Original Article

Preparation, Characterization, and Antibacterial Studies of N, O-carboxymethyl Chitosan as a Wound Dressing for Bedsore Application

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Abstract

Background: A study conducted on wound treatment by antibacterial wound dressings can reduce the need for using antibiotics to a minimum amount. These wound dressings can create a moist environment at the wound surface to speed the healing process up. In recent years, researchers have paid much attention to polymeric wound dressings. Chitosan can help heal the wounds because of its similar structure to glycosaminoglycans in the skin. In this regard, the aim of the present study was to fabricate and characterize a novel biolayer wound dressing based on the carboxymethyl chitosan polymer with ceramic nanoparticles as a reinforcement and antibacterial agent using the freeze-drying method. **Methods:** In this study, to make a flexible wound dressing from a biocompatible and biodegradable polymer, N-O-carboxymethyl chitosan, diopside was added to improve the mechanical and hydrophobic properties of the soft tissue and cell proliferation was fabricated. After making the samples, a variety of chemical and biological tests and analyses were performed on the samples, including scanning electron microscope and Fourier-transform infrared spectroscopy. **Results:** The results showed that the use of this wound dress significantly reduced the risk of infection at the wound site. **Conclusions:** An antibacterial product with the proper mechanical behavior as a soft tissue was produced and evaluated in this study. The chemical and biological investigation represented that the sample with 5 wt% magnetite nanoparticles has excellent characteristics and can be introduced as a wound dressing application.

Keywords: Carboxymethyl chitosan, freeze-drying, tissue engineering, wound healing

Introduction

The wound healing issue has always been an interesting subject of researchers and usual people. The ancient Egyptians were using a variety of compounds such as animal fats, honey, and plant fibers for the treatment of burns and diabetes wounds.[1-4] Maintaining the integrity of the skin in humans is vital to protect against water loss, bleeding, and counteracting

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the entry of microorganisms. Therefore, wound healing is carried out with advanced mechanisms and techniques such as electrospinning and freeze-drying (FD).[5-7] Wound healing is essential in terms of time and pain because skin regulates important functions in the body and has a protective role. Many factors such as immune system, moisture, the acidity of the wound, and biofilm fabrication under the wound site can prevent wound healing.[8-11] It should be noted that carboxymethyl chitosan (CMC) is one of the most compatible biopolymers combined with ceramic nanoparticles containing suitable antibacterial properties and biocompatible property in the soft tissue. Unfortunately, the healing process of chronic wounds can take months or years. Therefore, there is a vital need for special wound dress that maintains moisture and removes excess secretions from a particular environment and can control temperature and improve the pH in the healing process.[12-15] On the other hand, a proper wound dressing should be able to create a warm and moist environment on the wound surface with having suitable proliferation and differentiation of keratinocyte cells and thus it can cause growth factors (GFs) to contact with the wound surface and increase the rate of healing.[5-11,16-19] To make a wound dress with the above purposes, natural polymers have been considered due to their high biocompatibility, biodegradability, and similar properties to the body tissues in the wound healing process.[20-24]

Polymers used for skin applications should not cause damage or inflammation to the skin. Chitosan is a biopolymer that does not show any toxic effects, injury, or inflammation. Hence, it can be a suitable choice for treating wounds and burns.[25-27] Biofibers are made of chitin and chitosan, which effectively make absorbable structures and prepare fabrics for wound healing. Recently, chitosan and its derivatives have been proposed as a suitable candidate for bone scaffold since they are nontoxic, biocompatible, and biodegradable.[28-32] According to the results released by Lim *et al*.,[33] chitosan stops the growth of microorganisms on the wound. Since red blood cells and platelets have a negative charge, they bind to the positively charged chitosan, causing the blood to clot and prevent bleeding.[34-37] Due to the antibacterial properties of chitosan, it accelerates the wound healing process. In this study, to achieve the unique features of polymers and according to the mentioned characteristics, N-O-CMC is considered as the base polymer with the addition of bioceramic to evaluate its chemical and biological response.[4,18,33,38-41] Figure 1 shows the chemical structure of CMC, which has been used in the treatment of cancer, wound healing, and cosmetic problems. N-O-CMC is a chitosan derivative with interesting physical and biological properties such as moisture retention, gel formation, excellent biocompatibility, antibacterial activity, and significant chemical activity.

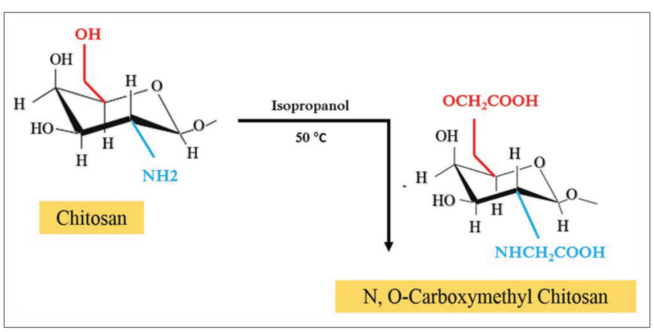

Figure 1: Chemical structure of N-O-carboxymethyl chitosan used in this study

Since polymers have poor mechanical properties, composite polymer preparation is one of the ways to improve their mechanical and chemical properties. The use of composites as biomaterials in the restoration of damaged living tissues is the subject of many recent studies in medical applications.[40-45] One of the most popular bioceramics in tissue engineering is the diopside, which contains sufficient amounts of silicon and magnesium ions with the chemical composition of $CaMgSi_2O_6$.[42,43] Several researchers worked on using different drugs on wound and bone to consider their effects, after short- and long-term follow-up periods.[44-53] In this regard, the aim of this study was to make a polymer-based wound dressing with ceramic and antibacterial nanoparticles using the FD method, which can be used to repair and regenerate damage on the skin due to its proper mechanical and biological properties.

Methods

Materials preparation

In this study, to make a flexible wound dressing from a biocompatible and biodegradable polymer, N-O-CMC, diopside was added to improve the mechanical and hydrophobic properties of the soft tissue and then cell proliferation was fabricated. Figure 2 illustrates the fabrication process of a novel soft tissue with antibacterial properties. A specific amount of CMC was solved in 100 mL distilled water with 1 vol% acetic acid (CH-COOH) and glutaraldehyde as a cross-linking agent. In the first step, according to the previous studies,[4,18] a novel tissue is fabricated and prepared using CMC solution containing a certain percentage of diopside nanoparticles. The obtained solution was placed in a homogeneous thermal magnetic stirrer for 1 h to make it homogenized. Then, the

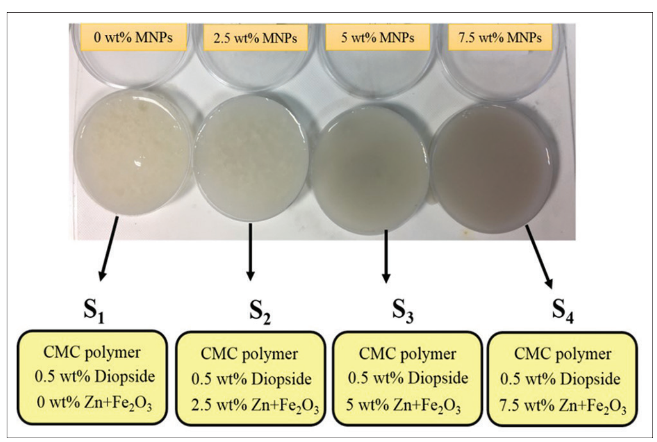

Figure 2: The preparation of soft tissue using the freeze-drying technique

samples were transferred to a -65°C freezer for 24 h. Afterward, they were placed in the freeze-drier for 48 h at -45°C.

Experimental analysis

In the present study, Fourier-transform infrared spectroscopy (FTIR) was used to investigate the structure of the synthesized nanocomposite samples containing various amounts of MNPs in order to study the structure and chemical bonds of the nanocomposites. Thus, the morphology of the samples was observed with a scanning electron microscope (SEM) (AIS2300C-20KV) at an accelerating voltage of 10 kV. To increase the electrical conductivity of the specimens and the images' sharpness, the specimens were coated with a skinny layer of gold.

Experimental testing

The degradation diagram shows that the sample with the lowest amount of MNPs had the most moderate amount of heavy ion absorption.[1] Therefore, it can be concluded that weight loss is more significant in biological evaluation. On the other hand, porosity is also crucial in terms of weight loss and weight gain. For instance, as the porosity is low, the weight might be low. The results showed that as the percentage of MNPs increases, water absorption and subsequent degradation rate increase.[1] These findings can be attributed to the possible chemical bonds between the nanoparticles and the CMC. Moreover, the presence of ion groups in the nanocomposite structure can support the formation of physical, hydrogen bonds, and ion interactions of the sample. Since the wound dress is supposed to be placed in the natural and biological environment of the body, it is necessary to study some characteristics related to the physiological conditions of the body to evaluate the layer formation of the samples in the biological environment. To determine the biological reaction of the nanocomposite sample in the solution, 1 cm of each sample is placed in the plastic with 10 mL simulated body fluid (SBF). Then, the falcons were placed in the water bath for 14 days at a constant temperature of 37°C. At the end of 1, 4, 7, 14, and 21 days, the suitable containers were removed from the water bath, and the corresponding pH for each sample was measured. To perform a biological test and check the nontoxicity, the pH of the samples was checked in the SBF solution. According to the previous studies,[24,33] chitosan and its derivatives have excellent antibacterial properties against Gram-negative and Gram-positive bacteria, as well as antibiotic-resistant bacteria. The biological property and antibacterial activity of the samples containing different weight percentages of MNPs were investigated in the presence of Gram-positive *(Staphylococcus aureus)* and Gram-negative (*Escherichia coli*) bacteria, which are the essential microorganisms in the wound environment as shown in Figure 3.

For the evaluation of the antibacterial response of the sample, we prepared a bacteria solution with a concentration of 0.5 McFarland, and then, we placed the samples in a test tube and incubator at 37°C for 18–24 h. Then, the antibacterial process needed 24 h of bacterial culture. To remove moisture, the synthesized wounds were transported into the molds of agar cultures (containing *E. coli* and *S. aureus* bacteria). At the end of the multivariate culture of Agar, they were placed in an incubator at 37°C ± 1°C for 18 h.

Results

The FTIR spectrum of CMC polymer with different compositions of ceramic and MNPs was shown in Figure 4. The significant peaks corresponded to O-H tensile vibration at 9158, also the C-H bond tensile vibration at 2077, and N-H flexural vibration at 1520 occurred. Polysaccharide-related peaks, including glycosidic vibrations, C-O, and C-O-C tensile vibrations, can be seen at 1188 range. According to Figure 4a-d, the peak (at 1480 peak) indicates the symmetrical axial deformation of the COO, which confirms the presence of carboxymethyl groups. On the other hand, the interaction of CMC chains with Fe ions causes a clear change from the carboxylate bond (1607/cm) to a higher number of descending waves (11,640/cm), which confirms the presence of nanoparticles in the polymer matrix.

Figure 5a-d illustrates the SEM images of the porous samples with homogenized and irregular distribution and dispersion of the MNPs. The examination of the immersed sample in the SBF solution after 21 days shows an absence of nanoparticles. It can be concluded that the calcium silicate bioceramic nanoparticles are more stable regarding their spherical shape surrounded by the polymer in the sample containing 5 wt% of MNPs. Furthermore, the SEM images indicate a successful complete network between the polymer and ceramic in the sample with 5 wt% MNPs, which can cause the polymer structure to remain constant.

The biodegradability results of the samples are shown in Figure 6. The weight loss results affected by the weight percentage of the MNPs are shown in Figure 6a-d. Figure 6a shows the SEM image of the samples after being placed in the PBS solution. A CMC polymer has surrounded the spherical particles in nanosystems. Figure 6b indicates clear crystalline plates for the sample containing 2.5 wt% MNPs. In addition, due to the interconnected structure and porosity ratio, the release rate of ions in this sample is lower. According to Figure 6c, ceramic nanoparticles with spherical spheres are well visible from the surface of the sample with the same roughness. Figure 6d illustrates the sample with the highest amount of MNPs consisting of transparent and white particles. In this Figure 6, the polymer surrounds the nanoparticles properly, and the dissolution rate is slightly higher than another sample, which leads to high dissolutions. The integrity of the skin in humans and animals is vital to protecting against water loss, bleeding, and counteracting the entry of microorganisms. For this purpose, wound healing in humans and animals is done by a complex and advanced mechanism. Wound healing is important in terms of time, as the skin regulates important functions in the body and has a protective role.

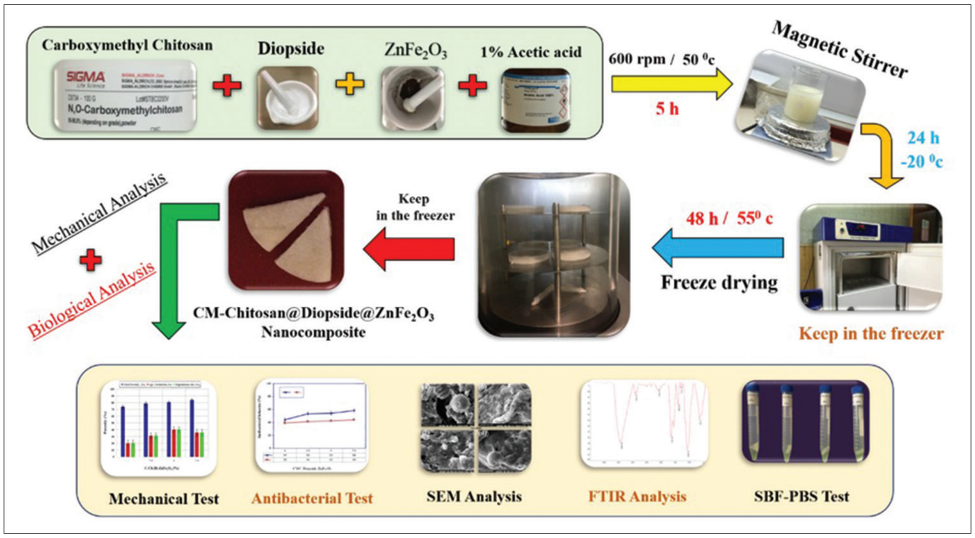

Figure 3: The process of fabrication and characterization of wound dressings by freezing-drying technique

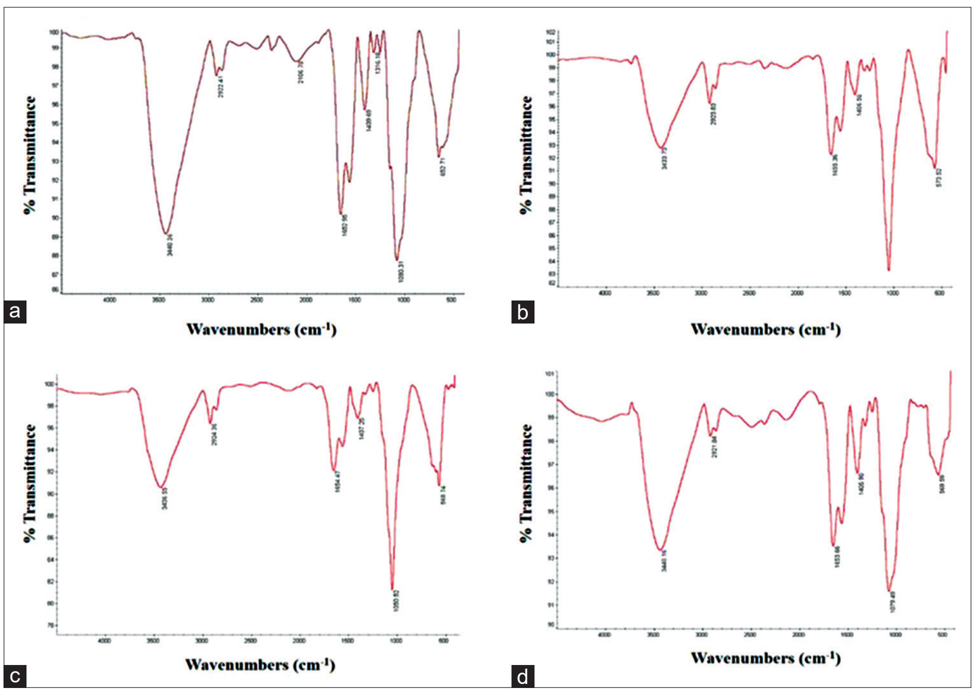

Figure 4: FTIR spectrum of polymer–ceramic samples with different concentrations; (a) 0 wt%, (b) 2.5 wt%, (c) 5 wt%, and (d) 7.5 wt% MNPs

As shown in Figure 7, the layer formation has been increased for the sample containing 5 wt%. Despite this, the sample containing 7.5 wt% MNPs presents a lower layer formation after soaking in the biological saline. Wound healing initially involves the homeostasis stage, in which the homeostasis phase begins immediately after the wound has formed. After the skin damage, arteries constrict and damage the platelets and activate the coagulation process. Then, it is followed by the formation of fibrin under the influence of small and local vascular components. Finally, plasma proteins and white blood cells enter the wound site. Figure 7 shows the pH results of the samples in the SBF after 21 days. As can be seen, there is no significant change in pH from the day 7^{th}.

Since one of the main objectives of this study is to provide antibacterial wound dress to prevent the possible infection on the wound, the antibacterial behavior of the sample is investigated.

Furthermore, the sample with the higher amount of MNPs has a controlled rate of destruction, suitable size, a higher percentage of porosity for cell growth, sterility, and ultimately creates new tissue that can replace with the damaged tissue inside the body. Figure 7 shows that the dissolution rate of the novel wound dress containing 5 wt% MNPs is higher than the sample with 7.5 wt% MNPs [Table 1]. Table 1 illustrates the percentage of the layer formation, as well as the rate of degradation of

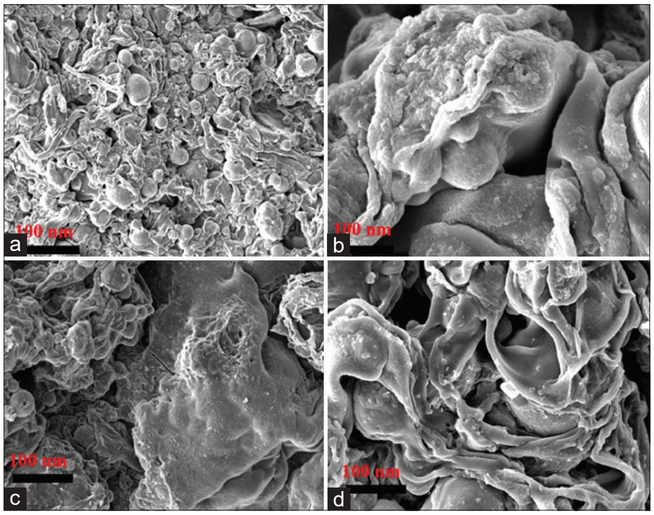

Figure 5: SEM images of polymer–ceramic samples with different weight fractions of (a) 0 wt%, (b) 2.5 wt%, (c) 5 wt%, and (d) 7.5 wt% MNPs after being placed in SBF solution for 21 days

bionanocomposite containing different weight percentages of MNPs.[1] Table 1 shows that the addition of MNPs to the base matrix can enhance mechanical and biological properties of the sample with 5 wt% MNPs. According to the porosity results, it is confirmed that the degradation rate and the layer formation in the wound dress increase up to the sample with 5 wt% MNPs.[53-56]

The pH of all solutions on the 1st day, like the pH of the SBF, was equal to 7.4. By passing the time and with the dissolution of the ceramic in the solution, and penetration of ions into the solution, pH started to be changed. As Figure 8 shown, most of the changes occurred in the 1st week. In the most significant one, after 5 days, the pH decreased from 7.4 to 7.2. The release of Si bonds, calcium, and iron ions from the structure of the samples, after immersion in the SBF solution, was significantly visible. Furthermore, according to the previous research,[1,13-17] the addition of MNPs nanoparticles has reduced pH. We can refer to Figure 9, which shows the ICP-AES analysis of the porous soft bionanocomposite wound dress. In addition, the wound condition with moisture hurts healing, as a lack of moisture on the surface of the wound reduces the activity of cells, reduces the blood oxygenation, and severely stops the wound healing. On the other hand, dehydration due to sodium depletion can delay all aspects of the healing process.

Discussion

Figure 9 shows the ionic concentration of the sample containing calcium and magnesium in the diopside-detected structure, using ICP-AES. The amount of calcium ions from 32 mM in the sample without MNPs increased to about 35 mM for the sample containing 7.5 wt% MNPs. It shows that the addition of MNPs may increase the calcium ion interaction from the sample to the solution based on the mass and density of the MNPs and calcium ions. In addition, the release of magnesium

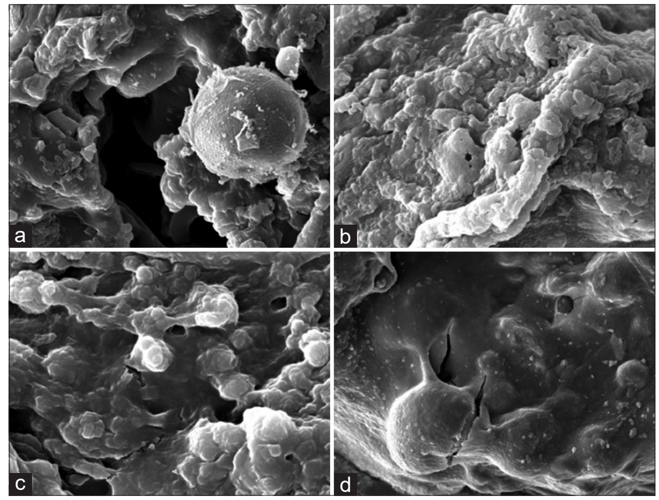

Figure 6: SEM images of polymer–ceramic samples with different weight fractions of (a) 0 wt%, (b) 2.5 wt%, (c) 5 wt%, and (d) 7.5 wt% MNPs after being placed in PBF solution for 21 days

Table 1: Biological properties (weight changes and layer formation) of nanocomposite

Sample	Degradation rate (%)	Layer formation (%)
S1	20	20
S2	31	31
S3	40	40
S4	36	36

ions has increased with a similar trend. The importance of antibacterial response of the wound dress can prevent or make a significant delay in the creation of biofilm under the wound and accelerate the wound healing process. The antibacterial activity of wound dress under *S. aureus* and *E. coli* is one of the most important microorganisms' environments. The obtained results of the antibacterial activity of wound dress containing MNPs are shown in Figure 10. The results show that the antibacterial activity of wound dress is affected by the concentration of MNPs, so that the antibacterial activity of the samples increases with increasing the concentration of MNPs. According to the results, bionanocomposite wound dress containing 5 wt% MNPs can effectively kill or prevent the entry of bacteria into the wound site. By taking the antibacterial activity and the SEM images results into account, it can be concluded that bionanocomposite wound dress containing 5 wt% MNPs presents a proper antibacterial and chemical properties with a sterile environment around the wound, that can increase cell growth and proliferation of fibroblast cells.[26,57-63] Maghsoudlou *et al*.[64] prepared novel chitosan reinforcement with antibacterial nanoparticles for bone tissue engineering application using the FD technique. Farazin *et al*.[65] fabricated a polycaprolactone–hydroxyapatite reinforced with titanium oxide using the FD technique. They presented a homogenized soft tissue with a dimension of 10 cm (diameter) and evaluated the mechanical and biological properties. However, the PCL-HA-TiO_2 nanocomposite

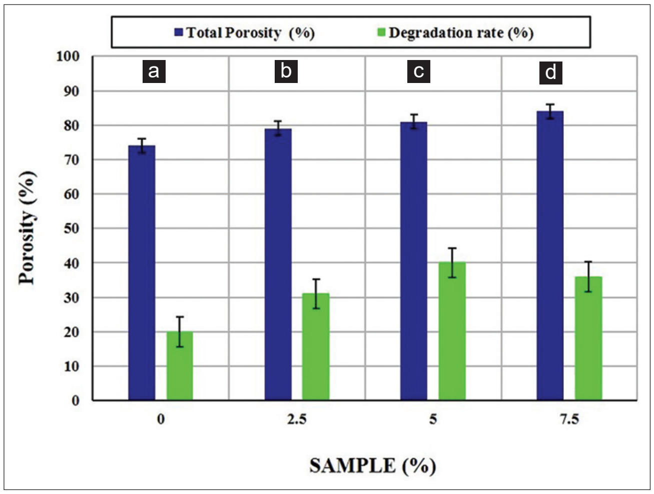

Figure 7: Comparison of porosity and degradation rate of nanocomposite with (a) 0 wt%, (b) 2.5 wt%, (c) 5 wt%, and (d) 7.5 wt% of MNPs

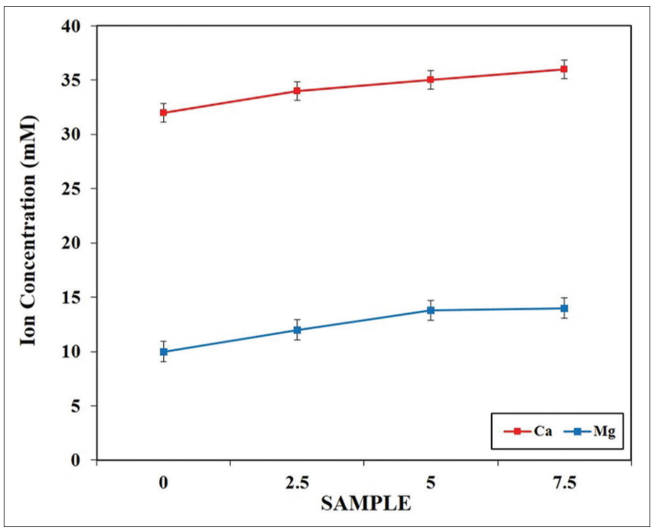

Figure 9: Graphs of the release of calcium, magnesium, and silicon ions

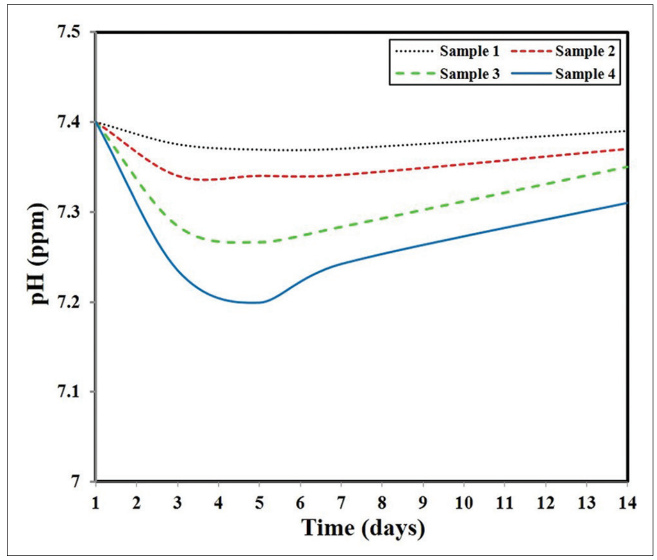

Figure 8: pH changes of the samples in the SBF saline after 14 days

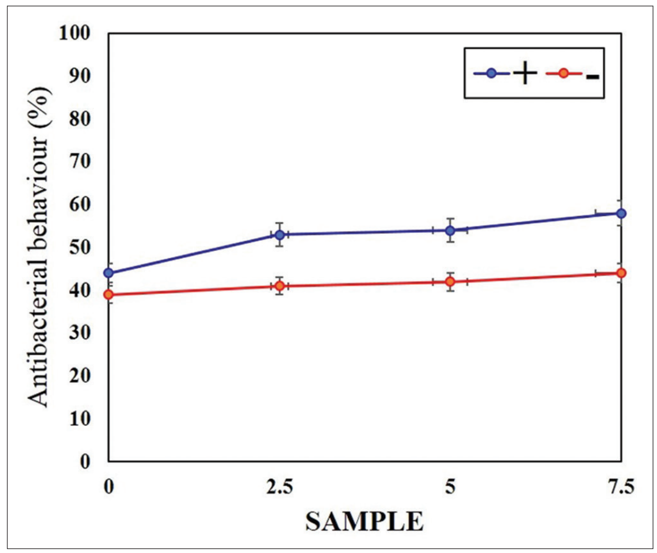

Figure 10: Antibacterial diagram of bionanocomposite wound dress with (a) 0 wt%, (b) 2.5 wt%, (c) 5 wt%, and (d) and 7.5 wt% of MNPs

has been fabricated using a similar technique with lower mechanical performance.[66] In another study, Farazin *et al*.[67] investigated the mechanical properties of the novel tissue using molecular dynamics with Materials Studio software. Hadisi *et al*.[68] fabricated hyaluronic acid-based silk fibroin and core–shell using the electrospinning technique for burn approaches.

In many cases, the number of living cells in damaged tissue is too small that they cannot repair damaged tissue, especially if the goal is to service very large damage. Therefore, it is necessary to increase the number of isolated cells from biopsied tissue. For this purpose, after biopsy of a specific part of the body, a small number of cells are isolated. Then, these cells are cultured and propagated by conventional methods to be used in the infected site. GFs act as signaling molecules between cells, such as cytokines and hormones that bind to a specific receptor on the surface of target cells.[41-43] They can also stimulate or inhibit the proliferation, differentiation, migration, adhesion, and expression of cell genes. These molecules are very vital in the wound healing process as they play an important role in inflammatory responses and promoting angiogenesis.[9,11,21,61]

Conclusions

In the present study, the nanocomposite wound dress was made of CMC as a polymer and diopside bioceramic to reinforce the chemical stability with different weight percentages (0, 2.5, 5, and 7.5 wt%) of MNPs, using the FD method. In fact, due to the polymer's hydrophilic properties composed of ceramic, and the poor mechanical properties of chitosan in the presence of water, wet environments, and inadequate hardness of this nanocomposite was observed. The morphological investigation on the porous nanocomposites was performed, and the SEM images represented the size and percentage of pores to evaluate the effects on the mechanical properties with the addition of MNPs. The amount and size of pores in composite scaffolds increased with the addition of 5 wt% MNPs that selected as the best structure in terms of the number of porosity and uniformity on the surface of the tissue. According to the obtained results, it can be said that increasing the magnetic phase in the polymeric part may increase the elastic modulus. Therefore, based on the other studies and evaluations, it can be concluded that the addition of diopside and MNPs can increase the tensile strength of the product, in which the addition of MNPs has a significant effect on the antibacterial property of the wound dress. It seems that CMC nanocomposite tissue containing 5 wt% of MNPs prepared by FD has a good potential application for wound healing. On the other hand, the ionic release on PBS presents the proper ability of the ions to solve the infection problems. The release of drugs or ions from the wound can treat the skin lesions, mainly the issue of inflammation or infection because of moisture. Therefore, some anti-inflammatory drugs can be loaded on the wound, simultaneously, to facilitate the process of tissue regeneration.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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