



CONSTRUCTION AND EVALUATION OF COMPOSITE SCAFFOLD PROPERTIES OF CELLULOSE BASE ENHANCED WITH CALCIUM ALGINATE CONTAINING PROPOLIS FOR USE IN SKIN TISSUE ENGINEERING



Maryam Asri¹

Azadeh Asefnejad^{2*}

Mitra Naeimi³

^{1,2}Department of Biomedical Engineering, Science and Research Branch, Islamic Azad University, Tehran, Iran.

³Email: asefnejad@srbiau.ac.ir

³Biomedical Engineering Department, Central Tehran Branch, Islamic Azad University, Tehran, Iran.



(+ Corresponding author)

ABSTRACT

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Ideal wound dressing materials should meet the requirements including maintaining a local moist environment, good surface absorbance for wound exudate, decreasing wound surface necrosis, and avoiding the lack of moisture in the wound. Common treatments for infections caused by skin diseases, resulting in extensive damage and often cause the death of people. In this project, the electrolytic solution of carboxymethylcellulose / calcium alginate / propolis was used to fabricate suitable fiber diameter and uniform distribution. Cellulose is suitable for the grafting of a myriad of chemical groups because of the ample availability of hydroxyl groups, and Propolis was selected in this research due to its excellent properties, such as accelerating wound healing and cell growth and antibacterial growth. The optimum electrospinning conditions were achieved at a voltage of 20 kV, with a spacing of 20 cm from the syringe to the collecting plate and a rate of 15 ml / h. Then, to evaluate the properties of the scaffold. Scanning electron microscopy (SEM), infrared Fourier transform (FTIR), mean of weight loss were performed. Finally, for testing the cellular survival of the samples MTT test was performed and a mechanical test was performed on the specimens. The scaffold degradation rate was linear and resulted in its complete destruction in 21 days. Cytotoxicity testing of scaffolds yielded acceptable results. A suitable structure for porosity and permeability was obtained in calcium carboxymethylcellulose/calcium alginate/propolis scaffold and this scaffold is a good candidate for application in skin tissue engineering.

Contribution/ Originality: In this project, the electrolytic solution of carboxymethylcellulose / calcium alginate / propolis was used to fabricate suitable fiber diameter and uniform distribution.

1. INTRODUCTION

The body's largest organ is the skin and the skin surface plays an important role in protecting the internal organs of the body. so any serious damage to this level can endanger the health of other organs [1]. Skin also acts as a barrier to protect internal organs against all types of environmental damage, including mechanical, chemical, and physical damage [2]. In addition to the exchange of oxygen with the outside of the body, and increases blood flow to the underlying tissues [2]. Removal of toxins from the body through transpiration is also another of the tasks of the skin [3]. When the skin is damaged, the wound is damaged in the affected area, which, if treated

improperly and uncontrolled, can lead to infection and build up of necrosis, and ultimately the destruction of the limb and surrounding tissues.

Currently, plastic surgery is an effective treatment for irreversible skin lesions, such as subacute wounds and severe acne, skin grafts and the transplantation of the skin. That is difficult and complicated to access to a healthy and quality skin which is capable of harvesting and replenishing at the damaged site. Also, the limitation in the area of the harvesting area is one of the other problems of this method and bearing financial burden for heavy surgeries by the patient and requiring surgeon's individual skills is one of the limitations of this method. Hence, the use of other suitable alternatives for repairing and treating the skin of the patient's skin has been proposed, including the use of high biocompatibility and superior biological efficiency scaffolds using tissue engineering. Through various engineering techniques. Polymeric and porous scaffolds produced by electrospinning, have been considered by researchers as a suitable strategy.

In wound healing, choosing the appropriate dressing, is especially important in tissue repair [4]. The purpose of wound dressing is to facilitate wound healing, preventing infection, preventing bleeding, helping to heal the wound, temperature insulation, retaining moisture and protecting the wound from mechanical damage [5, 6]. Dressing that has all the features of an ideal dressing is not produced yet [7]. Biopolymers are polymers that are produced by biological systems such as microorganisms, plants and animals, and have similar benefits to the host tissue and, on the other hand, have the ability to relate to the biological environment and avoid toxicity [8, 9]. Today, coatings made of natural polymers such as collagen, chitosan / chitin and alginate are available [10]. But the problem with the direct release of antibiotics from natural polymer coatings is that most natural polymers are hydrophilic and can not inhibit the rapid release of small antibiotic molecules unless they are linked to a very high degree of cross-linking, as well as natural polymers in the body [11]. The proteases are degraded, which leads to the release of active agents from the coating [5, 12]. Propolis shows strong bactericidal or bacteriostatic properties, especially the cinnamonic acid and flavonoid in the waxy lamb show antibacterial performance [10, 13]. In some cases, propolis extracts are more effective than commercially available drugs. Chemical decomposition of propolis also proves the presence of the following substances: water, collagen, carbohydrates, proteins (most of which are alumina and globulin), essential amino acids, acetylcholine, minerals such as calcium, iron, silicon and phosphorus, vitamins A, C, E, B1, B2, B3, B5, B6, B7, B8. The high percentage of sugar and minerals and the antibacterial properties of propolis, especially on *Staphylococcus aureus*, which is the common cause of wound healing [14, 15]. Carboxymethyl cellulose is an important ingredient in the preparation of biodegradable and biocompatible polymers that has applications in drug delivery and tissue engineering [16]. In addition to proper biocompatibility, the ability to make chemical changes in these molecules is an interesting range of ejaculations based on cellulose, which can be created from gel to porous sponge according to the type of application and type of crosslinking [17, 18].

Alginates are anionic and hydrophilic polysaccharides which exhibit excellent biocompatibility and biodegradability properties. The researches show that Calcium alginate (AlgCa) wound dressings maintain hydration and local protection when in contact with body fluids that are present in exudative wounds. In fact a high swelling capacity is a specific property of alginate [19].

The purpose of this study was to Construction and Evaluation of Composite Scaffold Properties of Cellulose Base Enhanced with Calcium Alginate Containing propolis Extract as a suitable substitute for skin tissue.

2. MATERIALS AND METHODS

2.1. Materials

Calcium alginate and Sodium Carbonate were obtained from Sigma-Aldrich (America), and carboxymethyl cellulose (CMC) and poly vinyl alcohol were purchase from Merck (German). Propolis was used from Manuka (New Zealand). penicillin, Dimethyl sulfoxide (DMSO), trypsin and DMEM culture medium were sourced were obtain from Gibco (America), Phosphate-buffered saline (PBS), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide (MTT) were sourced from Sigma-Aldrich Ltd. (USA). L929 cells were provided by the Institute of Pastor (Iran). All other chemicals were of reagent grade and were used without further purification.

2.2. Preparation of Polymer Solution and Electrospinning

First, the factors affecting the scaffold properties such as concentration, voltage, and distance must be determined and optimized.

First, the CMC polymer is dissolved slowly in distilled water / DMF on the mixer plate, and then the calcium alginate is added in 1: 1, 1: 2, and 1: 3 ratios. After dissolving CMC at 48 ° C and 14% w / w, bicarbonate solutions were prepared at concentrations of 20 and 25% by weight of calcium alginate at 45 minutes. Then, the polymer solution was prepared in optimum conditions at a rate of 15 ml / h and a distance of 20 cm to the collector and voltage of 20 kV. In this method, the pump is placed horizontally, then connected to the insulin hinge, and finally, attach the positive pole to the syringe to insert the load into the syringe solution, and place a sheet of aluminum foil on the collector opposite the syringe and The negative pole of the power supply is connected to it. After making the electrospun scaffolds of calcium alginate / CMC and alginate / CMC/ propolis nanofibers, their initial results, such as SEM images, were compared with the results of others, and it was found that the optimization results of the electrospinning process were properly restored according to the previous study.

2.3. Preparation of Propolis Extract

The most common materials used to prepare the extract in biological methods are ethanol, methanol, and water. In this study, Propolis samples were crushed and for extraction, 25 g of the sample accurately was weighed, then it was poured in a volumetric flask, And then the sample volume was shaken by ethanol 96% to 100 ml and the mixture was well mixed. This procedure was repeated once or twice for 3 days. The mixture was then kept in an incubator for one to two weeks and then the mixture was passed through a filter. And the filtered material was placed in a refrigerator for one day at a temperature of (1-4 ° C). The solution was then re-filtered. Then the remaining alcohol in the suspension was completely separated by a soxhlet extractor and a propolis alcohol extract was obtained. Since the ethanol solvent is evaporated at a lower temperature than water, in a few hours, completely separated from the solution and the extract of the propolis was collected in the compartment.

2.4. Fabrication of CMC / Calcium Alginate / Propolis composite Scaffold

In this section, a polymeric carboxymethyl cellulose solution and calcium alginate solution was prepared as in the previous example. Than 3% propolis extract was added and then put on magnetic stirrer to earn a uniform and homogeneous solution. Then the electrospinning process was done according to the defined parameters in the previous section.

2.5. Scanning Electron Microscope (SEM)

In order to investigate the morphology of polymer scaffolds in terms of pore size, fiber–fiber interconnection and fiber diameter, samples were prepared with 1 × 1 dimensions and coated with gold and were prepared by scanning electron microscopy. The porosity of scaffolds was investigated using SEM images as inputs, and an image processing program written in Matlab software.

2.6. ATR-FTIR Analyze

The chemical structures of electrospun scaffolds were characterized by ATR-FTIR spectroscopy (NEXUS 670, Thermo- Nicolet, USA). The transmittance of each sample was recorded with a resolution of 4 cm⁻¹ between 4000 and 400 cm⁻¹ at room temperature.

2.7. Study of Mechanical Properties

Precise measurements of the mechanical properties of scaffolds are essential for medical applications. Because this method can be used to ensure the tolerance of the sample against the forces involved during and after surgery. Therefore, due to the uniaxial stretch test on a variety of information fibers such as Young's modulus, yield stress, breaking stress, and fracture energy can be achieved. To do this test, the samples were repeated 6 times, the test speed was 60 mm / min and the sample length was 4 cm. According to ASTM-F1634 and ASTM-D882 standards, the electrospinning samples were considered 1.5 cm wide and 1 mm thick.

2.8. Contact Angle Measurement

The contact angles of the electrospun nanofibers were measured using the Video Contact Angle Optima surface analysis system (KRUSS G10, Sessile drop, Germany). The contact angle was measured at room temperature. Briefly, the rectangular-shaped samples (15 mm × 15 mm) were placed on the stage. A distilled water drop is put on five different sites of nano-fibrous membranes and the measured angles were averaged.

2.9. Cell Toxicity Test

The cytotoxicity test was performed according to ISO10993-5. The sterilization of samples was performed with immersion in 70% ethanol then dried and sterilized under UV light for 1 h on each side. Scaffolds were placed in 24 well plates. Then the samples were immersed in the DMEM medium. In this study, L929 fibroblast cells were used. The cells were cultured in sticky cultures from the Pasteur Institute of Iran - Department of Cell Bank.

The cell suspension was pipetted directly onto the scaffolds with an initial seeding density of 5000 cells/scaffold. At different intervals, the MTT test was performed to evaluate cell survival. After removing the culture medium and washing with PBS, about 400 µl culture medium was added with 40 µL of solution (MTT 5 mg/ml) to each well of the culture. The incubator was then kept at 37 ° C for 4 hours. Subsequently, the DMEM was removed slowly, and 200 µl of dimethyl sulfoxide (DMSO) was added to the well to dissolve the formazan dye. Then the absorbance of the solution was measured at 570 nm wavelength using an ELISA reader.

2.10. Statistical Analysis

All data presented are expressed as mean ± standard deviation (SD). Statistical analysis was carried out using one-way analysis of variance (ANOVA), followed by Tukey post hoc test for multiple comparisons, and significance was considered at $p \leq 0.05$.

3. RESULT AND DISCUSSION

3.1. Scanning Electron Microscopy (SEM)

Figure 1 was shown the electrospun samples of CMC / calcium alginate to determine the optimal concentration of polymer solution. In sample a, calcium alginate 20%, and in sample 2 calcium alginate 25% were considered. Electrospinning Parameter includes voltage, distance to the collector plate, and feeding rate, in both samples were defined and the only variable parameter was the concentration of the polymer solution. At first, the samples without propolis extract were used. As shown in Fig 1 (a and b), the surface of the sample, was more uniform than sample b, and the adhesion of the fibers is not observed in sample b. For this reason, the optimal concentration of 20% w/w was considered for the electrospinning process. Table 1 was indicated that fiber diameter decreased by adding propolis to the electrospinning process. The best nanofibers were observed in presence of propolis without adhesion between the fibers and the absence of beads.

In all samples, porosity is above 70%, Which is one of the requirements for tissue engineering scaffolds [20].



Figure-1. SEM of a. Calcium Alginate 20% / CMC , b. Calcium alginate 25% / CMC and c. Calcium alginate / CMC / propolis.

Table-1. The properties of nanofibers.

Sample	Minimum of fiber diameter (nm)	Maximum of fiber diameter (nm)	Average of fiber diameter (nm)	Porosity (%)
Calcium Alginate / CMC / Propolis	575.5	745.5	660±58.5	76.38

3.2. Infrared Spectroscopy in Fourier Transform Method

Figure 2 was illustrated FT IR spectrum of Calcium Alginate / CMC / Propolis sample. Peaks of calcium alginate were appeared around 3270, 1630 and 1014 cm^{-1} , due to the stretching of the hydroxyl, carboxyl and C-O-C groups. The peak of CMC is about 3400 cm^{-1} , which is due to tensile vibrations of OH groups and intra molecular and intermolecular hydrogen bonds. The peak of the C-H vibrations is 2922 cm^{-1} . Observation peaks in the region of 3336 cm^{-1} are attributed to the O-H bending bonds of phenolic compounds in the propolis.

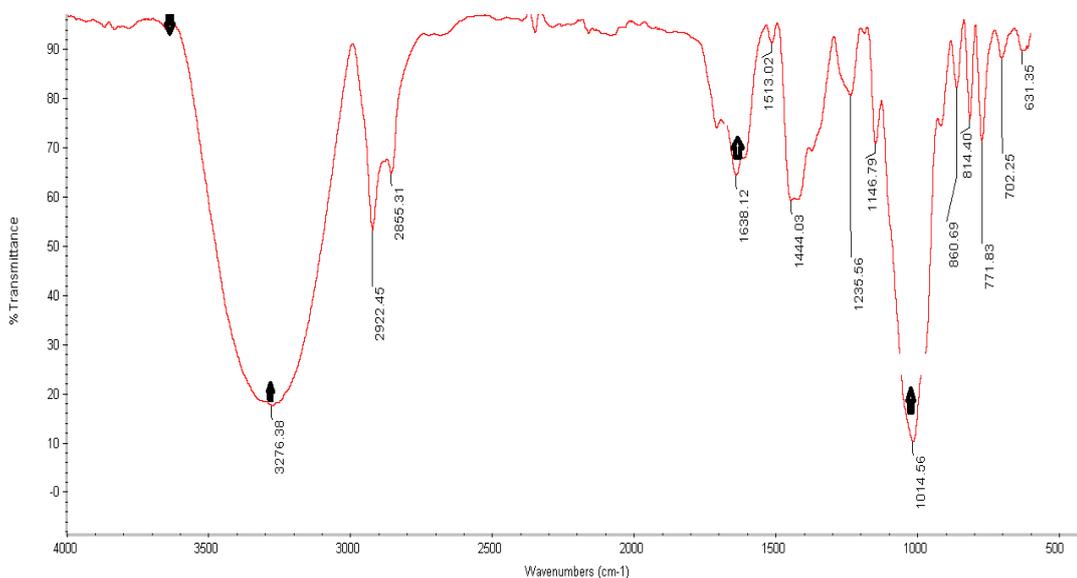


Figure-2. The FTIR spectrum of calcium alginate / CMC / Propolis.

3.3. Evaluation of Mechanical Properties

As shown in Table 2, calcium alginate increased the ultimate tensile strength (UTS) from 16.6 ± 1.2 MPa (CMC electrospun scaffold) to 17.1 ± 1.8 MPa (calcium Alginate / CMC scaffold). According to the previous search, alginate could enhance the mechanical properties of scaffolds [21]. Although the UTS of calcium Alginate / CMC / Propolis scaffold was decreased, Elongation at break of Calcium Alginate / CMC / Propolis scaffold was increased (29.8 ± 4.6 %) in compare of CMC (27.4 ± 4.7 %) and Calcium Alginate / CMC (22.5 ± 4.7 %).

Table-2. The mechanical properties of CMC, CMC / calcium alginate, CMC / calcium alginate / propolis.

Sample	Minimum of fiber diameter (nm)	Maximum of fiber diameter (nm)	Average of fiber diameter (nm)	Porosity (%)
Calcium Alginate / CMC / Propolis	575.5	745.5	660±58.5	76.98

3.4. Biodegradability Test

In order to investigate the degradation process, the sample weight loss in the phosphate buffer solution was investigated. The degradation chart for samples containing and without propolis during 21 days is shown in Figure 3. According to Figure 3, destruction in both groups containing and without propolis was linear and there was a significant difference between the two groups ($p \leq 0.05$).

Figure 3 was illustrated that calcium alginate / CMC / propolis electrospun nanofibers were losing weight more than calcium alginate / CMC electrospun nanofibers after 21 days. In fact the presence of propolis in the scaffold was to increase the rate of degradation.

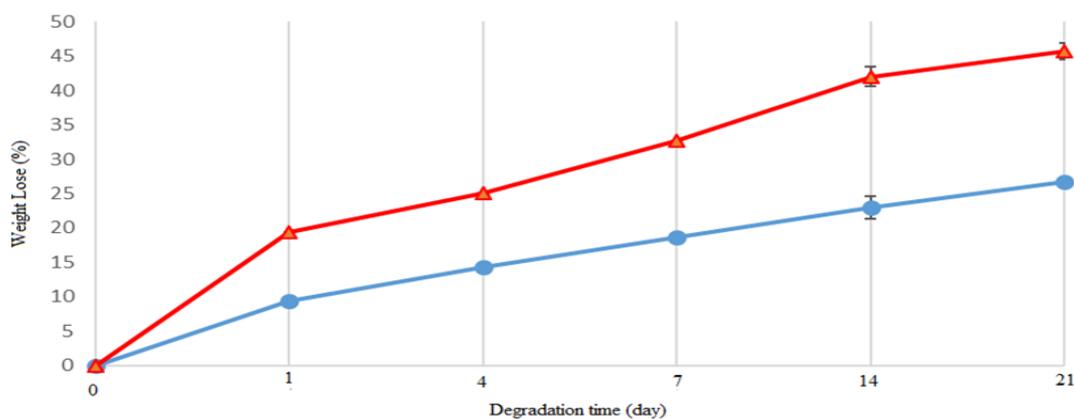


Figure-3. Degradation of calcium alginate / CMC and calcium alginate / CMC / propolis electrospun nanofibers.

3.5. Hydrophilicity of Electrospun Nanofibers

The contact angle of CMC, CMC / calcium alginate, and CMC / calcium alginate/propolis electrospun samples are shown in Figure 4. In general, if the contact angle of the scaffold is more than 90° , the surface is hydrophobic, and if it is less than 90° , the surface of scaffold is called hydrophilic [22]. Since CMC is a hydrophilic polymer [23] it is expected that by adding calcium alginate to the scaffold, the water contact angle is also increased, the results of this analysis confirmed this statement. With the addition of propolis extract, the contact angle of the scaffold was reached 23.87° , indicating lower water content.

3.6. Cell Cytotoxicity

In this section, the MTT test was investigated. Figure 5 showed the MTT value of the control group and scaffolds in 1 day, 3 days, and 5 days. The results of MTT showed that the growth of fibroblastic cells was done on the scaffolds, without any toxicity, and cell proliferation on all scaffolds increases with the lengthening of culture time, and the superiority of the CMC / calcium alginate/propolis scaffold to other scaffolds also becomes more significant.

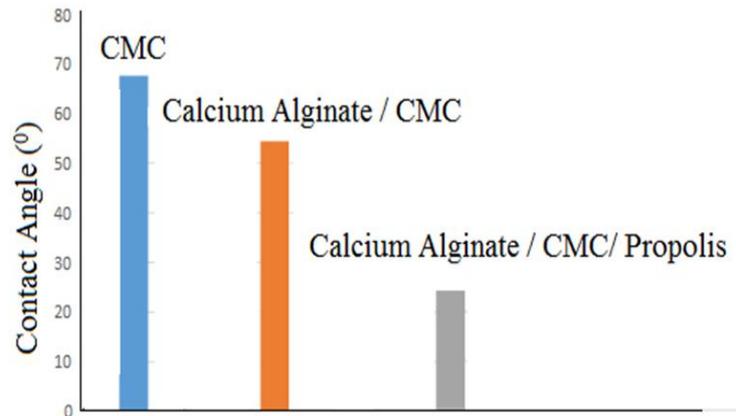


Figure-4. Contact angle of CMC, calcium alginate / CMC and calcium alginate / CMC / propolis electrospun nanofibers.

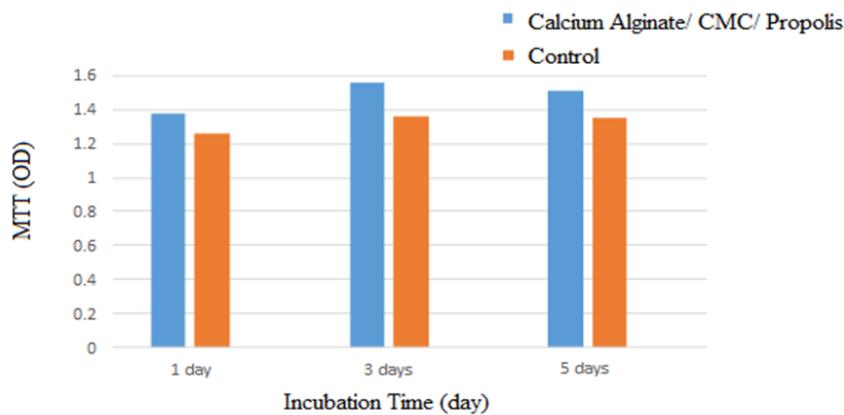


Figure-5. MTT of control sample and calcium alginate / CMC / propolis electrospun nanofiber.

4. CONCLUSION

In this study, electrospun scaffolds of calcium alginate / CMC and alginate / CMC/ propolis nanofibers were fabricated. The SEM figures were demonstrated the smooth and bead free nanofibers were made. The mechanical strength, water absorption, and porosity were suitable for scaffolds as skin tissue engineering applications. Contact angle measurements were illustrated that the addition of propolis and calcium alginate / CMC was created a hydrophilic surface which made scaffold suitable for cell attachment and proliferation. So it seems that alginate / CMC/ propolis is a good candidate for skin tissue engineering.

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