

Improved Biomedical Properties of Chitosan/Alginate Composites by Chemical Immobilization of Gelatin Layer

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Abstract

Introduction: Recently, creating bioactive wound dressings from natural polymers is of importance. Also, there is high need for a device to staunch blood flow in deep wounds such as liver and spleen wounds, and would absorb in the body on its own. This device must be extremely safe, act fast, and adjust with local and general body health.

Objective: This project designed surface modification for films made from natural Chitosan-Alginate polymers containing multiple ratios via chemically immobilizing the gelatin polymer.

Material and methods: The scaffolds were synthesized by electrospinning method. In this regard, polymers were dissolved in the solvent and then graphene oxide was added into polymeric solution with a ratio of 2% and 4%. The parameters of the scaffold evaluated via scanning electron microscopes (SEM), Fourier transform infrared spectroscopy (FTIR), X-ray Diffraction (XRD), contact angle, alizarin red staining, and alkaline phosphatase (ALP). For evaluation of the cell behavior on the scaffolds, the MG-63 was used.

Result: Chitosan-Alginate (C-A) solutions were prepared in 8:2, 7:3 and 5:5 weight percentages. Afterwards, these solutions were used to make composite films to use as solvent casting. Gelatin (G) was immobilized onto C-A films using the crosslinking reagents, which included Glutaraldehyde. The effects of C-A ratio, gelatin concentration, amount of crosslinking agent and duration of immobilization process on the actual immobilized layer were investigated. The films were characterized by Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR), Differential Scanning Calorimetry (DSC) and Zeta Potential. SEM images demonstrated that immobilization onto 7:3 and 5:5 weight percentages ratios of C-A, resulted in a more consistent gelatin layer compared to 8:2. FTIR spectroscopy, which showed the appearance of amid peak in modified films. In DSC curves, suppression in Glass Transition Temperature (T_g) in modified films was detected. Also, Zeta potential decreased as the amount of gelatin layer on C-A films was increased.

Conclusion: The newly developed (C-A-G) Composite films by simple, yet effective method of immobilization can be used for various biomedical applications like tissue engineering and wound healing.

Keywords: Gelatin; Alginate; Chitosan; Glutaraldehyde; Immobilization; Surface modification

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1. Introduction

Nowadays many research has been done on the natural polymers as an ingredient of scaffolds. Chitosan and Alginate are considered among natural polymers as scaffold. Chitosan is broadly used in tissue engineering, considering its biocompatibility, biodegradability, bioactive functional groups and similar chemical structure to Glucose Amino Glycans (GAGs). Other reasons to use Chitosan include: low price, accessibility, antitoxicity, antibacterial activity, bioinertness, and affinity to bind with proteins [1,2]. Chitosan has polar NH₂ and OH groups that make the polymer cationic. Positive charge of Chitosan is favorable for cell adhesion, however, this prevents cell spreading; therefore, Chitosan is usually used in composite form with other polymers such as Alginate to reduce the positive charge and improve cell-scaffold interaction. [3-5]. Another biodegradable polysaccharide is Alginate, which is biocompatible and has been broadly used in cell culture studies. [6] Carboxyl groups in Alginate bind to cationic groups and form a gel structure. Hydrogel of Alginate is useful in tissue engineering applications, but because of Alginate's low mechanical strength, it is usually used in combination with other polymers [7]. By using a combination of composite materials desired properties can be achieved, while the weakness points of each component is eliminated. Gelatin as a biocompatible protein is inexpensive, has low antigenicity and high bio absorption. Using gelatin in tissue engineering scaffolds leads to preferable cell adhesion, proliferation and migration [8]. Recent studies showed that gelatin will enhance the activation of macrophages and help stop bleeding [9]. Biological responses of biomaterials are mostly determined by their surface chemistry and structure. Therefore, modification must bring biological properties to surface by altering surface hydrophobicity/hydrophilicity, surface potential, functional groups, roughness and structure [10]. Materials can be modified by chemical, physical or biological modification methods [11]. Polymers are easily combined with cells or biomolecules by chemical or physical interaction, and form biologic functional groups [12]. Active biological groups can

be physically or chemically immobilized onto polymer substrates. In the process of immobilization, a micromolecule sits on the surface either for a short time or permanently [13]. In physical immobilization, molecules are physically absorbed or entrapped in the polymer structure and chemical immobilization takes place by covalent binding [14]. Presence of functional groups such as COOH, NH₂ and OH are essential for covalent binding, otherwise the surface must be modified to allow covalent binding with biomolecules [15].

In this study, Chitosan-Alginate films were made by solvent casting. Since RGD sequence in Gelatin is recognized by receptors in cell membrane, gelatin was immobilized onto surface of composite films to improve interactions between cell membrane and material's surface. Immobilization was achieved by using chemical method which is relatively simple and does not engage complicated laboratory equipments. However, this method for surface modification of C-A films has not been studied adequately so far. In the end, composite films were characterized by SEM, FTIR, DSC and Zeta potential tests.

2. Materials and Methods

2.1. Materials

Chitosan used in this study was purchased from Chitotech with a deacetyl degree of 91.8 %. Alginate powder with 100000 g/mol wt% was supplied by BDH-Prolabo. Gelatin, glutaraldehyde and Acetic acid (96%) were purchased from Merk.

2.2. Preparation of chitosan-alginate films

Chitosan and Alginate powder was dissolved in acetic acid 1.5 % (w/w) to obtain 2wt% polymer solutions in which the ratio of C/A was 7:3, 8:2 and 5:5 in terms of weight percentages. After stirring for 72 hours on a 800 rpm stirrer, the homogenised solutions were poured into Petri dishes (with 5cm diameter) and then dried at 25°C to prepare C-A dense films.

2.3. Gelatin preparation and immobilization

Gelatin solutions were prepared by dissolving Gelatin powder in acetic acid 1.5 % (w/w). Weight percentages used in this experiment are presented in

Table 1. Homogenous solutions were attained by using a 250 rpm stirrer for 4 hours.

The size of composite films used for immobilization was 2 cm in length and 2cm in width.

For Gelatin immobilization Chitosan-Alginate films were immersed in 19ml Gelatin solution for certain periods of time (mentioned in Table 1), at room temperature, Meanwhile, 1ml glutaraldehyde was added in presence of gentle shaking. The ratios of C/A and wt% of glutaraldehyde used in this experiment are also showed in Table 1. To observe the effect of crosslinker, control samples were prepared in similar conditions, and were dissolved only in crosslinker solution in the same time frame. Afterwards, the coated films were immersed in acetic acid 0.001ml for 5 minutes followed by rinsing with distilled water to wash out the homopolymers. After the drying stage, the coated film was immersed in a NaOH aqueous solution (1N) to neutralize the remaining acetic acid.

2.4. Scanning electron microscopy (SEM)

Morphology of samples and effect of variable parameters in immobilized layer was studied via SEM. Cross section and longitudinal section of samples were obtained prior to gold coating.

2.5. weight measurement of immobilized layer

One of the most common methods to verify the presence of immobilized layer in modified films is to measure the weight of films before and after modification. 24 samples with 2cm in length and 2cm in width were prepared to measure their weight (W_0) with 0.0001 g precision. Then, these samples were modified by gelatin immobilization according to Table 1 and each experiment was repeated three times for accuracy. After drying, the weights of modified films were measured again (W_1). Graft amount was calculated using equation 1 [16].

$$\text{Graft amount (mg/cm}^2\text{)} = (W_1 - W_0) / A \quad (1)$$

2.6. Zeta potential analysis

Zeta potential of samples were measured to compare surface charge before and after immobilization, plus to prove the presence of gelatin in modified films. Control samples and modified films were prepared in

5×5 cm² and zeta potential test was carried out in neutral pH and room temperature.

2.7. FTIR spectroscopy

FTIR is a standard method to identify various functional groups in a compound that leads to recognizing chemical structure and bindings. This test was another way to prove the success of immobilization process.

2.8. DSC analysis

DSC will complement the investigation of thermal behavior in the modified scaffolds. This analysis was carried out on pure gelatin and modified films in the constant rate of 40 °C/min.

3. Result and Discussion

3.1. Morphology studies of films

SEM images of modified films compared to the control samples, indicating immobilization of uniform gelatin layer on the surface of the films with the conditions that are listed in Table 1. The uniformity of immobilized gelatin on the surfaces of samples with different conditions was not equal. In samples that modified with 2% gelatin solution (Figure 1), a uniform layer of gelatin was immobilized. By using increased amount of gelatin (4% gelatin solution), saturated layer of gelatin was immobilized and also many chains of gelatin penetrated into films, creating a three-part combination [17]. Whereas the goal in this project is the creation of uniform gelatin layer on the surface, and also optimum penetration of gelatin chains in bulk. Hence, the film's main combination was kept as Chitosan and Alginate. The scanning electron microscopy results showed that the immobilization of Gelatin on Chitosan-Alginate films with 7:3 and 5:5 ratio were more uniform than films with 8:2 ratio. Since the apparent mechanical properties, such as flexibility of films, with 7:3 ratio were better than 5:5 ones, films with 7:3 ratio were selected for further testing. Also, the amount of 2% Gelatin and 0.01% of glutaraldehyde was enough to immobilize thin layer of Gelatin. Duration process is a critical factor in immobilizing the Gelatin layer [10]. SEM images showed that modified samples with 1-hour duration

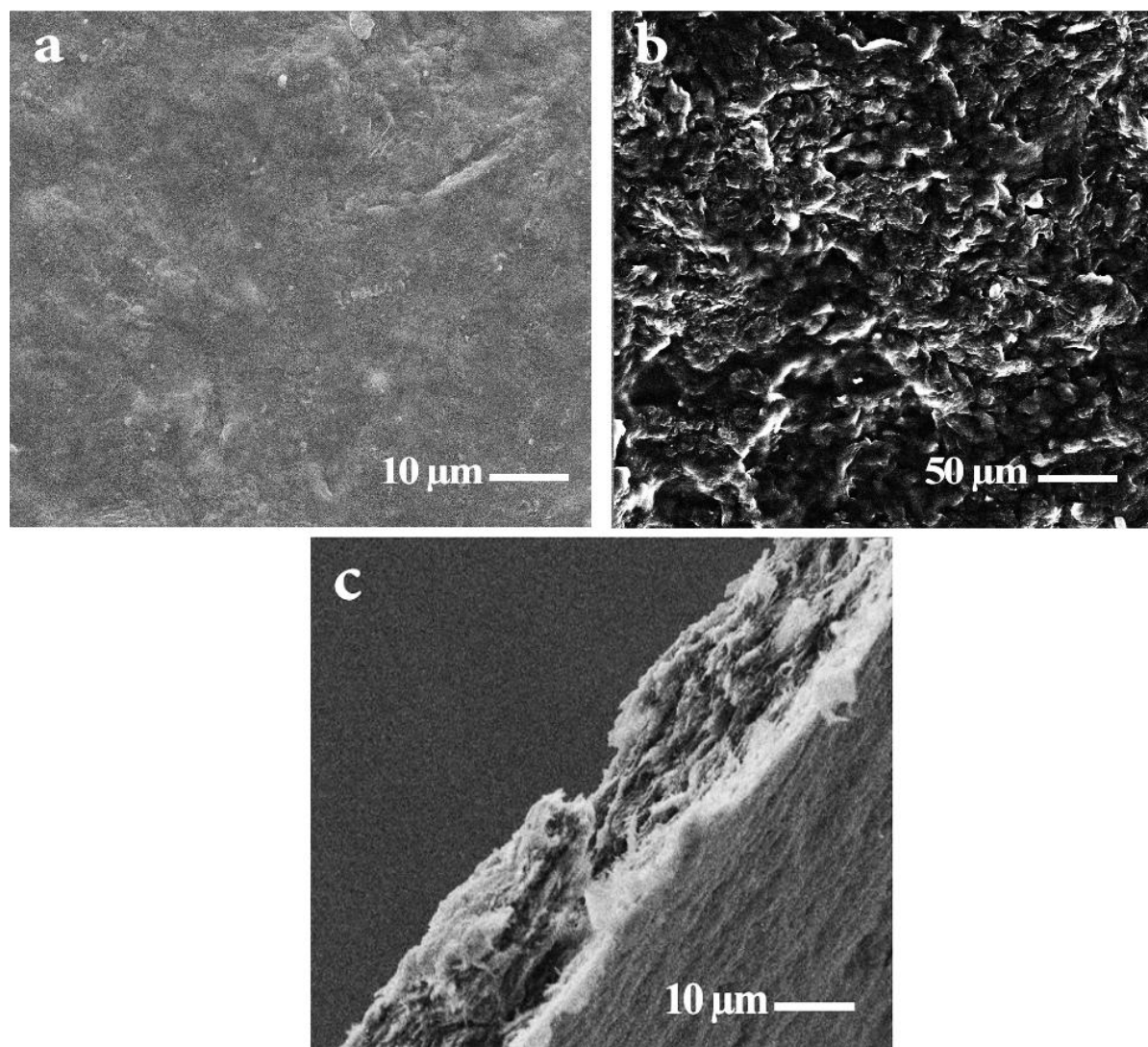


Figure.1. SEM studies of Chitosan-Alginate films with 7:3 ratios, 0.01% of glutaraldehyde, 1-hour duration, (a) control, (b) 2% gelatin solution (c) cross-sectional studies of chitosan-alginate films with 7:3 ratios, 0.01% of glutaraldehyde, 1-hour duration, 2% gelatin solution.

had a more uniformed layer of Gelatin immobilized on their surfaces. The first hour, gelation chains may influence the film structure and increase the film's strength. Another important factor in creating the film's strength is the duration time that the bulk of films are cross-linked. Cross-linking of Gelatin chains may also occur on the surface of the films resulting into an immobilized continuous and strong layer. With more time the films became brittle and less flexible [10]. So the appropriate immobilization time was determined to be one hour. SEM cross-section image (Figure 1(c)) shows that the gelatinous layer on both sides of the surface of the film are immobilized,

and the ends of gelatin chains have penetrated into bulk of the film. Thus, a continuous line is formed that is a gelatin concentration gradient, which increases from bulk to surface of the film.

3.2. Study of immobilized gelatin weight

The measurements of the amount of immobilized gelatin on every film is shown in Figures 2. Figure 2(a) shows the changes in the amount of immobilized gelatin, based on the changes in C-A ratios. Other immobilization conditions including the Gelatin concentration, duration of immobilization, and wt% of glutaraldehyde were the same. As seen in Figure

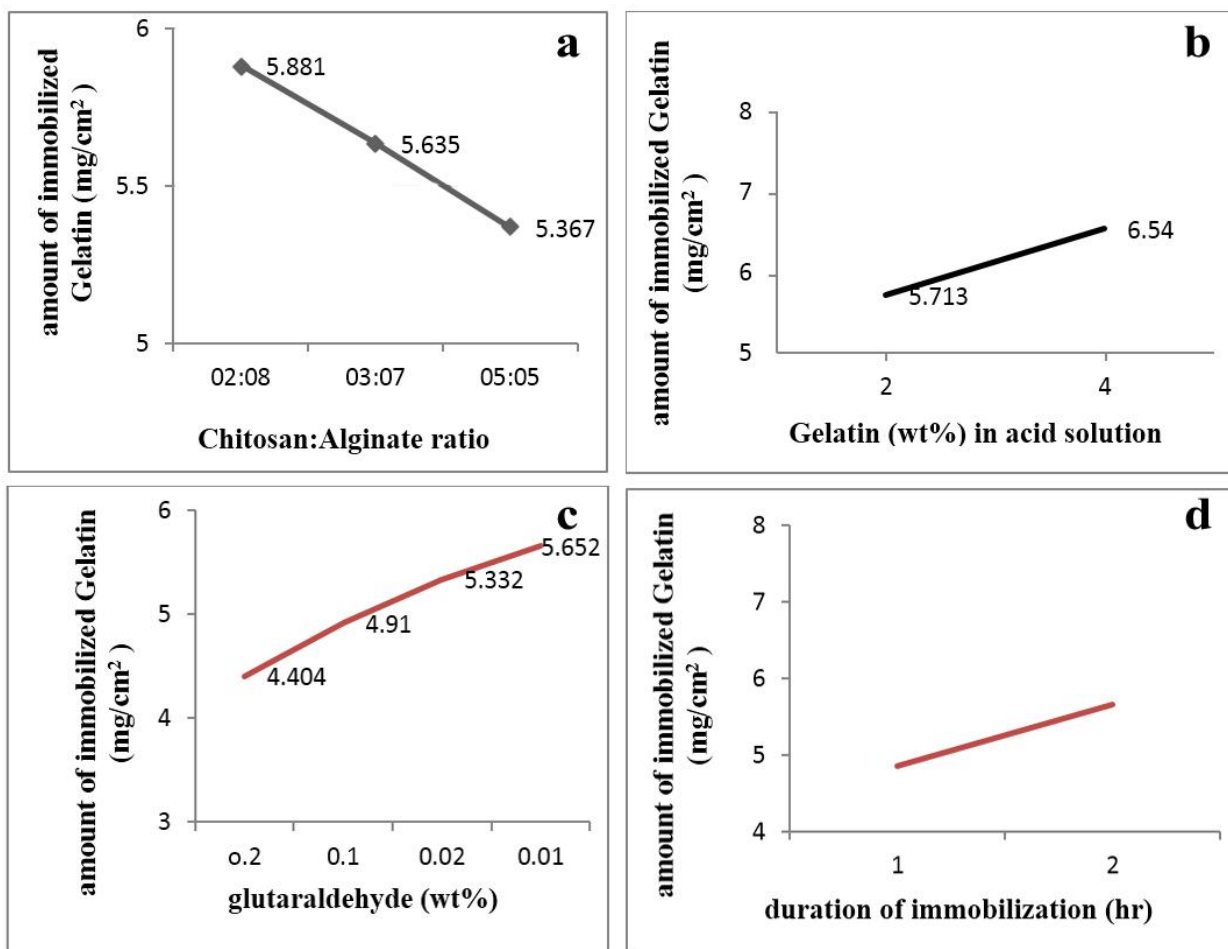


Figure 2. the curves of the amount of immobilized gelatin based on the, (a) changes in C-A ratios, (b) gelatin concentration, (c) changes of glutaraldehyde percentages, and (d) duration of the process changes.

2(a), with a reduction of Chitosan, amount of immobilized Gelatin also declined. These changes can be attributed to the chemical composition of the polymer. In higher Chitosan percentage, active groups that react with Gelatin increase, therefore, more Gelatin was immobilized on the surface [18]. The effect of Gelatin concentration on the amount of immobilized Gelatin is shown in Figure 2(b). All parameters relating to the modification of the films, except Gelatin concentration, were considered equal in this study. These results show that by increasing Gelatin concentration from 2% to 4%, the amount of immobilized Gelatin is increased. Figure 2(c) shows the amount of immobilized Gelatin based on the changes of glutaraldehyde percentage. For this study, films with different percentages of crosslinking agent and glutaraldehyde were modified, while the other

parameters of study were kept the same. As illustrated in Figure 2(c), increased concentration of glutaraldehyde reduced the amount of immobilized gelatin. Because by increasing glutaraldehyde percentages, the active groups of surfaces were crosslinked before the gelatin chains reacted with them. Figure 2(d) shows the changing of the amount of the immobilized Gelatin in relation to duration changes, while all other parameters were accounted for and kept the same. The results (2(d)) showed that in 1 hour duration of the process, more Gelatin was immobilized. During the first hour, Gelatin chains penetrated into the films, and the immobilized Gelatin layer was formed. But after 2 hours of reaction, the amount of immobilized Gelatin decreased. This was to the acidic crosslinking solution that caused gelating to dissolve in the solution over time.

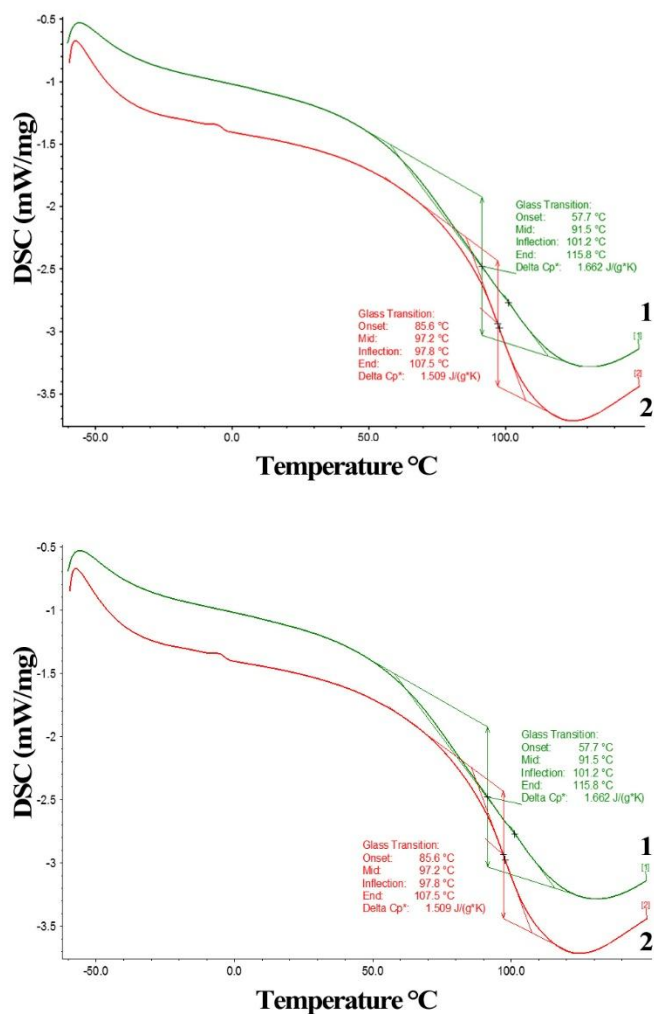


Figure 3. DSC curves, (a) Chitosan-Alginate films with 7:3 ratio, 0.2% of glutaraldehyde, 2 hour duration (1) control (2) 4% Gelatin solution, (b) (1) Chitosan-Alginate films with 7:3 ratio, 0.2% of glutaraldehyde, 2 hour duration and 4% Gelatin solution (2) Gelatin powder.

3.3. Zeta potential

The results of zeta potential of samples are shown in Table 2. Since the ratio of Chitosan was more than Alginate in the samples, the film's total surface charge was positive [4]. In modified sample B, the zeta potential is 18.8 mv. Sample C had modified conditions similar to sample B, yet higher Gelatin concentration. The Zeta potential of sample C was reduced to 15 mv. Due to the presence of carboxyl group in Gelatin, the charge of this polymer is negative [19]. Type B gelation, which is produced

through the basic process and is used in this study, is negatively charged. Therefore, by immobilizing Gelatin on the surface of C/A films the Zeta potential decreased. According to the results of Zeta potential, immobilizing of Gelatin on the C/A films shows that by increasing Gelatin percentages in solution, the amount of immobilized Gelatin is also increased.

3.4. DSC experiments

DSC curves in Figure 3 shows the Tg of modified films, gelatin powder and control samples. Tg is the temperature where the polymer is turned from glassy state to pasty state, and at this degree polymer chains loose free [20]. Tg in control sample was 109.1 °C, and the Tg of gelatin powder was 97.8 °C. The Tg decreased to 101.2 °C in modified sample. Results showed that the presence of gelatin is constant in modified film. The decrease in temperature was due to the presence of gelatin in modified films with 4% gelatin solution and 2% glutaraldehyde. By comparing the results from DSC experiments of pure gelatin and modified films with 4% gelatin solution (b), similar thermal properties of these two samples is significant and indicates the presence of high percentage of gelatin in modified films with 4% gelatin solution.

3.5. FTIR test results

The results of FTIR test is shown in Figure 4. A peak at about 3100-3500 cm^{-1} is the result of -NH and -OH groups. -OH group is present in the chemical structure of C-A film and Gelatin. -NH group in the chemical structure of Gelatin. So this thin band peak is present in all studied samples. Curve number 2 is for modified film with 4% Gelatin solution. Its peak is larger than the control sample's peak (curve number 3). This is due to the presence of a large percentage of Gelatin, and so, -NH group in the sample. Peaks shown in 2920 cm^{-1} and 2865 cm^{-1} , are due to -CH₂ and -CH₃ groups of Chitosan and Gelatin respectively, which is also observed in every sample. When comparing the curves of control sample and modified samples, these peaks are larger in modified samples. This is because of the presence of Gelatin and more -CH₂ and -CH₃ groups in modified film. The peaks at 1698 cm^{-1} and

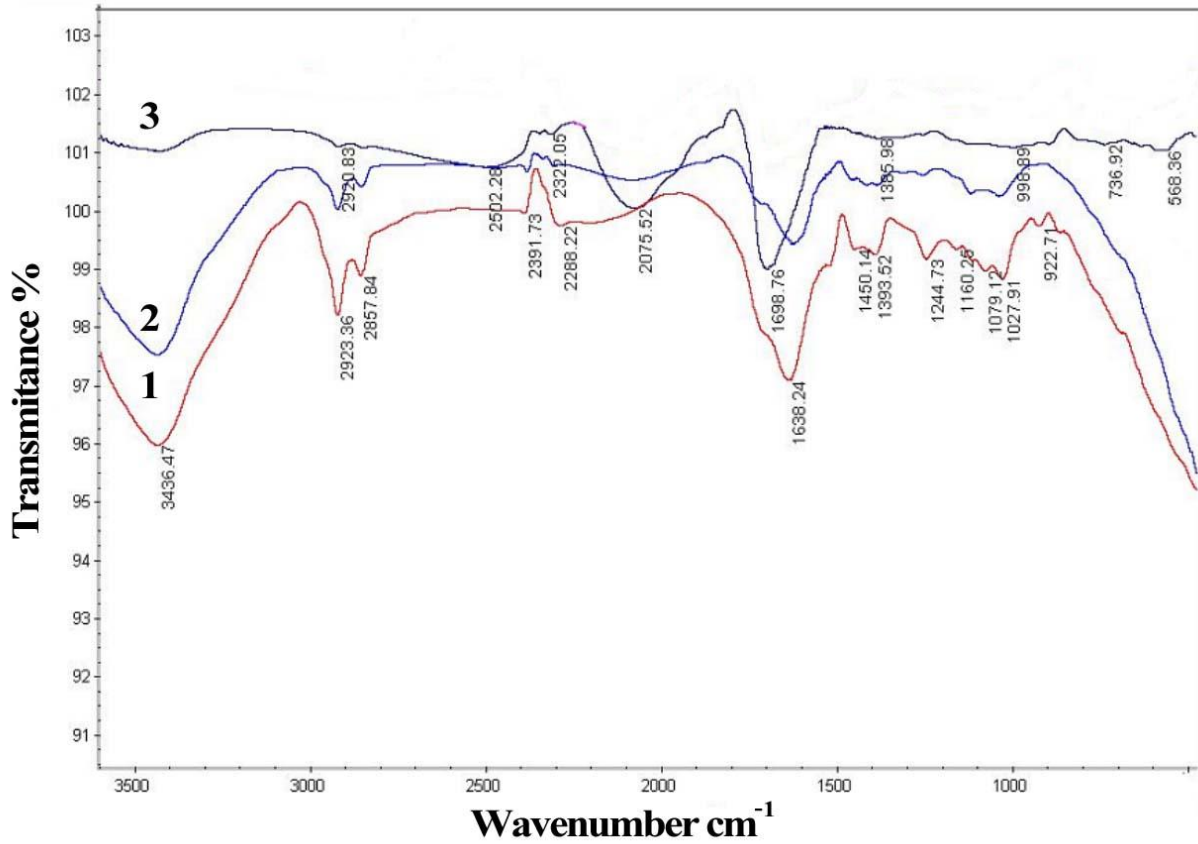


Figure 4. FTIR spectra of (1) gelatin powder (2) Chitosan-Alginate films with 7:3 ratio, 0.2% of glutaraldehyde, 2 hour duration and 4% Gelatin solution (3) control.

Table 1. Parameters used in immobilization (ratio of C/A, concentration of gelatin solution, concentration of glutaraldehyde and time duration)

Time [hr]	Glutaraldehyde [wt%]	Gelatin [wt%]	C/A	C/A	C/A
1	0.1	2%	2:8	3:7	5:5
1	0.01	2%	2:8	3:7	5:5
2	0.1	2%	2:8	3:7	5:5
2	0.01	2%	2:8	3:7	5:5
1	0.2	4%	2:8	3:7	5:5
1	0.02	4%	2:8	3:7	5:5
2	0.2	4%	2:8	3:7	5:5
2	0.02	4%	2:8	3:7	5:5

Table 2. zeta potential measurements of chitosan-alginate films with 7:3 ratio, 0.01% of glutaraldehyde, pH=7,A) control, 2 hours duration ,B) 0.01% gelatin solution, 2 hours duration, C) 2% gelatin solution, 1 hours duration

Zeta potential [mV]	A	B	C
	19.4	18.8	15

1638 cm^{-1} in control sample (curve number 3) are dedicated to the presence of C=O group in Chitosan and Gelatin respectively. This peak is identified in curve number 2, yet it shifted to 1638 cm^{-1} because of amid group in the Gelatin.

4. Conclusion

Surface modification of C-A films via chemically immobilization of Gelatin was done successfully. Surface morphology of modified samples by SEM showed immobilized Gelatin on the surface of samples. Effective Parameters on the amount of immobilized Gelatin, which included Chitosan-Alginate ratio, Gelatin concentration, amount of crosslinking agent and duration of immobilization process, were studied and the results are shown as curves. The results of Zeta potential test showed that by Gelatin immobilization that has negative surface charge, on the surface of the sample and increases its value, the charge of the modified films decreased. In the DSC test, the Tg of modified samples compared to control sample decreased. The temperature fall was due to the presence of Gelatin on modified films. In the results of FTIR test shift in peaks of modified sample relative to the control sample indicated the immobilization of gelation on modified sample.

Conflict of interest

The authors declare that they have no conflict of interests.

Acknowledgments

None declared.

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