3D-printed gelatin-alginate hydrogel dressings for burn wound healing: A comprehensive study. Int J Bioprint 2022; 8:110-115.
10. Mehrabi A, Jalise SZ, Hivechi A, Habibi S, Kebria MM, Haramshahi A, *et al.* Evaluation of inherent properties of the carboxymethyl cellulose (CMC) for potential application in tissue engineering focusing on bone regeneration. Polym Adv Tech 2024; 35: e6258.
11. Pan X, Cheng S, Zhang C, Jiao Y, Lin X, Dong W, *et al.* Mussel-inspired magnetic pullulan hydrogels for enhancing catalytic degradation of antibiotics from biomedical wastewater. J Chem Eng, 2021; 409: 128203.
12. Teixeira MO, Marinho E, Silva C, Antunes JC, Felgueiras HP. Pullulan hydrogels as drug release platforms in biomedicine. J Drug Deliv Sci 2023; 89: 105066.
13. Cao d, Wu p, Cheng Q, He C, Chen Y, Zhou J. Ultrafast fabrication of self-healing and injectable carboxymethyl chitosan hydrogel dressing for wound healing. ACS Appl Mater Interfaces 2021; 13: 24095-24105.
14. Jing H, Huang X, Du X, Mo L, Ma C, Wang H. *F*acile synthesis of pH-responsive sodium alginate/carboxymethyl chitosan hydrogel beads promoted by hydrogen bond. Carbohyd Polym 2022; 278: 118993.
15. Zhao Y, Li R, Liu Y, Song L, Gao Z, Li Z, Peng X. An injectable, self-healable, antibacterial, and pro-healing oxidized pullulan polysaccharide/carboxymethyl chitosan hydrogel for early protection of open abdominal wounds. Int J Biol Macromol 2023; 250: 126282.
16. Cardoso P, Glossop H, Meikle TG, AburtoMedina A, Conn CE, Sarojini A, *et al.* Molecular engineering of antimicrobial peptides: Microbial targets, peptide motifs and translation opportunities. Biophys Rev 2021; 13: 35-69.
17. Rai A, Ferrão R, Palma P, Patricio T, Parreira P, Anes E, *et al.* Antimicrobial peptide-based materials: Opportunities and challenges. J Mat Chem B 2022; 10: 2384-2429.
18. Zhang X, Qin M, Xu M, Miao F, Merzougui C, Zhang X, *et al.* The fabrication of antibacterial hydrogels for wound healing. Eur Polym J 2021; 146: 110268.
19. Moosazadeh Moghaddam M, Farhadie B, Mirnejad R, Kooshki H. Evaluation of an antibacterial peptide-loaded amniotic membrane/silk fibroin electrospun nanofiber in wound healing. Int Wound J 2023; 20: 3443-3456.
20. Ramandi M, Piranfar V, Nadoushan M, Sarshoori J, Misialek M, Heiat M, *et al.* Dose-response effects of the CM11 as a short cationic antimicrobial peptide on histopathological and biochemical changes in mice. Cur Cheml Biol, 2017. **11**: p. 150-157.
21. Zhang X, Meng Y, Shen W, Dou J, Liu R, Jin Q, Fang S. pH-responsive injectable polysaccharide hydrogels with self-healing, enhanced mechanical properties based on POSS. React Func Polym. 2021;158:104773.
22. Zhang M, Chen G, Lei M, Lei J, Li D, Zheng H. A pH-sensitive oxidized-dextran based double drug-loaded hydrogel with high antibacterial properties. Int J Biol Macromol, 2021; 182: 385-393.
23. Emami Z, Ehsani M, Zandi M, Daemi H, Ghanian MH, Foudazi R. Modified hydroxyapatite nanoparticles reinforced nanocomposite hydrogels based on gelatin/oxidized alginate via Schiff base reaction. Carbohydr Polym Tech 2021; 2: 100056.
24. Li J, Jingjing S, Jiaheng L, Mengbo X, Bingjie C, Jingan L. A hyaluronic acid/chitosan composite functionalized hydrogel based on enzyme-catalyzed and Schiff base reaction for promoting wound healing. Int J Biol Macromol, 2024; 255: 128284.
25. Wu Y, Xu W, Li J, Zhong Z, Huang L, Li S. Influence of tannic acid post-treatment on the degradation and drug release behavior of Schiff base crosslinked konjac glucomannan/chitosan hydrogel. Eur Polym J 2024; 202: 112592.
26. Mehrabi A, Karimi A, Mashayekhan S, Samadikuchaksaraei A, Milan PB. *In situ* forming hydrogel based on thiolated chitosan/carboxymethyl cellulose (CMC) containing borate bioactive glass for wound healing. Int J Biol Macromol 2022; 222: 620-635.
27. Alasvand N, Simorgh S, Kebria MM, Bozorgi A, Moradi S, Sarmadi VH, *et al.* Copper/cobalt doped strontium-bioactive glasses for bone tissue engineering applications. Open Cerami 2023; 14: 100358.
28. Davachi SM, Haramshahi SMA, Akhavirad SA, Bahrami N, Hassanzadeh S, Ezzatpour S, *et al.* Development of chitosan/hyaluronic acid hydrogel scaffolds via enzymatic reaction for cartilage tissue engineering. Mater Today Commun 2022; 30: 103230.
29. Chu Y, Huang L, Hao W, Zhao T, Zhao H, Yang W, *et al.* Long-term stability, high strength, and 3D printable alginate hydrogel for cartilage tissue engineering application. J Biomed Mater 2021; 16: 064102.
30. Lan X, Liang Y, Erkut EJ, Kunze M, Mulet-Sierra A, Gong T, *et al.* Bioprinting of human nasoseptal chondrocytes-laden collagen hydrogel for cartilage tissue engineering. The Faseb J 2021; **35**: e21191.
31. Martorana A, Lenzuni M, Contardi M, Palumbo FS, Cataldo S, Pettignano A, *et al.* Schiff base-based hydrogel embedded with *in situ* generated silver nanoparticles capped by a hyaluronic acid–diethylenetriamine derivative for wound healing application. ACS Appl Mater Interfaces 2024; 16: 20186-20201.
32. Yi X, He J, Wei X, Li H, Liu X, Cheng F. A polyphenol and ε-polylysine functionalized bacterial cellulose/PVA multifunctional hydrogel for wound healing. Int J Biol Macromol 2023; 247: 125663.
33. Chang KC, Chen WC, Chen CH, Chen JC. Chemical cross-

linking on gelatin-hyaluronan loaded with hinokitiol for the preparation of guided tissue regeneration hydrogel membranes with antibacterial and biocompatible properties. Mat Sci Eng: C 2021; 119: 111576.
34. Hegazy HA, Moon HH, Lee DH, Bhang SH, Kim YC, Song C. Preparation of polyaspartamide-based adhesive hydrogels via Schiff base reaction with aldehyde-functionalized dextran. Mat Adv 2023; 4: 1989-1997.
35. Wang L, Yang K, Li X, Zhang X, Zhang D, Wang L, et L. A double-crosslinked self-healing antibacterial hydrogel with enhanced mechanical performance for wound treatment. Acta Biomate 2021; 124: 139-152.
36. Liu F, Wang L, Zhai X, Ji S, Ye J, Zhu Z, *et al.* A multi-functional double cross-linked chitosan hydrogel with tunable mechanical and antibacterial properties for skin wound dressing. Carbohydr Polym 2023; 322: 121344.
37. Chen Y, Wang Q, Li D, Mensah A, Qiu Y, Ke H, *et al.* Mussel-inspired double cross-linked hydrogels with desirable mechanical properties, strong tissue-adhesiveness, self-healing properties and antibacterial properties. Mate Sci Eng C 2021; 120: 111690.
38. Zhao Y, Li R, Liu Y, Song L, Gao Z, Li Z, *et al.* An injectable, self-healable, antibacterial, and pro-healing oxidized pullulan polysaccharide/carboxymethyl chitosan hydrogel for early protection of open abdominal wounds. Int J Biol Macromol. 2023; 250: 126282.
39. Li Z, Li G, Xu J, Li C, Han S, Zhang C, *et al.* Hydrogel Transformed from Nanoparticles for Prevention of Tissue Injury and Treatment of Inflammatory Diseases. Adv Mater 2022; 34: 2270119.
40. Li C, He X, Li Q, Lv M, Shen J, Jin L, *et al.* A photothermal-response oxygen release platform based on a hydrogel for accelerating wound healing. NPG Asia Mater 2023; 15: 3-5.
41. Jang MJ, Bae SK, Jung YS, Kim JC, Kim JS, Park SK, *et al.* Enhanced wound healing using a 3D printed VEGF-mimicking peptide incorporated hydrogel patch in a pig model. Biomed Mater 2021; 16: doi: 10.1088/1748-605X/abf1a8.
42. Guan G, Lv Q, Liu S, Jiang Z, Zhou C, Liao Wl. 3D-bioprinted peptide coupling patches for wound healing. Mater Today Bio, 2022. 13: 100188.
43. Amani J, A. Barjini K, Moghaddam M, Asadi A. *In vitro* synergistic effect of the CM11 antimicrobial peptide in combination with common antibiotics against clinical isolates of six species of multidrug-resistant pathogenic bacteria. Protein Pept Lett 2015; 22: 940-951.