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Carboxymethyl cellulose/sodium alginate hydrogel with anti-inflammatory capabilities for accelerated wound healing; *In vitro* and *in vivo* study

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ABSTRACT

Recently, managing the chronic skin wounds has become increasingly challenging for healthcare professionals due to the intricate orchestration of cellular and molecular processes involved that lead to the uncontrollable inflammatory reactions which hinder the healing process. Therefore, different types of wound dressings with immunomodulatory properties have been developed in recent years to effectively regulate the immune responses, enhance angiogenesis, promote re-epithelialization, and accelerate the wound healing process. This study aims to develop a new type of immunomodulatory wound dressing utilizing carboxymethyl cellulose (CMC)/sodium alginate (Alg)-simvastatin (SIM) to simultaneously enhance the inflammatory responses and the wound healing ratio. The CMC/Alg-SIM hydrogels exhibited appropriate swelling ratio, water vapor transmission rate, and desirable degradation rate, depending on the SIM content. The fabricated dressing showed sustained release of SIM (during 5 days) that improved the proliferation of skin cells. According to the *in vitro* findings, the CMC/Alg-SIM hydrogel exhibited controlled pro-inflammatory responses (decreased 2.5- and 1.6-times IL-6 and TNF-α, respectively) and improved secretion of anti-inflammatory cytokines (increased 1.5- and 1.3-times IL-10 and TGF-β, respectively) in comparison with CMC/Alg. Furthermore, the CMC/Alg-SIM hydrogel facilitated rapid wound healing in the rat model with a full-thickness skin defect. After 14 days post-surgery, the wound healing ratio in the CMC/Alg hydrogel group (~93%) was significantly greater than the control group (~58%). Therefore, the engineered CMC/Alg-SIM hydrogel with desired immunomodulatory properties possesses the potential to enhance and accelerate skin regeneration for the management of chronic wound healing.

1. Introduction

The skin serves as a crucial barrier, protecting organs from environmental and physiological stress, but its integrity can be compromised by various agents, leading to conditions like inflammation and chronic wounds (Barros et al., 2021; Biglari et al., 2019). Several crucial components such as growth factors, cytokines, and mitogens are responsible for improving the wound healing process (Bello and Phillips, 2000;

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Hosseini Hooshiar et al., 2024). In addition, recent research has highlighted the vital role of targeted drug administration at the wound site to enhance the healing process and immune response (Zhang et al., 2020).

Recent progress in tissue engineering has led to the development of hydrogel-based structures with ideal chemical, physical, and biological properties capable of regenerating soft tissues (Capanema et al., 2018; Heydari et al., 2021a; Peng et al., 2022). In this context, Alginate (Alg)-based hydrogels, recognized for their biocompatibility and biodegradability, are particularly effective in chronic wound management and can encapsulate a range of therapeutic agents (Ehterami et al., 2019; Raus et al., 2021). These hydrogels dissolve slowly, a rate that can be controlled by modifying the alginate's molecular properties (Li et al., 2023; Shen et al., 2023; Zhang et al., 2023), and are available in various forms such as foams and nanofibers (Hegde et al., 2022; Hu and Lin, 2022; T. Wang et al., 2023).

Carboxymethyl cellulose (CMC), a derivative of cellulose, enhances hydrogels' absorption and drug-release capabilities due to its biocompatibility and cost-effectiveness. It has shown promise in improving the wound microenvironment, aiding in cellular processes crucial for healing, especially in diabetic patients (Alibak et al., 2022; Zhu et al., 2023). CMC has been developed for various purposes such as tissue engineering, drug delivery, wound dressing, and plant breeding (Chang et al., 2022; El-Samad et al., 2022).

Simvastatin (SIM) is a medication used to lower lipids that functions by competitively inhibiting hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, an enzyme that converts HMG-CoA to mevalonate (Yasasvini et al., 2017). Studies have shown that SIM can enhance the production of vascular endothelial growth factor (VEGF), thereby promoting angiogenesis. Additionally, it could decrease oxidative stress, improve microvascular function, and enhance immunomodulation, all of which contribute to more efficient wound healing (Heydari et al., 2021b; Rezvanian et al., 2021). Furthermore, SIM exhibits anti-inflammatory properties in both acute and chronic wounds via decreasing pro-inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α) and increasing anti-inflammatory cytokines such as tissue growth factor-β (TGF-β), interleukin-10 (IL-10), and VEGF. It also inhibits the migration of leukocytes through the endothelial cells by reducing the expression of adhesion molecules (ICAM-1), leading to reduced inflammation (Mahmood et al., 2023; Rezvanian et al., 2021).

In the current investigation, a non-toxic chemical crosslinking technique was employed to fabricate hydrogel wound dressings composed of CMC and Alg, with the inclusion of SIM. The physicochemical properties, drug release behavior, *in vitro* cell viability, anti-inflammatory factors, and *in vivo* wound healing application of the fabricated film dressings were evaluated using different tests.

2. Materials and methods

2.1. Materials

Sodium alginate (Alg), carboxymethyl cellulose (CMC), hematoxylin and eosin (H&E) staining, 3-(4,5-Dimethylthiazole) 2-yl)-2,5-diphenyltetrazolium bromide (MTT), Citric Acid (CA), ethanol, and lipopolysaccharide (LPS) were purchased from Sigma Aldrich, USA. Additionally, Dulbecco's modified Eagle's medium (DMEM-high), bovine fetal serum (FBS), streptomycin, and penicillin were purchased from Bioidea, Iran. The synthesized cDNA, RNA extraction kit, and SYBR Green PCR products were provided by Biofact, Korea. The Elisa kits for tissue growth factor-β (TGF-β), IL-6, IL-10, and TNF-α were purchased from KPG Co., Iran. L929 fibroblast cell line and human keratinocyte (HaCaT) cell line were purchased from Royan cell banks, Iran.

2.2. Preparation of the CMC/Alg– SIM hydrogel film

Initially, 30% w/w of Alg and 70%w/w of CMC in 10 ml distilled water were prepared separately, and then mixed and stirred for a duration of 5 h at 4 °C. Then, the concentration of 2% (w/v) SIM dissolved in 1 ml of ethanol was prepared and slowly added to a glycerol (4 v/v%) solution at a constant rate of 0.01 ml/min. Afterward, the drug solution and CMC/Alg compound were mixed at 30 °C and stirred using a mechanical stirrer with speed of 50 rpm at 25 °C for 1 h. Citric acid (CA), as a chemical cross-linked agent, was then added to the above mixture at a concentration of 1% (w/v) relative to the total weight of polymers. The stirring process was continued for an additional 30 min at 30 °C (Mojahedi et al., 2024). Subsequently, films of the CMC/Alg and CMC/Alg-SIM were prepared by casting the solution into the Petri dishes. These films were then dried at temperature of 42 °C for a duration of 48 h in an oven.

2.3. Characterization of hydrogel film

2.3.1. Fourier transform infrared (FTIR)

To obtain more information about the functional groups and chemical bonds in the samples, FTIR spectrophotometers (Tensor, Bruker, Germany) were applied between the wavelength of 4000 to 400 cm^{-1}.

2.3.2. Wettability

The wettability of the films was also evaluated using water contact angle at room temperature, (n = 3), to determine hydrophilicity of the Alg, CMC, CMC/Alg, CMC/Alg-SIM samples.

2.3.3. Zeta potential

The zeta potential of the Alg, CMC, CMC/Alg, CMC/Alg-SIM samples was also determined using zeta sizer (Horiba SZ-100, Japan). Samples were dissolved in Double-distilled water (DDW) and subjected to homogenization for 30 min utilizing an ultrasonic probe (Hielscher, UP400Sfor, Germany) to achieve homogeneity and then measured by the instrument.

2.3.4. Mass swelling ratio

Following cutting into 2 × 2 cm^2 segments, the hydrogel films were dried to a constant weight (W_0). The hydrogel sheets were immersed into the phosphate buffer saline (PBS; pH ~7.4) and removed at regular intervals (24 h) to measure their weight (W_t). Then, the swelling rate was calculated using equation (1) (Eq. (1)) (Heydari et al., 2018):

$$Swelling\ rate\ (\%) = \frac{W_t - W_0}{W_0} \times 100 \quad \text{(Eq. 1)}$$

2.3.5. Water vapor transmission rate (WVTR)

According to earlier research (Khorasani et al., 2018), a bottle with a diameter of 34 cm was used to measure the WVTR. The samples, measuring 35 mm in diameter, were placed on top of a 34 mm-diameter vehicle containing 15 ml DDW. The bottles were then put in an incubator that was adjusted at 37 °C and 35% relative humidity. After incubating for 24 h, the bottles were removed and weighed again. Using Eq. (2), the water vapor transmission rate (WVTR) was determined (Shi et al., 2023):

$$WVTR\left(\frac{g}{m^2}\right) = \frac{W_i - W_f}{A} \quad \text{(Eq. 2)}$$

Where, W_i and W_f are the initial and final weights of bottles, respectively, and A is the permeation surface of samples.

2.3.6. Biodegradation behavior

Biodegradability analysis was utilized to evaluate the degradation rate of hydrogels. For this purpose, first the weight of dried hydrogel (W_0) was measured and recorded, and then, they were immersed in 10 ml of PBS solution (n = 3) and incubated in an incubator at 37 °C and CO_2 concentration of 5% for 28 days. The degree of degradation for the samples was then calculated via measuring their weight on days 1, 3, 7,

14, 21 and 28 days (W_t) after immersing in PBS)pH ~7.4) according to Eq. (3) (Amirian et al., 2021; Liu et al., 2021; Shafizadeh et al., 2023).

$$Degree\ of\ degradation\ (\%) = \frac{W_0 - W_t}{W_0} \times 100 \quad \text{(Eq. 3)}$$

2.4. Drug release

For evaluation of SIM release, hydrogels (2.5 × 2.5 cm^2) were incubated in 10 ml PBS (pH~7.4) and moved into shaker incubator to calculate the drug entrapment efficiency for 1 day at 37 °C. After that, the CMC/Alg-SIM sample and CMC/Alg (as the blank sample) were placed in a dialysis bag with a cutoff of 12 kDa (n = 5). The bag was sealed and placed in a flask containing 100 ml of PBS (pH = 7.4) under continuous stirring (100 rpm) at 37 °C. 1 ml aliquot of the released medium was removed at desired intervals and analyzed at 242 nm using a UV–Visible spectrophotometer. Fresh PBS was then added at the same intervals. Based on the calibration curve and total drugs entrapped, we calculated the amounts of SIM released at each interval time (Heydari et al., 2018, 2021b).

2.5. In vitro cell experiments

2.5.1. Cells cytotoxicity

Contact method was used to culture L929 fibroblast cell line on CMC/Alg and CMC/Alg-SIM samples with dimension of 1 × 1 × 0.1 cm^3. Separately, L929 fibroblast cell line and human keratinocyte (HaCaT) cell line were cultured in Dulbecco's altered Eagle's medium (DMEM), supplemented with 10% fetal bovine serum and streptomycin at 37 °C in the presence of 5% CO_2. All hydrogels were sterilized by incubation into PBS for 30 min and UV presentation for 40 min for each side. Subsequently, 10^4 cells were seeded on the samples and incubated at 37 °C in the presence of 5% CO_2. The medium was changed each day. Cytotoxicity was inspected after 1, 3, and 5 days of culturing utilizing MTT assay. After removing the culture medium, MTT solution (0.5 wt% in PBS) was added to each well and incubated at 37 °C for 4 h. DMSO was subsequently added to facilitate solubilizing formazan, and the optical density (OD) for each sample was assessed using a microplate reader (Hiperion, Show: MPR4) at a wavelength of 560 nm. The determination of relative cell viability was determined by applying Eq. (4) (Heydari et al., 2023, 2024):

$$Relative\ cell\ survival\ (\%control) = \frac{OD_{Sample} - OD_{Blonk}}{OD_{Control} - OD_{Blonk}} \times 100 \quad \text{(Eq. 4)}$$

Where OD_{sample}, OD_{blank} and $OD_{control}$ are optical densities of CMC/Alg and CMC/Alg-SIM samples, DMSO (blank), and TCP (control), respectively.

For the 1st, 3rd, and 5th days following L929 cell seeding, the nuclei of the L929 cells were observed using DAPI (4′, 6-diamidino-2- phenylindole) staining to determine the percentage of live cells. Subsequent to the removal of the medium from the wells at specific intervals, the CMC/Alg and CMC/Alg-SIM hydrogels were rinsed with PBS. Following this, the samples were immersed sequentially in gradient ethanol (50%, 70%, 90%, and absolute ethanol) in a duration of 20 min for each. The samples were then fixed in the 2.5% glutaraldehyde solution after the ethanol had been removed. In the next step, the samples were washed with PBS and underwent a 30 s DAPI staining (Asghari-Vostakolaei et al., 2023). Live L929 cells pictures were acquired using a fluorescent microscope (Olympus BX51/Japan).

2.5.2. Immunomodulation behavior

Inflammatory factors were assessed employing RAW-264.7 and disc-shaped hydrogels with a diameter of 10 mm (n = 3). The samples were seeded with macrophages (10^6 cells/well) for 24 h, then stimulated with LPS (100 ng/ml) for an additional 24 h. The control group (just cells stimulated with LPS) was also studied in a similar way (Farshid et al., 2023). The quantification of cytokine levels in the functionalized media was achieved by means of ELISA protocol. To determine the concentrations of TNF-α, IL-6, IL-10, and TGF-β, commercially available Elisa kits were employed (Heydari et al., 2024; Sadeghi-Soureh et al., 2020).

2.6. In vivo wound healing and histological assessment

The *in vivo* part of this research was approved by the Institutional Ethics Board of Department of Animal Use and Care Administration at Isfahan University of Medical Sciences (Ethic Code: IR.MUI.RESEARCH.REC. 1401.389). Sprague-Dawley (SD) rats (male, 18 weeks old, 270 g) were chosen for the assessment of wound closure. Pentobarbital (2.2 mg/100 g) was used to anesthetize the rats before they were shaved and cleaned with iodine. A full-thickness square wound with a 1 cm diameter was made on the rats back, and the wound was then sealed with hydrogel. The control group was designated as having untreated wounds in a similar manner. At defined intervals (0, 7, and 14 days), photographs of the injury were collected, and Image J software was used to determine the surface area (A_0 and A_1) (Shaheen et al., 2022). The rate of wound healing was determined according to the Eq. (5):

$$Wound\ healing\ rate\ (\%) = \frac{A_0 - A_1}{A_0} \times 100 \quad \text{(Eq. 5)}$$

After 14 days, the dorsal tissue was taken from the wound site, 4% paraformaldehyde was used to fix it for 24 h, paraffin was used to embed it, and 5 μm pieces of the tissue were cut out. According to the manufacturer's instructions (Sigma-Aldrich, USA), H&E staining was carried out to look at granulation tissue development and epithelialization.

2.7. Statistical analysis

The one-way analysis of variance (ANOVA) test was used for the statistical analysis of the data. Graph-Pad Prism Software (Version 9) was applied to perform the Tukey-Kramer post hoc test, which was used to evaluate the significance between groups. A p-value of less than 0.05 was considered statistically significant (Mirjalili et al., 2022).

3. Results

This study introduces a novel based on Carboxymethyl cellulose (CMC)/Sodium alginate (Alg) Hydrogel with Anti-inflammatory capabilities for Accelerated Wound healing application. CMC/Alg hydrogels containing Simvastatin were prepared via a chemical crosslinking process via Citric acid (CA) for chronic wound healing application.

3.1. FTIR

Fig. 1a presented the FTIR spectrum of CMC/Alg and CMC/Alg-SIM. In the FTIR spectrum of CMC/Alg sample, the peak at 1613 cm^{-1} is attributed to the stretching vibrations of carbonyl compound (C═O) that is a characteristic band of Alg. C–H bending vibrations of aromatic nuclei are shown in peaks at 1418, 816, and 693 cm^{-1} (Han and Wang, 2017). The peak at 1033 cm^{-1} indicates the C–O stretching vibrations that confirms the presence of Alg in CMC/Alg. The peak at 2972 cm^{-1} corresponds to the C–H stretching vibrations of Alg. The stretching vibrations related to O–H group can be seen in the wave number of 3419 cm^{-1} (Han et al., 2018). The presence of a board peak of C═O at 1724 cm^{-1} confirms the chemical interaction between hydroxyl and ester groups during cross-linking process via CA agent (Capanema et al., 2018). FTIR spectrum of CMC/Alg-SIM illustrated peaks at around 3548, 2958, and 2879 cm^{-1} which are related to the stretching vibrations of OH and CH groups of SIM, respectively (Han et al., 2018; Rezvanian et al., 2016). Besides, peaks at 1619 and 1703 cm^{-1} are correspond to the C═O and C═C asymmetric stretching vibrations of the CMC and SIM, respectively (Ning et al., 2019; Rosyida et al., 2019; Ruan et al., 2019).

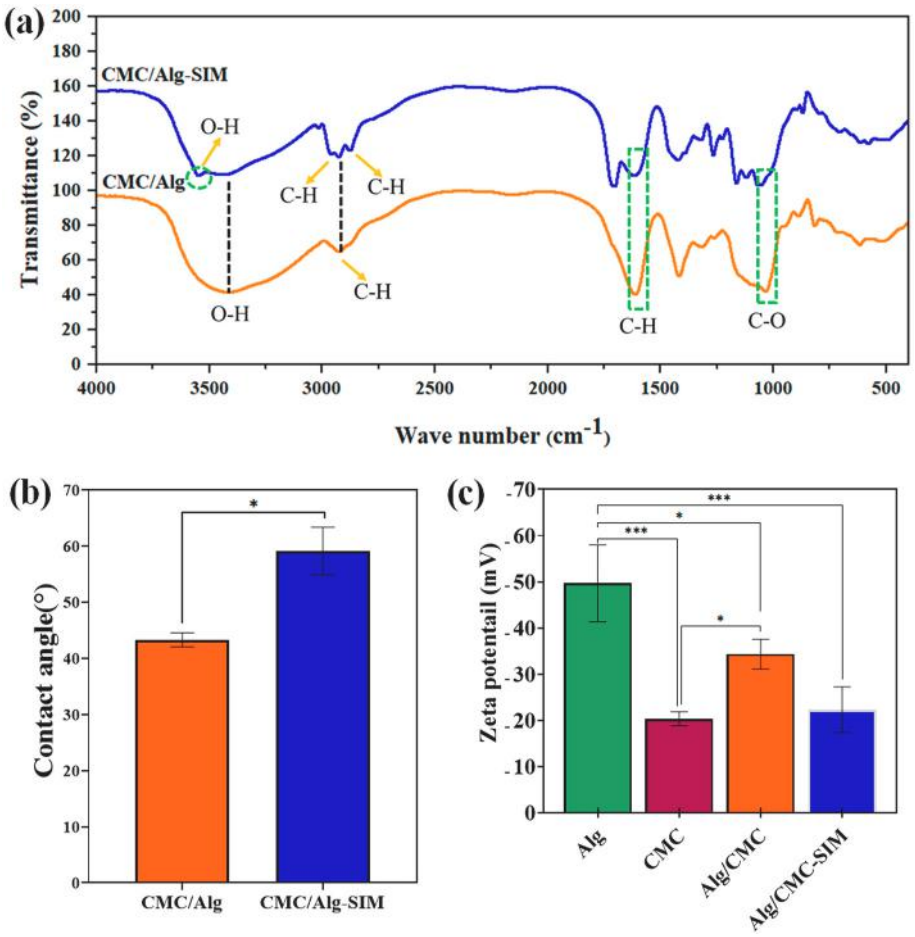

Fig. 1. a) Chemical characterization by using FTIR spectra of CMC/Alg and CMC.Alg-SIM, b) the water contact angle of CMC/Alg and CMC.Alg-SIM after 5 s, and c) zeta potential of Alg, CMC, CMC/Alg, and CMC/Alg-SIM for evaluation surface charge. The results are reported as the means (n = 3) ± standard deviation. (*: $p < 0.05$ and ***: $p < 0.001$).

3.2. Physical characterization

3.2.1. Hydrogel surface hydrophilicity

The contact angle value is influenced by the morphology, surface potential, and chemical structure of a biomaterial (Liu et al., 2014). Whereas SIM is a drug with a hydrophobic nature, pure CMC and alginate have been indicated to be substances with hydrophilic properties. The contact angle of CMC/Alg and CMC/Alg-SIM hydrogels was measured at room temperature. Results showed that the contact angles for the CMC/Alg and CMC/Alg-SIM samples were 43.3 ± 2° and 59.1 ± 4°, respectively ($p < 0.05$) (Fig. 1b). Therefore, the addition of SIM to the structure of CMC/Alg hydrogel led to a little decrease from its hydrophilicity.

3.2.2. Zeta potential

Fig. 1c displays the zeta potential value of CMC, Alg, CMC/Alg, and CMC/Alg-SIM hydrogel, which was prepared in this experiment. This observation demonstrates that with the addition of CMC, with zeta potential of −20.3 ± 2 mV, to the Alg, a negatively charged natural polymer, the zeta potential of Alg was decreased from −49.6 ± 16 mV to −34.3 ± 8 mV (for the complex). This phenomenon is due to the chemical interactions between Alg and CMC, facilitated by the inclusion of CA as a chemical crosslinking agent. Furthermore, through the incorporation of SIM into the polymer hydrogel, the resulting material attains a favorable quantity of negative charges (−22.34 ± 9 mV), which enables interactions with proteins.

3.2.3. Mass swelling ratio

The swelling capacity of samples is one of the most imperative and crucial criteria for wound dressing agents (Hooshiar et al., 2024). Fig. 2a illustrated the changed hydrogel volume of CMC/Alg and CMC/Alg-SIM samples subsequent to immersion in PBS during 24 h. As shown in Fig. 2a, the weight of the hydrogel membranes increased to 883 ± 24% during immersion in PBS for 24 h while simultaneously preserving their morphological stability.

3.2.4. Water vapor transmission rate

A significant characteristic of wound dressings is their water vapor permeability (Shakiba-Marani and Ehtesabi, 2023). A wound dressing with a water vapor transfer rate (WVTR) of 2000–2500 g/m^2 is necessary to prevent the wound bed from becoming dry, losing water, and generating exudate (Gruppuso et al., 2022). As shown in Fig. 2b, CMC/Alg hydrogel had a WVTR of 2100–2300 g/m^2. Additionally, SIM did not significantly enhance water vapor permeability.

3.2.5. Biodegradation behavior

The biodegradation behavior of hydrogels measured during 28 days was found to be dependent on hydrogel composition (Fig. 2c). In this study, O–H chemical groups of CMC and Alg made these hydrogels candidates for hydrolysis degradation in PBS solution. According to the Fig. 2c–a 3-stage weight loss pattern was observed. At the initial stage of degradation, the hydrogel followed the continuous degradation. As time increased, in the second stage the polymer structure breaks the long chains into shorter ones via the hydrolysis process. In the third stage, the

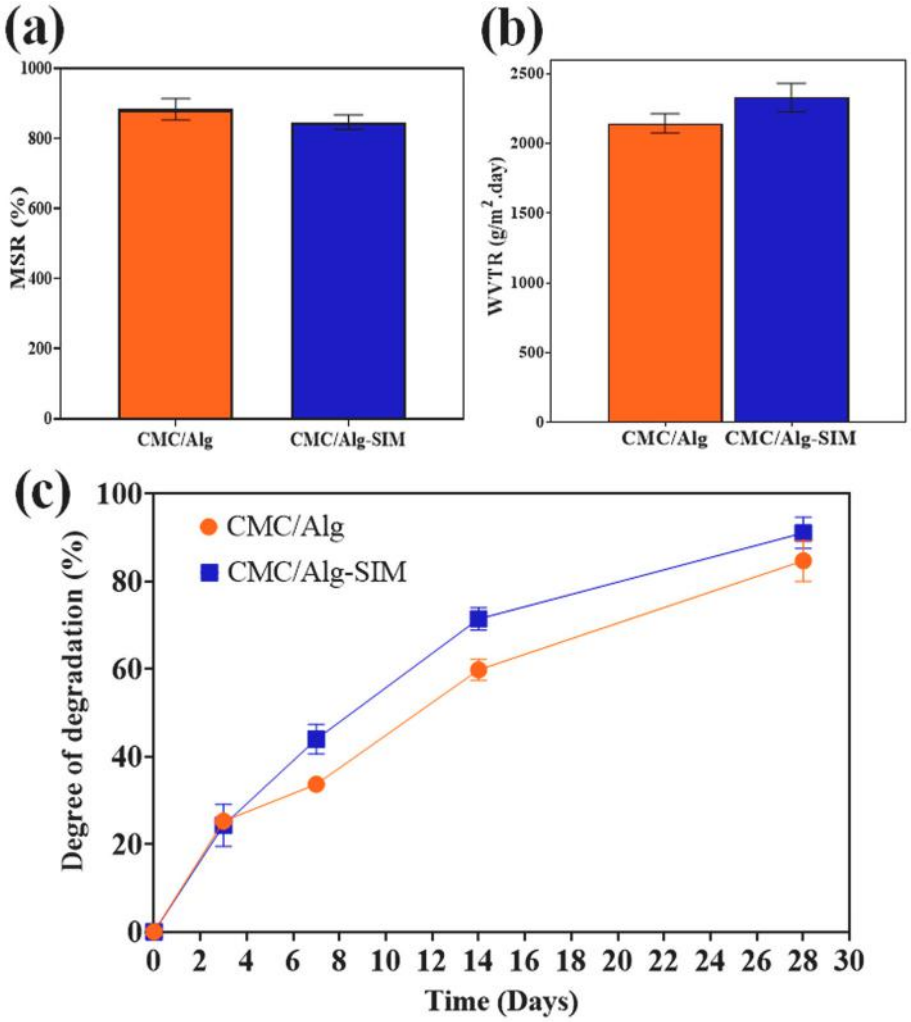

Fig. 2. Physical characterization of CMC/Alg and CMC/Alg-SIM hydrogels, a) The mass swelling ratio in PBS (pH ~7.4) for 24 h, b) Water vapor transmission rate (WVTR) in DDW during 24 h, and c) the weight loss of samples during 28 days-soaking in PBS (pH ~7.4). The results are reported as the means (n = 3) ± standard deviation. The statistical analysis did not show any significance in statistical analysis.

exit of smaller chains from the bulk of the hydrogel led to the intensification of weight loss. During the first 3 days, all the samples followed the same trend with degradation rate of about 24% but the releasing of SIM process resulted in the faster degradation of CMC/Alg hydrogel.

3.3. SIM release behavior

To assess the potential value of CMC/Alg hydrogel as a carrier for drug delivery, an evaluation was conducted on the sustained-release properties of SIM. The observation of SIM released from CMC/Alg hydrogel was achieved through the monitoring of absorbance at 242 nm, as depicted in Fig. 3a. The results of the drug release profile showed sustained release for 5 days. This phenomenon can be attributed to the surface wettability of the polymer and drug composite, as well as the enhanced rate of drug release diffusion. In other words, the addition of SIM, as a hydrophobic drug, results in a low possibility of drug interaction and release by water molecules (Singla et al., 2019).

3.4. Biological evaluation

3.4.1. Cell viability

CMC/Alg containing SIM samples can be utilized for wound healing due to their appropriate swelling behavior, water vapor transmission rate, controlled drug delivery, and degradation rates. To evaluate the cytocompatibility of the samples for *in vivo* wound healing, a facile approach was employed. During wound contraction and ECM remodeling, fibroblasts and keratinocytes play a major role. As a result, L929 and HaCaT cells were selected first to co-culture with the membranes as prepared. To determine the metabolic activity of cells on CMC/Alg and CMC/Alg-SIM hydrogels after 1, 3, and 5 days of culture, MTT assays were conducted (Fig. 3b and c). Following 5 days of culture, there was a substantial increase in cell survival, indicating that the samples were not cytotoxic. In the experiment, the viability of the cells in CMC/Alg with and without drug was comparable to that of the control group, and even exhibited improvement. Interestingly, the survival rate of L929 cells treated with CMC/Alg-SIM hydrogel (115.7 ± 3% control) was more than that of CMC/Alg sample (106.1 ± 5% control), indicating that SIM release had positive effect on cell survival.

Furthermore, the HaCaT cells survival rate of CMC/Alg-SIM hydrogel (132.5 ± 7% control) was similar to CMC/Alg sample (128.1 ± 3% control), indicating that SIM release does not have negative effect on the cell survival (Fig. 3c).

For confirmation of the MTT assay, DAPI staining was performed on L929 cells. The fluorescent images of cells stained with DAPI, as shown in Fig. 3d, offer valuable insights into cell viability and proliferation. Furthermore, these images support the observed trend of increasing cellular viability over a period of 5 days, as determined by the MTT assay.

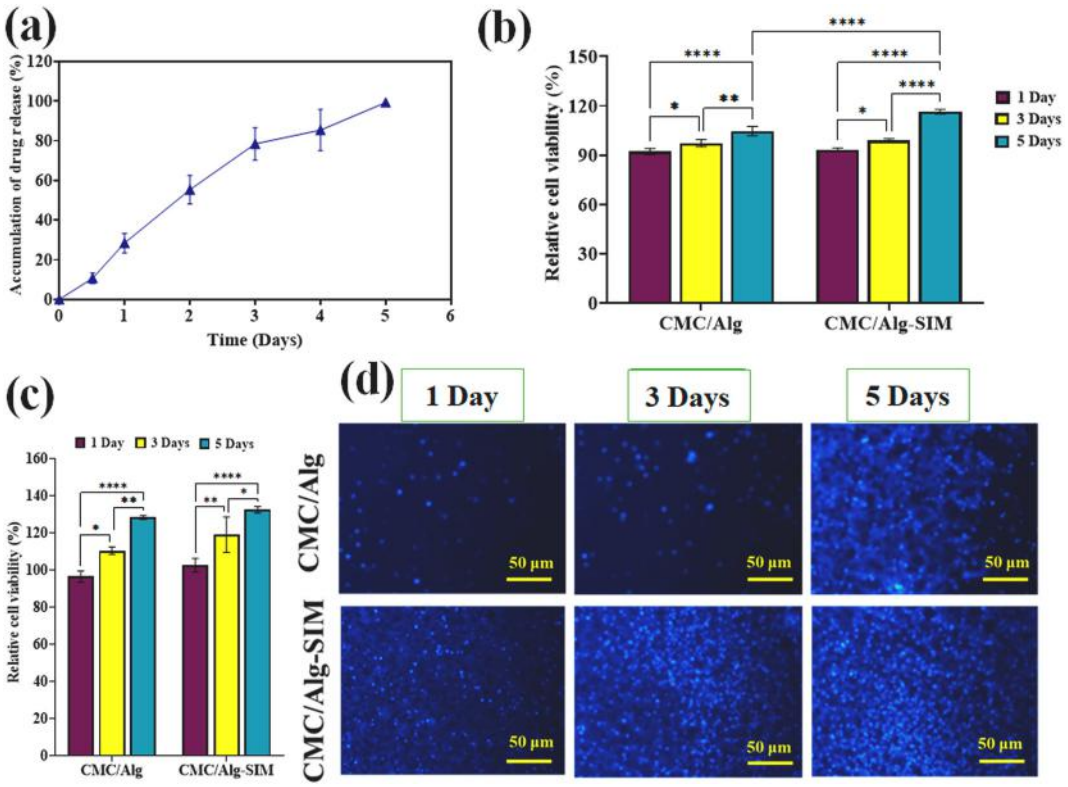

Fig. 3. a) Accumulative SIM release of CMC/Alg-SIM in PBS (pH ~7.4) during 5 days, b) Relative L929 cell viability of CMC/Alg and CMC/Alg-SIM hydrogels measured using the MTT assay during 1, 3, and 5 days and normalized against the control (TCP), c) Relative HaCaT keratinocytes cell viability of CMC/Alg and CMC/Alg-SIM hydrogels measured using the MTT assay during 1, 3, and 5 days and normalized against the control (TCP), and d) CLSM *DAPI staining* images of L929 cells after adhesion on the surface of CMC/Alg and CMC/Alg-SIM hydrogels during 1,3, and 5 days, the untreated cells were used as the control group. *Scale bar:* 50 µm. The results are reported as the means (n = 3) ± standard deviation. (*: $p < 0.05$, **: $p < 0.01$, and ****: $p < 0.0001$).

3.4.2. *Immune cells response*

As shown in Fig. 4, pro-inflammatory (IL-6 and TNF-α) and anti-inflammatory (IL-10, TGF-β) parameters were measured in LPS-stimulated cells that were seeded on CMC/Alg and CMC/Alg-SIM for a duration of 48 h. The highest concentrations of IL-6 and TNF-α were observed in the TCP and CMC/Alg samples without the administration of any medications, as depicted in Fig. 4 (a, b). This suggests that CMC/Alg does not possess the capability to decrease the expression levels of pro-inflammatory cytokines. Conversely, when SIM was added to the hydrogel structure, it was demonstrated that the pro-inflammatory mediators were decreased ($p < 0.05$) in comparison to CMC/Alg and the control group. For example, the TNF-α levels for the control, CMC/Alg, and CMC/Alg-SIM were approximately 40.24 ± 5, 40.28 ± 6, and 15.28 ± 8 pg/ml, respectively.

In contrast to the control and CMC/Alg groups, the CMC/Alg-SIM sample exhibited significantly higher levels of cytokines (IL-10) and growth factor (TGF-β) associated with the anti-inflammatory phase (M2 phase), as depicted in Fig. 4 (c, d) ($p < 0.05$). The TGF-β levels for the TCP, CMC/Alg, and CMC/Alg-SIM samples were approximately 49.65 ± 10, 60.2 ± 12, and 73.55 ± 4 pg/ml, respectively.

Also, Fig. 4e shows that every result showed that sustained release of SIM from CMC/Alg hydrogels could successfully trigger macrophage polarization to M2 phenotype and moderate inflammatory responses.

3.5. *In-vivo assay*

The impact of CMC/Alg hydrogels on the *in vivo* skin wound healing was evaluated through the creation of a wound defect (1 × 1 cm^2 full-thickness skin wound) in a rat model. The healing ratio of the wounds, after treatment with CMC/Alg-SIM hydrogel and in the control group (no treatment), was measured at specific time points (0, 7, and 14 days) and recorded in Fig. 5a.

In comparison to the control group (approximately 29%), the application of CMC/Alg-SIM hydrogels led to a significant enhancement in wound repair, with a wound closure rate of approximately 76% after 7 days of treatment. Notably, a conspicuous recovery of the wounds was observed at day 14, where the CMC/Alg-SIM hydrogel exhibited the highest wound contraction speed, resulting in a wound closure of about 94%, surpassing the control group's closure rate of approximately 59% (Fig. 5b).

To gain a more comprehensive understanding of the fundamental mechanism the wound healing resulting treated with CMC/Alg hydrogels, H&E staining assay of wound skin was performed, as shown in Fig. 5c after a post-treatment period of 14 days. The results demonstrated that the epidermis grew thinner in CMC/Alg-SIM group than the control group (~0.65 times), indicating less fibrosis occurred. Additionally, the vascularization (~3 times) was observed in CMC/Alg-SIM group compared to the control group, which should be attributed to the contribution of SIM and interaction with skin cells (Fig. 5 d, e).

4. Discussion

Insufficient angiogenesis, extended immune response, and other irregularities can result in chronic wounds that are hard to heal or lead to nonfunctional scars. Therefore, it is essential to utilize bioactive dressings in combination with medical treatment in order to accelerate the healing process of chronic wounds (Heydari et al., 2024). In this study, evaluation chemical, physical, and biological behavior of hydrogel wound dressings composed of CMC and Alg, with the inclusion of SIM to improve chronic wound healing process.

The contact angle of a biomaterial is modified by its geometry, surface potential, and chemical structure(Liu et al., 2014). Hydrophobic

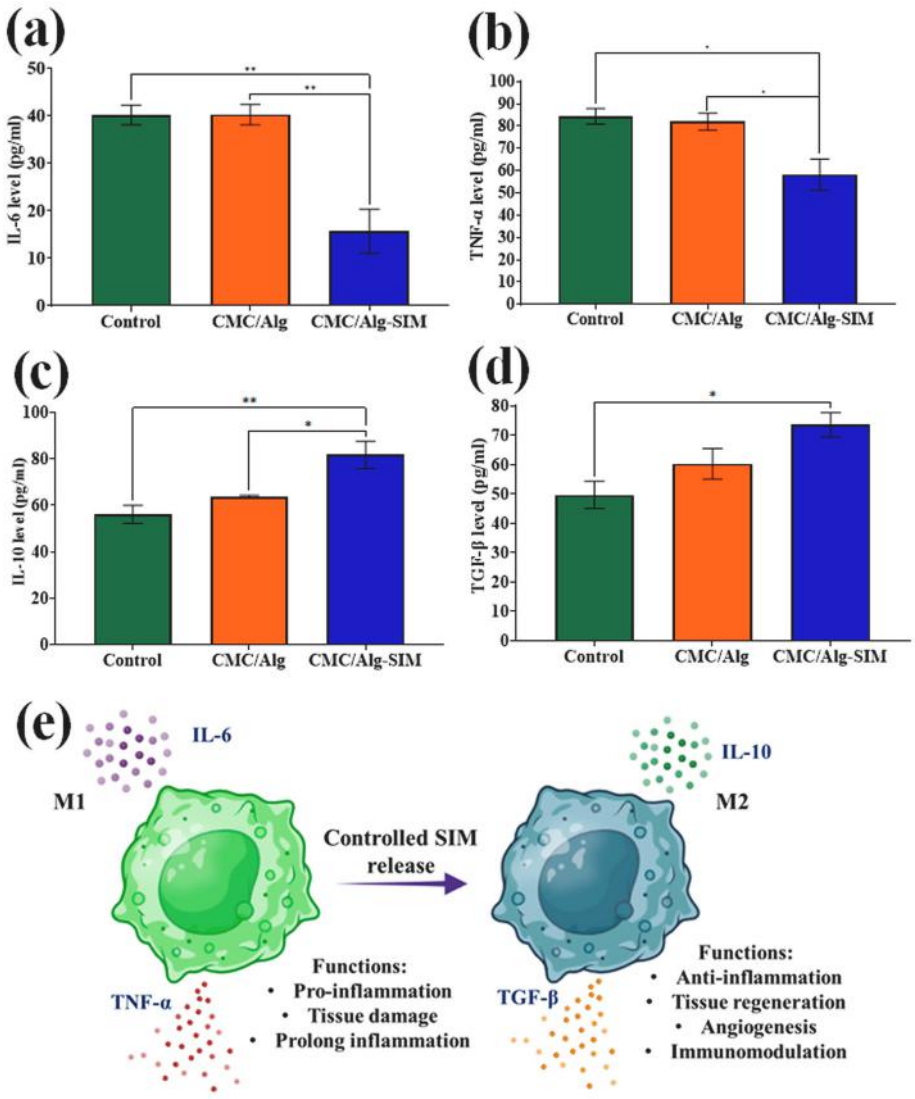

Fig. 4. *In vitro* immune response of *RAW 264.7* macrophage for 48 h, ELISA analysis of macrophage cytokines a) IL-6, b) TNF-α, c) IL-10, d) TGF-β, and e) the schematic of the effect of SIM release on immune cells response. The results are reported as the means (n = 3) ± standard deviation. (*: $p < 0.05$ and **: $p < 0.01$).

drugs like SIM have been shown to reduce the interaction between polymers or hydrogels and water molecules, as reported in other studies as well (Ahmadi et al., 2020; Cisneros et al., 2021). A desirable water contact angle for the purpose of wound healing applications lies in the range of 50–60°, a parameter of significance for cellular interaction, such as adhesion, proliferation, and migration (Movahedi et al., 2020).

Following, by evaluation zeta potential the results demonstrated that the chemical interactions between Alg and CMC, facilitated by the inclusion of CA as a chemical crosslinking agent. Furthermore, through the incorporation of SIM into the polymer hydrogel, the resulting material attains a favorable quantity of negative charges, which enables interactions with proteins. It seems that this change is caused by the surface charge of SIM, which with its presence has led to the reduction in the surface charge of the CMC/Alg-SIM hydrogel (Wang et al., 2014). This, in turn, fosters the adsorption of proteins and promotes cell adhesion, ultimately contributing to the advancement of skin cell tissue engineering (Chang and Wang, 2011). The ability of samples to swell is a highly important and critical factor for wound dressing agents. The swelling ratio results of current study showed hydrogels could be used to absorb exudate from the wound site (Chen et al., 2022). Moreover, the incorporation of SIM to CMC/Alg hydrogel does not have significantly effect on mass swelling ratio ($p > 0.05$).

Similarly, He et al. (2021) evaluated the physical behavior of composite dual-crosslinked hydrogel based on natural product sodium alginate and carboxymethyl cellulose (CMC) that formed by SA crosslinking with glutaraldehyde (GA) and calcium ion (Ca^{2+}). They demonstrated that by the addition of CMC to hydrogel structure the swelling ratio increased from about 200% to 1265% in 250 min.

Water vapor permeability in another important parameter in wound dressing developed. CMC/Alg hydrogel with or without drugs have suitable WVTR to prevent dryness and loss of water and exudate formation in the wound bed. Similarly, Abbasi et al. (2020) investigated the effect of adding Alg to PVA hydrogel films led to an improved water vapor permeability rate of 2260 g/m^2. As a result, our findings suggested that CMC/Alg and CMC/Alg-SIM could be used as wound dressings.

The ideal biomaterial scaffold for skin tissue engineering application should have a suitable degradation rate along with new skin tissue regeneration (Amirian et al., 2021). In this study, as shown in degradation results, a 3-stage weight loss pattern was observed. At the initial stage of degradation, the hydrogel followed the continuous degradation, and this was caused by the swelling water molecules penetration in the polymer structure. As time increased, in the second stage the polymer

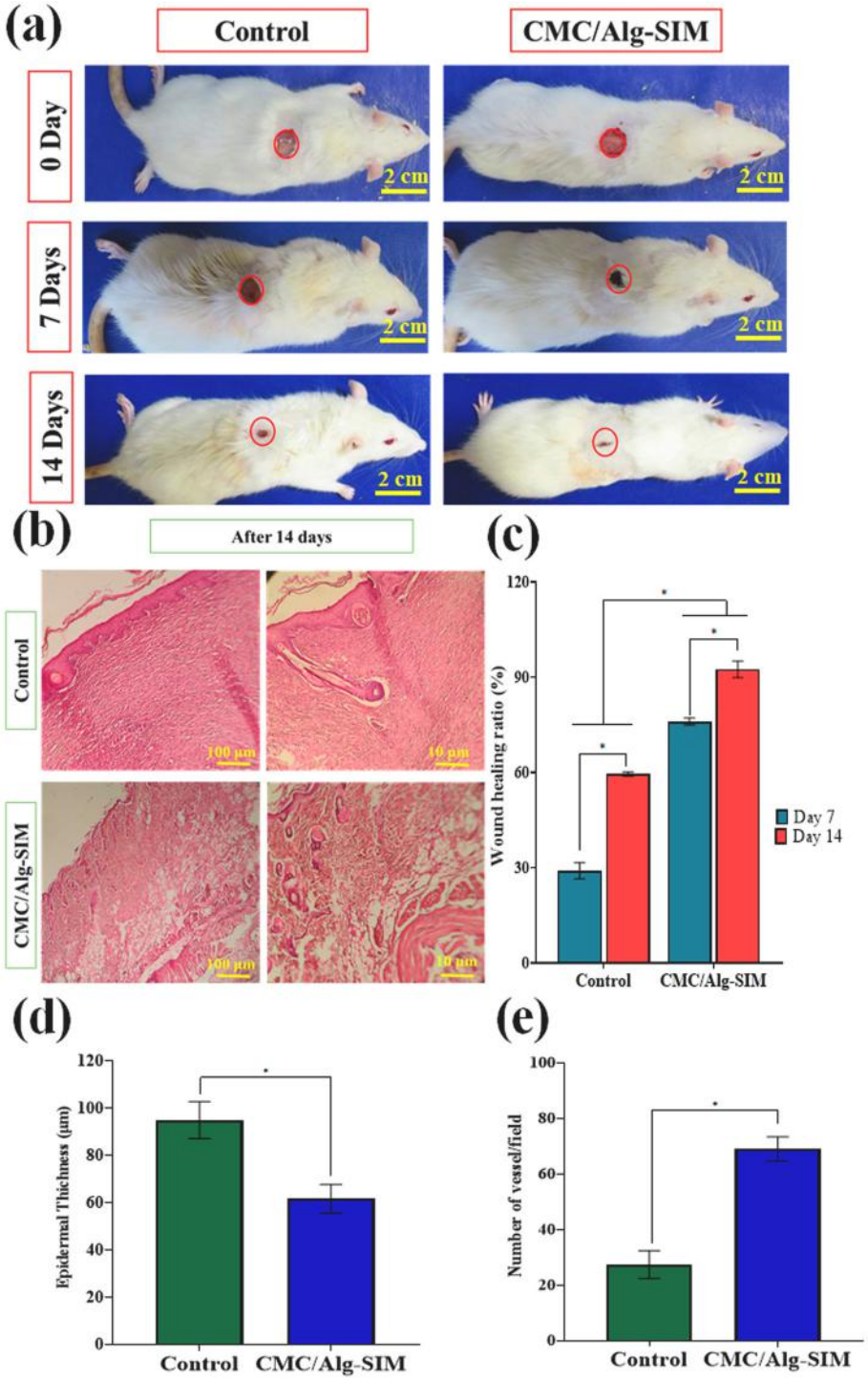

Fig. 5. *In vivo* wound healing study of CMC/Alg-SIM in a rat models: a) Images of wounds treated with CMC/Alg-SIM hydrogel at 0, 7, and 14 days, in comparison to the control (untreated wound). b) Histopathological assessment of wound sections under various circumstances using H&E staining after 14 days. *Scale bar:* 2 cm, *Scale bar*: 10 μm and 100 μm. c) Quantitative evaluation of the percentage wound healing during 0, 7, and 14 days. d) The mean thickness of the epidermis after 14 days, and e) the mean diameter of the blood vessels at 14 days after treatment. The results are reported as the means (n = 3) ± standard deviation. (*: $p < 0.05$).

structure breaks the long chains into shorter ones via the hydrolysis process. In the third stage, the exit of smaller chains from the bulk of the hydrogel led to the intensification of weight loss (M. Wang et al., 2023). Similarly, Maroufi et al. (Maroufi and Ghorbani, 2021) evaluated *in vitro* biodegradation process of the chitosan-based hydrogel containing curcumin and demonstrated that by adding drug molecules to hydrogel, the degradation rate increased. Therefore, the controlled degradation of CMC/Alg-SIM might be promising for skin tissue engineering and controlled drug delivery at wound site.

According to SIM released from CMC/Alg hydrogel profile it can be said that the results aligns with previous studies proposed that the direct relation between degradation profile and drug delivery system and influences the released profile of drugs (Sharma et al., 2015).

Previous studies have indicated that a sustained release of the anti-inflammation factor occurred within 3 days following injury, which proves to be highly suitable for chronic injuries (Puertas-Bartolomé

et al., 2019). In this context, there is a specific desire for the sustained release of SIM, particularly due to the rapid efficacy of the formulations in the presence of an infection.

The cell behavior showed that the CMC/Alg with or without SIM followed ideal cell survival and cell proliferation in L929 and HaCat cell line. Similarly, Yahia et al. (2023) investigated gelatin-based hydrogels with SIM and platelet-rich plasma (PRP) and reported that the hydrogel containing SIM followed the suitable L929 cell viability and proliferation.

Macrophages play a crucial role in several stages of wound healing, including the inflammatory and remodeling phases. Based on the current study findings, continuous release promoted M2 macrophage polarization and resulted in an upregulation of M2 macrophage mediators (Louiselle et al., 2021). Similar to this research, another study investigated the effectiveness of gelatin-based hydrogels with SIM and platelet-rich plasma (PRP) for tissue regeneration (Yahia et al., 2023). They demonstrated that SIM significantly enhanced the generation of vascular tissues while also exerting an anti-inflammatory effect by reducing the release of IL-6 and IL-8. In general, every result showed that sustained release of SIM from CMC/Alg hydrogels could successfully trigger macrophage polarization to M2 phenotype and moderate inflammatory responses.

Based on our findings, we chose the CMC/Alg-SIM for *in vivo* research due to its favorable physical characteristics, biocompatibility, and superior immunomodulatory responses. It is commonly known that wound healing is a controlled process that involves a wide range of elements that must collaborate in a well-planned and coordinated manner, including growth factors, various cell types, extracellular matrix, blood vessels, and others (Deng et al., 2022; Ling et al., 2021).

Blood vascularization, which promotes nutrition, oxygenation, and waste transfer, is essential for wound healing process. *In vivo* wound healing results demonstrated that the CMC/Alg-SIM hydrogel could enhance wound healing with less fibrosis and scar formation.

Similarly, Mahmood et al. (2023)created composite functional wound dressing (CS/G/TA) based on κ-carrageenan (κ-CRG) and tannic acid (TA) were prepared via ionic gelation with the aid of aluminum chloride and were co-encapsulated with SIM and Geranium oil (GO) to improve the process of wound healing. The *in vivo* findings demonstrated that the CS/G/TA hydrogel film showed excellent wound healing effect through improved angiogenesis, epidermal regeneration, controlled the thickness of the dermis, and down regulation in the expression of inflammatory markers compared to the control group.

In general, the presence of SIM is critical to enhance wound healing process.

5. Conclusion

In conclusion, we have successfully manufactured an immunomodulatory CMC/Alg-SIM hydrogel for the purpose of promoting wound healing process. The obtained hydrogels presented an appropriate swelling capacity, as well as a water vapor transmission rate, degradation profile, and adjustable biological properties. The introduction of the SIM led to a different profile in terms of the water wettability and degradation ratio of the CMC/Alg. Interestingly, CMC/Alg-SIM hydrogel demonstrated its superior biofunction by enhancing the biocompatibility, proliferation, macrophages activation, anti-inflammatory and pro-inflammatory modulation, and immune cells response. Additionally, *in vivo* assays revealed that the CMC/Alg-SIM hydrogel could promote angiogenesis and accelerate the process of wound healing. In general, the presence of SIM is critical to enhance anti-inflammatory response, angiogenesis, and accelerate wound healing process. The fabricated CMC/Alg-SIM hydrogel offers a unique and controlled release of SIM, making it a promising wound dressing for the enhanced repair of chronic skin wounds.

CRediT authorship contribution statement

Seyed Mohammad Reza Hosseini: Writing – original draft, Methodology, Investigation. **Parisa Heydari:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Conceptualization. **Mahtab Namnabat:** Writing – original draft, Methodology, Investigation. **Reyhaneh Nasr Azadani:** Writing – original draft, Methodology, Investigation. **Fateme Azimi Gharibdousti:** Writing – original draft, Investigation. **Elmira Mousavi Rizi:** Writing – original draft, Investigation. **Arezoo Khosravi:** Writing – review & editing, Methodology. **Atefeh Zarepour:** Writing – review & editing, Visualization, Methodology. **Ali Zarrabi:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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