Development of Chitosan/Gelatin/Keratin Composite Containing Hydrocortisone Sodium Succinate as a Buccal Mucoadhesive Patch to Treat Desquamative Gingivitis

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Abstract
The aim of this research was to develop chitosan/gelatin/keratin composite containing hydrocortisone sodium succinate as a buccal mucoadhesive patch to treat desquamative gingivitis, which was fabricated through an environmental friendly process. Mucoadhesive films increase the advantage of higher efficiency and drug localization in the affected region. In this research, mucoadhesive films, for the release of hydrocortisone sodium succinate, were prepared using different ratios of chitosan, gelatin and keratin. In the first step, chitosan and gelatin proportions were optimized after evaluating the mechanical properties, swelling capacity, water uptake, stability, and biodegradation of the films. Then, keratin was added at different percentages to the optimum composite of chitosan and gelatin together with the drug. The results of surface pH showed that none of the samples were harmful to the buccal cavity. FTIR analysis confirmed the influence of keratin on the structure of the composite. The presence of a higher amount of keratin in the composite films resulted in high mechanical, mucoadhesive properties and stability, low water uptake and biodegradation in phosphate buffer saline (pH = 7.4) containing $10^4$ U/ml lysozyme. The release profile of the films ascertained that keratin is a rate controller in the release of the hydrocortisone sodium succinate. Finally, chitosan/gelatin/keratin composite containing hydrocortisone sodium succinate can be employed in dental applications.

Keywords: Chitosan, gelatin, keratin, hydrocortisone sodium succinate, composite, drug release

Introduction
Periodontal disease, such as lichen planus, is a general term that describes different pathological conditions that cause weakening, inflammation, and erosion of alveolar bone and gingival tissue. Desquamative gingivitis is an oral manifestation of lichen planus where the appearance of gingiva becomes red, glazed and swollen. Most patients that are suffering from desquamative gingivitis can be treated by topical corticosteroids, such as immunosuppressive drugs [[1]]. Hydrocortisone sodium succinate is a water soluble derivative of hydrocortisone, a synthetic corticosteroid with anti-inflammatory, antivirus and anti-coma properties, and is administered in case of desquamative gingivitis [[3]]. Local administration is preferred because not only a high activity of steroids can be achieved in situ, but also, systemic side effects can be minimized or avoided [[5]]. Interaction of such systems with mucus-coated surfaces or mucous membrane enhances the residence time of the dosage form at the site of absorption. Topical buccal therapy is one of the most preferred strategies amongst mucoadhesive delivery systems, as it has been reported to be useful in controlling ulcerative and inflammatory mucosal diseases with steroid anti-inflammatory drugs. Also, the delivery of drugs into the oral cavity can prevent the first-pass effect. This can be an operative method to deliver drugs that are expected to be effective in the oral cavity [[6]]. Various mucoadhesive systems in the form of microparticles/discs [[8]], tablets [[9], [9]], paste [[10]] and films [[11]] containing different drugs such as bethamethasone disodium phosphate [[8]], mycophenolate mofetil [[12]], hydrocortisone acetate [[5]], clobetasol 17 propionate [[9]], curcumin [[10]], triamcinolone acetonide, and Licorice [[11]]. Amongst mucoadhesive systems, films are preferred due to their flexibility, low thickness and comfort [[13]]. Also, compared to oral gels, such films can remain in the lesion...
for a longer period of time [14]. Moreover, they can deliver a more accurate concentration of the drug to the target region [16]. An ideal mucoadhesive film should not induce sensitivity, dryness, or bad taste in the mouth, and it should provide comfort to the patient along with enough flexibility, elasticity, and resistance to fracture due to actions that induce mechanical compression, such as mastication [17]). Furthermore, it should have acceptable mucoadhesive properties with a limited swelling ratio to remain in the mouth properly for a specific period of time [15], [18].

Various formulations have been employed as mucoadhesive delivery systems including hydroxypropylmethyl cellulose, carboxyvinyl polymer, and polycarbophyl [5], poly(sodium methacrylate, methylmethacrylate), with hydroxypropylmethylcellulose and MgCl₂ [9], pectin, gelatin and sodium carboxymethyl cellulose [10], polyelectrolyte complexes of chitosan and gelatin [19] amongst which the latter has attracted considerable attention nowadays [20] due to mucoadhesiveness of chitosan that is related to its cationic polyelectrolyte structure [27], non-toxicity, stability, biocompatibility, and sterilizability of this natural polymer [28] along with biocompatibility, plasticity, adhesiveness and desirable cell adhesion of gelatin [29] making such formulation an appropriate candidate for the fabrication of mucoadhesive buccal films with reliable characteristics [19]. In order to control the biodegradation, keratin could be added to the biodegradable formulations used for drug delivery systems [30]. Keratin is the major structural fibrous protein providing outer covering, such as hair, wool, feathers, nails, and horns of mammals, reptiles, and birds. Cysteine units are abundant in its chemical structure, which can be oxidized to form inter and intra molecular disulfide bonds leading to a strong three-dimensional polymeric network [31]. In contrast to chitosan, keratin is known to have secondary structure resulting in a denser structure compared to chitosan, therefore, keratin can make the drug release slower. The results obtained by Tran et al. [33] clearly indicate that drug release can be controlled and adjusted at any rate by judiciously selecting the concentration of keratin in cellulose/chitosan/keratin composites. Keratin has been used solely or in combination with chitosan [34]. When keratin is used as the dominant matrix [36] or at a high concentration, the films obtained are too fragile [37].

In this study, the combination of chitosan, gelatin and keratin was used to produce a hybrid mucoadhesive film for buccal delivery of hydrocortisone sodium succinate. In fact, to combine the advantages of chitosan, gelatin, and keratin, fabrication of composite films was examined. To the best of our knowledge, there is no report on the application of such a formulation for mucoadhesive drug delivery. Physicochemical, mechanical, stability, biodegradation, in vitro release and mucoadhesive properties of the produced composite films were also evaluated.

Experimental procedures

Materials

Chitosan (medium molecular weight, deacetylation degree: 75–85%) and lysozyme (50000 U/mg) were obtained from Sigma-Aldrich. Type B gelatin (for microbiology, bloom value 90–130) and glycerol (98% reagent grade) were supplied by Merck Co. (Germany). Hydrocortisone sodium succinate was provided by ROTEXMEDICA (Germany). All other chemicals and solvents, such as acetic acid, acetone, ethylecellulose, and sodium hydroxide, were of analytical grade. Keratin was chemically extracted from waste wool fibers, which were originated from New Zealand Merino wool with a mean diameter of 21 µm.

Films preparation

Formulation optimization

All films were obtained by casting the solutions of chitosan and gelatin with different ratios. First, chitosan (2%w/v) and gelatin (1%w/v) solutions were prepared by dissolving each polymer in acetic acid aqueous solution and deionized water, respectively. Also, 25 wt % (based on polymer’s dry weight) of glycerol, as a plasticizer, was
added to each solution. Then, solutions were stirred at 50 °C to achieve a homogeneity mixture. To obtain composite films, gelatin solution was added drop-wise to the chitosan solution at different ratios according to Table 1, and the pH of the resulting mixture was adjusted to 5–5.5 using 1 M NaOH, then stirred for 2 h at 50 °C, followed by casting and evaporation. Based on the literature, a polyelectrolyte complex of chitosan and gelatin can only form at a pH value above the gelatin isoelectric point (i.e. 4.7) to maintain the negative net charge of gelatin and below the pKₐ of chitosan (i.e. 6.5) to prevent chitosan precipitation [38].

Table 1. Different ratios of chitosan/gelatin composite film to determine the optimized ratio.

<table>
<thead>
<tr>
<th>Sample code</th>
<th>CH:G(w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:0</td>
</tr>
<tr>
<td>2</td>
<td>4:1</td>
</tr>
<tr>
<td>3</td>
<td>3:1</td>
</tr>
<tr>
<td>4</td>
<td>2:1</td>
</tr>
<tr>
<td>5</td>
<td>1:1</td>
</tr>
<tr>
<td>6</td>
<td>1:2</td>
</tr>
<tr>
<td>7</td>
<td>1:3</td>
</tr>
<tr>
<td>8</td>
<td>1:4</td>
</tr>
<tr>
<td>9</td>
<td>0:1</td>
</tr>
</tbody>
</table>

Keratin extraction
Wool fibers were washed in a 60 °C solution containing 1 g/l nonionic detergent and 1%w/v sodium carbonate for 30 min at L:G = 40:1 ratio. Then, the fibers were rinsed several times and dried. Subsequently, Soxhlet extraction was carried out with petroleum ether for 12 h in order to remove the fatty matter from the fibers. After drying, the fibers were rinsed thoroughly with distilled water, dried and chopped into small pieces (2–3 mm).

In the next step, clean, defatted chopped wool fibers were immersed in a solution of Tris–HCl (pH = 8.5) containing 8 M urea, 0.1 M sodium dodecyl sulfate and 0.5 M sodium metabisulphite at a liquor to fiber ratio of 20:1 for 4 h at 60–65 °C. After centrifuging and filtration, the obtained keratin solution was dialyzed against deionized water using cellophane tubing of 12–14 kDa molecular mass cutoff for 3 days with frequent water changes, and finally, it was freeze dried for 48 h to prepare the keratin powder [[40]]. It is worth mentioning that Cilurzo et al ascertained that the extracted keratin did not induce any appreciable cytotoxicity at any of the concentrations or time-points tested [[41]].

Preparation of drug loaded chitosan/gelatin/keratin composite
After optimizing the chitosan/gelatin ratio, keratin was added at different ratios to the complex. Thus, 0.5, 1, and 1.5% (W/V) of keratin powder was added to the gelatin solution and stirred (300 rpm) for 30 min to obtain a homogeneous solution. The samples were labeled CHGK1, CHGK2 and CHGK3, respectively. 100 mg of the drug was then added to the obtained solution and stirred (300 rpm) for 15 more minutes. The prepared solution was transferred to the separatory funnel and added to the chitosan solution drop-wise for composite film preparation as described previously.

Preparation of the second layer
Ethylcellulose was used as a backing layer to control the release rate of the samples. To prepare the second layer, ethyl cellulose was dissolved in acetone (5%w/v) first. To enhance the mechanical properties of the film, glycerol was added to the solution (25%w/w of the dried weight of ethyl cellulose), and stirred at 300 rpm for 2 h. In fact, the incorporation of glycerol into polymer films eliminates their fragility and improves their
flexibility. Then, a certain volume of the solution equal to the first layer was poured on glass petri dish and allowed to dry at room temperature.

Film characterization

Compositional characterization
FTIR spectra of the films were collected using a Nicolet Nexus 670 after preparation of KBr pellets. The FTIR spectra were recorded in a wavenumber range of 4000 to 400 cm\(^{-1}\) at a resolution of 4 cm\(^{-1}\).

Morphological observations
Microstructure and morphology of the prepared films was examined by scanning electron microscopy (SEM; Philips XL30). The imaging was carried out at the acceleration voltage of 20 kV after gold (Au) coating.

Mechanical properties
To investigate the mechanical properties, the films were cut to the pieces of 2.5 cm × 10 cm. Mechanical properties measurements were carried out using a universal testing machine (Instron 5566) at a cross speed of 25 mm/min, according to ASTM D882–02. The thickness of each sample was measured by a thickness gauge (SDL), and the cross sectional area was calculated. The tensile strength and elongation at break were determined by the following formulas \([42]\). The values were expressed as mean ± standard error \((n = 5)\).

\[
\text{Tensile strength (MPa)} = \frac{\text{Force at break (N)}}{\text{Cross sectional area (mm}^2\text{) \times 100}}
\]

(1)

\[
\text{Elongation at break (%) } = \frac{\text{Increase in length (mm)}}{\text{Original length (mm) \times 100}}
\]

(2)

Swelling/water uptake capacity
Films were cut into 1 cm × 1 cm pieces and weighed to obtain the dry weight. Samples were then immersed in 10 ml artificial saliva (KinHydrate, pH = 6.8) and incubated at 37 °C. After 24 h of immersion, the samples were weighed subsequent to removing the water on the surface by filter paper. Then, the samples were transferred to an oven, kept at 60 °C for 4 h and desiccated for 48 h. The percentage of water uptake (swelling) and matrix erosion or dissolution (%DS) were calculated using the following equations:

\[
\%\text{SW or } W_{\text{uptake}} = \frac{W_w - W_d}{W_d} \times 100
\]

(3)

\[
\%\text{DS } = \frac{W_d - W_d'}{W_d} \times 100
\]

(4)

Where \(W_d\) and \(W_w\) are dry and wet weight of the samples, respectively. \(W_d'\) is the weight of the samples after drying in the oven \([43]\). The values were expressed as mean ± standard error \((n = 5)\).
Moisture content and total soluble matter
The samples were weighed \((W_0)\) and subsequently dried in an oven at 100 °C for 24 h. Films were then reweighed \((W_d)\), to measure their moisture content \((MC)\):

\[
\%MC = \frac{W_0 - W_d}{W_0} \times 100
\]

Total soluble matter \((TSM)\) was determined by immersing the dried samples in 10 ml of distilled water at ambient temperature for 24 h. Subsequently, the samples were dried again at 100 °C for 24 h to find the weight of dry matter insoluble in water. TSM was calculated using the following equation:

\[
\%TSM = \frac{W_1 - W_2}{W_1} \times 100
\]

where \(W_1\) is the initial dry matter and \(W_2\) is the undissolved dry matter after immersion.

Stability in PBS and biodegradation in lysozyme
The samples were weighted in the dry state \((W_1)\). Then, they were immersed in the phosphate buffered saline (PBS) \((pH = 7)\) with (for biodegradation assay) and without (for stability assay) \(10^4\) U/ml lysozyme for 1–7 days at 37 °C. The samples were weighed again after removing the water on the surface by filter paper \((W_t)\). The percentage of stability \((%ST)\) and biodegradation \((%BD)\) were calculated by the following formulations:

\[
%ST = \frac{W_t}{W_1} \times 100
\]

\[
%BD = \frac{W_1 - W_t}{W_1} \times 100
\]

It is worth mentioning that after each measurement, the solution was replaced with the fresh media to retain consistency in the pH and enzyme activity [[44]]. The values were presented as mean ± standard error \((n = 5)\).

Surface pH of films
Surface pH was measured to evaluate the possible damage to mucus by the films. Samples were immersed in artificial saliva \((pH = 6.8)\) for 2 h. Samples were taken out and the pH of each film was recorded by placing the probe of pH meter in contact with the wet sample [[14]]. The values were expressed as mean ± standard error \((n = 5)\).

MTT assay
The cell proliferation on the prepared films was determined according to ISO 10993–5 using MTT (3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The prepared films were first disinfected by immersion in 70% v/v ethanol solution for 30 min and were subsequently kept under UV for 1 h. The samples were then dried in a vacuum oven and washed with sterile PBS at least five times prior to use. To produce the extracts for each film, the samples were incubated in 1 ml of RPMI 1640 culture medium (Sigma) supplemented
with 10% (w/w) fetal bovine serum (FBS, GIBCO) while shaking for 3 and 7 days. Culture medium (RPMI and FBS) kept under the same condition was employed as the negative control.

To conduct MTT assay, L929 fibroblast cells were seeded into 96-well plates at a density of $1 \times 10^4$ cells/well and cultured under standard aseptic conditions. Then, the culture medium in each well was replaced with 90 µL of extraction media together with 10 µL FBS and was incubated for another 24 h. Then, 10 µL of a 0.5 mg/mL MTT solution was added to each well followed by incubation for 4 h at 37 °C. Formed formazan crystals were dissolved by the addition of 100 µL of isopropanol (Sigma) to each well. Subsequently, after 15 min of slow shaking, the optical density was recorded using a multiwell microplate ELISA reader at a wavelength of 545 nm. Finally, the obtained data was normalized with regard to the negative control.

In vitro release of hydrocortisone sodium succinate from buccal patch

The drug release rate from the mucoadhesive film was investigated through immersing samples in 10 ml of PBS (pH = 7.4) at 37 °C. The drug release was evaluated by a UV spectrophotometer (SHIMADZU, UV-160) at a wavelength of 247 nm and at time intervals of 1, 2, 3, 4, 5, 6 and 24 h. The values were expressed as mean ± standard error ($n = 3$).

Mucoadhesive force and residence time

The required tensile load to separate the mucoadhesive patch from the mucus membrane is one of the criteria for mucoadhesive performance [46]. Mucohesive force measurement was performed on a sheep buccal mucus membrane as a model membrane. The mucus membrane was separated from underlying connective tissue and washed thoroughly by PBS (pH = 7.4). The apparatus was made in Shahid Beheshti University of Medical Sciences, School of Pharmacy in Tehran, Iran [47]. The mucus membrane was fixed on a circular surface with a diameter of 15 mm. The other surface with the same diameter was attached to a balance using a string. Films, with a diameter of 15 mm, were attached to the upper disk and they pressed onto the mucus membrane manually for 120 s, and then, pulled with a constant speed of 0.5 mm/s. The detachment force was considered as the mucoadhesion force.

The detachment time was also considered as the mucoadhesion residence time [43], [46]. The values were expressed as mean ± standard error ($n = 5$).

Statistical analysis

Statistical analysis was carried out using SPSS software. One-way analysis of variance (ANOVA) and Tukey's test were performed to determine statistically significant differences between the experimental groups. $p < .05$ was considered as statistical significance. Error bars indicate the standard deviation from the mean value.

Results and discussion

SEM observations of chitosan, gelatin, and gelatin/chitosan composite films

The micrographs of chitosan, gelatin, and gelatin/chitosan composite films are shown in Figure 1. As it can be observed in this figure, the composite films display homogeneous surfaces with superb structural unity. Also, gelatin/chitosan composite films exhibit a compact, uniform, dense, and homogenous appearance. It is noticeable that no interfaces are seen in the blend, ascertaining a high compatibility between components due to associative interactions to form polyelectrolyte complex (PEC).
Figure 1. SEM micrographs (cross section view) of (A) chitosan, (B) gelatin, and (C) chitosan/gelatin (3:1) composite films.

Formulation optimization

Mechanical properties

Mechanical properties of different samples are presented in Figure 2. In order to compare samples, the data values are shown in column charts of tensile strength and elongation at break.

Figure 2. (A) Tensile strength and (B) Elongation at break of the film with different ratios of chitosan/gelatin.

The mechanical evaluation of films is of great importance for its application in the buccal cavity. Chitosan is rigid, and it is expected that the flexibility of the films would increase by adding gelatin [[38]].

It is reported that the mechanical properties of chitosan films, or composites containing chitosan, are dependent on the molecular weight of polymer, pH of the film forming solution and its degree of deacetylation. Moreover, the type of acid to dissolve chitosan, drying condition and the amount of water in the film affect these properties [[48]].

Electrostatic interactions and hydrogen bonds between chitosan, which is an amino polysaccharide, and gelatin are effective on the tensile strength and elongation at break [[54]]. In Figure 2, it is observed that the tensile strength (TS) of gelatin increases as a result of the addition of chitosan due to its rigidity. Strong interaction
between gelatin and chitosan is a reason for this behavior \([55]\)]. The backbone of gelatin contains free negatively charged carboxyl groups, enabling it to blend with the cationic ammonium groups of chitosan to form a network. Also, composite films have lower TS compared to chitosan film, which is attributed to the reduction of chitosan crystallinity by the presence of gelatin \([39]\)]. Sample 3 (CH:G = 3:1) exhibits an average TS of 92.78 MPa which is statistically higher than the other samples \((p < .05)\), and therefore, it was chosen as an optimal sample. It shows that the best compatibility of chitosan and gelatin occurs at this ratio, which corresponds well with other reported literature \([56]\).

Moisture content and solubility

Moisture content is a factor attributed to the total void volume occupied by water molecules in the network microstructure of the film, whereas solubility is attributed to the hydrophilicity/hydrophobicity of the material. According to the results given in Table 2, gelatin film displays the highest moisture content. TSM value is a parameter of the resistance of films against water. Gelatin films are completely soluble in water. It is important to point out that composite films are notably less soluble than gelatin, which could arise from interactions, such as electrostatic forces between positively charged chitosan and negatively charged gelatin at the operating pH (5.5).

Table 2. Moisture content and total soluble matter of different films.

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Moisture content (%)</th>
<th>Total soluble matter (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.75 ± 0.6</td>
<td>10.42 ± 0.7</td>
</tr>
<tr>
<td>2</td>
<td>12.59 ± 0.6</td>
<td>16.25 ± 0.6</td>
</tr>
<tr>
<td>3</td>
<td>12.78 ± 0.4</td>
<td>16.40 ± 0.5</td>
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<tr>
<td>4</td>
<td>12.77 ± 0.6</td>
<td>17.57 ± 0.5</td>
</tr>
<tr>
<td>5</td>
<td>11.56 ± 0.5</td>
<td>17.92 ± 0.6</td>
</tr>
<tr>
<td>6</td>
<td>6.44 ± 0.4</td>
<td>21.43 ± 0.5</td>
</tr>
<tr>
<td>7</td>
<td>11.38 ± 0.3</td>
<td>22.29 ± 0.7</td>
</tr>
<tr>
<td>8</td>
<td>11.54 ± 0.6</td>
<td>29.38 ± 1.4</td>
</tr>
<tr>
<td>9</td>
<td>12.67 ± 0.5</td>
<td>100.00 ± 0.0</td>
</tr>
</tbody>
</table>

Stability and biodegradation properties

The results of the stability test in PBS (pH = 7.4) at 37 °C and biodegradation test in PBS (pH = 7.4) in the presence of lysozyme at 37 °C are displayed in Figure 3. It should be noted that gelatin film nearly dissolves within a few hours; therefore, this film is not tested later on. As can be observed, stability of the films decreases in the first 4 days, and then, it reaches a plateau and the films reach a constant stability and display a rational trend. One of the possible explanations of this observation lies in the high solubility of the gelatin in aqueous environment. This leads to lower stability of gelatin film in comparison with the films containing chitosan. Also, by increasing chitosan in formulations, the stability increases and the complex has higher stability than gelatin films. Composite films are much more stable than gelatin film due to the formation of a polyelectrolyte complex, but they show lower stability compared to the chitosan film because of the high solubility of gelatin in an aqueous environment.
Figure 3. (A) Stability of the films in PBS and (B) Biodegradation of the films in PBS with lysozyme at 37 °C in the period of 1–7 days.

Since lysozyme is one of the most abundant enzymes in human body, especially in the oral cavity, the degradation of films by this enzyme is substantial. Figure 3(B) shows that samples with a higher amount of gelatin have a higher amount of biodegradation, owing to severe hydrolysis of gelatin macromolecules as a hydrophilic polymer \([59]\).

The β-1,4 N-acetyl-glucose amino groups of chitosan chains can be hydrolyzed by lysozyme, leading to the release of amino sugars that can either enter the metabolic pathway of glycosaminoglycan and glycoprotein or be excreted. Furthermore, the hydrophilic nature of chitosan allows for water penetration in the matrix more rapidly than the rate of degradation, and so, chitosan swells before degradation \([58]\).

The degradation rate is high in the first 4 days and is reduced in the following days. It seems that fast weight reduction at the beginning is related to gelatin hydrolysis, and slow weight loss in the following days is due to chitosan degradation by the lysozyme \([61]\).

Films containing drug and keratin

SEM observations of chitosan/gelatin composite containing keratin

SEM micrographs of chitosan/gelatin composite film containing keratin are shown in Figure 4. As it can be observed, keratin is rather uniformly distributed throughout the composite film. Meanwhile, some aggregates of the keratin are seen in the composite film. The addition of keratin leads to a more compact, aggregated and irregular structure than the chitosan/gelatin composite film in Figure 1(C).
FTIR analysis

To evaluate the structure of a drug and its presence in the films, FTIR spectra of drug as well as chitosan/gelatin composite containing a drug and keratin are presented in Figure 5.

The characteristic peaks of chitosan, gelatin and their complex were depicted completely in previous articles [39]. The spectrum of the drug exhibits a broad absorption band at 3435 cm⁻¹ which results from 0-H stretching vibrations. The peak of 2931 cm⁻¹ is assigned to stretching vibrations of aliphatic C-H. The sharp peak at 1720 cm⁻¹ is assigned to the ketone group and the 1655 cm⁻¹ absorption band corresponds to a carboxylate group in the drug structure. Also, 1057 cm⁻¹ is a characteristic peak of a C-O-C stretching band [62].
In composites, the characterization peaks of the drug can be seen with slight differences. The peak situated around 3376 cm\(^{-1}\) is related to the overlapping of O–H and N–H. The spectra of the composite containing the drug exhibit a shorter peak at 3418 cm\(^{-1}\). This shift may be related to hydrogen bond of O–H and N–H group existing in the drug and composite structure. Besides, the presence of a drug in the composite structure results in the shift of the drug's absorption bands from 1655 and 1720 cm\(^{-1}\), respectively, to 1645 and 1711 cm\(^{-1}\). It seems that ketone and carboxylate groups, which are deactivator groups, absorb electrons on the drug structure and are able to attract hydrogen that is abundant in the composite.

Characterization peaks of a composite containing keratin powder are approximately identical to a composite without keratin. Thus, by adding keratin to the matrix, no basic alteration occurs to the composite structure. The slight variations in saccharide and amide regions are due to the presence of keratin in the composite system. For example, stretching vibrations of C–O at 1080 and 1152 cm\(^{-1}\) in three component systems increases in comparison with chitosan/gelatin composite.

Observations indicate that the presence of a drug has no significant effect on complex structure. Furthermore, keratin does not cause any chemical degradation to the structure, but it cooperates in the bonds existing within structure.

Mechanical properties

Mechanical properties of the samples are shown in Figure 6(A) tensile strength and (B) elongation at break. Some authors believe that the addition of keratin to chitosan results in increased flexibility and elongation at break. However, the opposite trend was reported for tensile strength ([34]). Besides, the existence of chitosan, gelatin and keratin represents more acceptable properties compared to chitosan/gelatin and chitosan/keratin composite or even keratin film ([64]). For keratin film, a tensile strength of about 0.25 has been reported that is about 0.2 of the tensile strength that was obtained in this project ([19]).
By comparison of chitosan/gelatin (3:1) composite films with and without a drug, it can be concluded that the tensile strength and elongation at beak were reduced in the composites containing a drug due to the disintegration of composite matrix. On the other hand, by adding keratin to the composite formulation, the interactions between the drug and composite improve, and therefore, tensile strength, and elongation at break increase by increasing keratin percentage. This result affirms the influence of keratin in composites. Judging from the reports, a composite film containing 1.5% keratin represents the most desirable mechanical properties.

Swelling, water uptake and erosion percentage
As it is shown in Figure 7, samples containing 1% keratin absorbs more water in artificial saliva compared with the other samples.

![Figure 7. Water uptake of the films after different times (1, 2, 3, 4, 5 and 6 h) immersion in artificial saliva.](image)

Similar to the mechanical test results, CHGK1 that has 0.5% keratin has identical behavior to CHG (0% keratin). It seems that a small concentration of keratin has no effect on the final properties of the film. In CHGK2 and CHGK3, the water uptake percentage is more than the other two samples. The isoelectric point (PI) of keratin is about 4.9. Thus, at pH = 6.8 this polymer is negatively charged. Keratin molecules with a negative charge repel each other, and this repulsion causes water to penetrate inside the polymer matrix and induce swelling \([31, 64]\). On the other hand, by increasing keratin from 1 to 1.5%, swelling decreases. The reason for this happening may be the presence of keratin molecules amongst chitosan chains. Hence, keratin molecules separate chitosan chains to prevent amine group repulsion \([34]\). This phenomenon is an affirmation of previous tests, which explained that at a higher percentage of keratin, more proper interaction occurs between the keratin and chitosan/gelatin complex.

Water uptake percentage was also measured after 24 h (for film saturation). The results are shown in Figure 8. The same trend is observed in this figure.

![Figure 8. Water uptake percentage of the films after 24 h immersion in artificial saliva.](image)
The sample's erosion is calculated after 24 h and depicted in Figure 9. For all samples, a positive digit was the result because of the low thickness of the films. Due to low thickness of films, fragmentation may happen. Approximately all samples were affected by this phenomenon. The greatest weight reduction occurs in CHGK2 (1%). This may be because of a higher degree of swelling in this film compared to others. High swelling causes vacancies in the film’s matrix and makes the film more brittle against mechanical stress.

![Figure 9. Erosion or dissolution (DS %) of the films after 24 h immersion in artificial saliva.](image)

**Stability in PBS and biodegradation in lysozyme**

Stability was carried out in the period of 1–7 days and 0–6 h at 37°C in PBS at pH = 7.4. As it is depicted in Figure 10, throughout the first few hours, the stability of the film containing 1% keratin is higher. This is related to the higher swelling ratio of this film compared to the other films. However, after 4 h, CHGK3 (1.5% keratin) shows more stability.

![Figure 10. Stability of the different films in PBS at pH = 7.4 for (A) 0–6 h and (B) 1–7 days.](image)

It is obvious that even after 24 h, CHGK3 (1.5%) is the most stable film due to its logical swelling in comparison with the other samples. This logical swelling prevents the film from experiencing fragmentation, and the film preserves its structure.
The biodegradation of samples in PBS (pH = 7.4) in the presence of $10^4$ U/ml lysozyme is depicted in Figure 11. The film containing 1.5% keratin has the least degradation in the first 6 h. It seems that an interaction between keratin macromolecules and chitosan/gelatin composite, like hydrogen bonds, prevents enzyme penetration and film degradation. As a matter of fact, these particles are applied as barriers against enzymatic hydrolysis. It is impressive that even CHGK1 (0.5%), the film with the least amount of keratin, has less degradation than the chitosan/gelatin composite (CHG). Therefore, keratin enhances the film resistance to enzymatic degradation.

Figure 11. Biodegradation rate of the chitosan/gelatin composite film containing different ratios of keratin in PBS at pH =7.4 in the presence of 104 U/ml lysozyme in a period of (A) 0–6 h and (B) 1–7 days.

Even after passing days, the trend of degradation (Figure 11(B)) is identical to the first diagram (Figure 11(A)). It is obvious that the film containing 1.5% keratin shows the least biodegradation ratio.

Surface pH of samples
As it is shown in Table 3, all pHs are about 6.3–6.5, proving that films didn't change the pH of the environment to acidic or basic nature. Thus, no harmful effect to mucus is expected [[14], [66]].

Table 3. Surface pH of chitosan/gelatin composite film containing different ratios of keratin.

<table>
<thead>
<tr>
<th>Surface pH</th>
<th>Sample code</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.35 ± 0.0702</td>
<td>CHG</td>
</tr>
<tr>
<td>6.46 ± 0.2011</td>
<td>CHGK1</td>
</tr>
<tr>
<td>6.38 ± 0.1041</td>
<td>CHGK2</td>
</tr>
<tr>
<td>6.36 ± 0.0929</td>
<td>CHGK3</td>
</tr>
</tbody>
</table>

MTT assay
The results obtained by MTT assay for different films were compared with the control group (polystyrene well) as shown in Figure 12.
According to the results, the cell viability on the gelatin film is similar to the control group. Gelatin is constituent of some amino acids, such as arginine, glycine and aspartic acid (RGD sequence), that stimulate cell adhesion and migration. Also, nonspecific cell interaction occurs between chitosan positively charged ammonium sites at physiological pH and negatively charged cell membrane surfaces. Therefore, the gelatin/chitosan composite film appears to be favorable for cell adhesion, and the biological activity of chitosan is enhanced upon blending with gelatin.

It is worth mentioning that no statistical difference in cell viability is detected between the chitosan/gelatin composite film and the other films, but the number of cells decreases significantly ($p < .05$) in chitosan/gelatin composite film containing keratin in comparison with chitosan/gelatin composite film, which could be due to the reduction in the hydrophilicity. Meanwhile, the slightly higher number of cells on the surface of the films after 10 days ascertains their good affinity and biocompatibility for cells.

In vitro drug release
As it is depicted in Figure 13, the drug release profile in all samples is linear. Thus, the mechanism of drug release is diffusion [67].

To discuss the results of drug release thoroughly, the profile of release in the first 20 h is described below (see Figure 14). In the first 3–4 h, burst effect occurs, which releases about 80% of the drug concentration. Thus, there is only little amount of the trapped and remaining drug in all films. The degree of drug release from CHGK2 is the highest among samples. Due to the predominant diffusion mechanism of drug release in this system, excessive diffusion of water in CHGK2 (1%) leads to the dissolution of the drug and sudden release of hydrocortisone sodium succinate. After initial burst release, owing to the sudden decrease in drug concentration, the entanglement of polymer chains decreases, which results in greater release in the following hours due to the generation of vacancies [14].
On the other hand, CHGK3 (1.5%) has the smallest slope in the graph, which indicates slower drug release. In the CHGK3 film, the final release is lower than the other films. It appears that by increasing the keratin ratio, the interaction between drug and keratin particles increases, which results in drug entrapment in the system. In the film containing 1.5% keratin, lower pore formation and less erosion contributes to slower drug release \([68]\).

In comparison with previous research studies, the presence of a backing layer in the system increases the release time remarkably. For example, Abruzzo et al. reported a release period of about 30 min for their chitosan/gelatin system \([19]\). This backing layer causes unilateral release and prevents excessive swelling.

In some research about release kinetics, burst release is a phenomenon generally seen in delivery devices of different types, form and compositions. The burst effect might be suitable for specific applications such as targeted delivery, encapsulated flavors, wound treatment and pulsatile release. Notwithstanding, it is also likely to cause negative impacts such as short \textit{in vivo} half-life, local/systemic toxicity and shortened release profile that requires more periodic dosing \([69]\). Burst release is frequently associated with surface properties of host material, heterogeneous distribution of drugs within the polymer matrix, device geometry and intrinsic dissolution rate of drug, heterogeneity of matrices (pore density). However, some works have been carried out to develop mechanism based mathematical models for burst release. It is noticeable that for better predicting and understanding the burst release, it would be useful to develop models to clarify the mechanisms of burst release \([70]\).

Surface degradation and bulk degradations are two general types of degradation. In a surface-degrading polymer, degradation is limited to the external surface of the device \([71]\). In a bulk-degrading polymer, degradation happens homogeneously throughout the material \([72]\). Water is a crucial factor amid hydrolysis and therefore water intrusion into the device is of remarkable significance for the research of degradation kinetics as well as release kinetics. The degradation of semicrystalline polymers occurs in two steps:

- The first step comprises of water infusion into the amorphous areas with random hydrolytic scission of labile bonds, such as ester bonds
- The second step initiates when most of the amorphous areas are degraded. As degradation results in the scission of polymer chain, the change in the average molecular weight of the polymer could be utilized to quantify the degradation process over time. Employing gel permeation chromatography (GPC), the degradation process is generally examined by plotting the average molecular weight of the degraded biomaterial vs time. The following two equations are broadly employed to explain the degradation kinetics:

$$\text{Zero - order : } \text{Mw}(t) = \text{Mw}_0 k_{\text{deg}} t$$
Pseudo – first order: \( M_w(t) = M_{w0}e^{\text{-}k_{dgr}t} \)

where \( M_w(t) \) and \( M_{w0} \) are the average polymer molecular weight at time \( t \) and zero. \( k_{dgr} \) is related to the apparent degradation rate constant of the polymer [[70]].

**Mucoadhesive force**

When a mucoadhesive patch fabrication is claimed, its adhesion to mucus for a distinctive period of time should be guaranteed. Figure 15 shows the column graph of the mucoadhesive force of the films. Although hydrophilic molecules, like chitosan, shows mucoadhesive properties, excessive swelling of these molecules can form a loose, slippery layer on the mucus, which limits its application. However, by adding a polymer with the opposite charge to cationic chitosan and the formation of a three dimensional network, swelling can be controlled [[68]].

Previous studies expressed that the addition of a high amount of gelatin also reduces the mucoadhesion force of chitosan/gelatin composite due to its high swelling ratio. Thus, the chitosan/gelatin ratio should be optimized in the composite to achieve the best results. Furthermore, it was mentioned that high proportions of cationic chitosan chains could react with sialic acid and sulfate parts of mucus glycoproteins, which have a negative charge in the oral cavity [[19]]. Thus, the mucoadhesive force in chitosan/gelatin film is the result of this reaction.

![Figure 15. Mucoadhesive force of the chitosan/gelatin composite film containing different ratio of keratin.](image)

It should be stated that the bridge structure (disulfide bonds) in biological systems is of great importance for binding films to the mucus surface. Thiol polymers, or thiomers, are basically mucoadhesive and have thiol groups in their side chains. By thiol/disulfide exchange* reactions or a simple oxidation, disulfide bonds can be formed between these polymers and subdomains that are rich in cysteine*. Thus, thiomers mimic the natural mechanism of secreted mucus by forming covalent disulfide bonds [[73]]. In fact, mucus structure can be summarized to repetitive arrays, carboxylic regions and amino ends. More than 10% of carboxylic ends are cysteines. Also, amino ends have regions rich in cysteine, which are responsible for forming mucin oligomers in disulfide bonds.

Based on disulfide exchange, disulfide bonds form between mucin glycoproteins and mucoadhesive thiol polymers, which leads to covalent interaction [[6]].

As demonstrated in Figure 15, with increasing keratin content, the mucoadhesion force increases. The film containing 1.5% keratin displays the highest mucoadhesion force. It seems that, besides the interaction of the polyelectrolyte complex and mucus, keratin molecules, with cyteine regions, can form bonds with glycoproteins and develop the mucoadhesion of the composite.
Mucoadhesion residence time

Different parameters affect the mucoadhesion time. One of them is the ability of polymers to absorb water. A polymer that can maintain its structure against water can adhere longer on mucus. Another significant factor is the type of film-forming polymer and polymer blend uniformity. Some researchers believe that physical and chemical properties of the polymer have a substantial effect on polymer residence time on mucus while others argue that there is no relation between mucoadhesive residence time and mucoadhesive force, and highly adherent polymers do not necessarily adhere on mucus for a long time ([43]). They believe that surface charge density and flexibility of the chain are the major factors leading to high adhesion strength. Residence time also depends on the solubility rate of the applied polymers, and the presence of a hydrophobic polymer in the blend lengthens the mucoadhesion residence time ([46], [74]).

Figure 16 represents residence time of chitosan/gelatin composite containing different ratios of keratin. CHGK3 (1.5%) has the longest residence time on mucus amongst all samples. On the other hand, the chitosan/gelatin composite without keratin shows the shortest residence time. Based on the results of the swelling test, keratin decreases the swelling rate in the chitosan/gelatin composite, and a balanced swelling ratio leads to increased adherence of polymer to mucus.

Amongst the three films containing keratin, CHGK2 (1%) has the shortest residence time on mucus because of its excessive swelling. On the other hand, the film containing 0.5% keratin (CHGK1) adheres for a longer time in comparison with CHGK2 (1%) due to its better stability in PBS, as it was previously shown.

Conclusions

A chitosan/gelatin film was formed by casting in different ratios. To optimize the best proportion of the components, different characteristics, such as tensile, swelling, stability and biodegradation, in lysozyme were tested. It was found that the chitosan/gelatin composite film with the ratio of 3:1 was chosen as the optimum sample for fabricating the mucoadhesive patch. Keratin, which was extracted chemically from wool, was incorporated in the chitosan/gelatin composite in three different ratios together with hydrocortisone sodium succinate as an anti-inflammatory drug for the treatment of desquamative gingivitis. FTIR spectra confirmed the presence of the drug and keratin in the system without any destruction in composite structure, and the existence of some specific interactions between them was also noted. The tensile strength, as well as elongation at break, also increased by increasing keratin extent. The film containing 1.5% keratin was recognized as the most stable sample in PBS after 24 h due to its low water absorption. In addition, keratin acted as a barrier to enzyme diffusion to composite structure and enzymatic hydrolysis because of the formation of hydrogen bonds. Thus, with an increase in the amount of keratin, the biodegradation rate decreased. By evaluating the surface pH of samples, it was concluded that these films cannot damage mucus surface. The release profile demonstrated that keratin acts as a rate controller in the system. Based on the interactions between keratin and
drug particles, some drugs were trapped in the system even after 120 h. The results of the mucoadhesion test demonstrated that higher concentrations of keratin results in a higher mucoadhesive strength that is related to the formation of disulfide bonds between keratin and mucus glycoproteins. Moreover, the presence of keratin increased the mucoadhesion time. Eventually, chitosan/gelatin (3:1) composite containing 1.5% keratin displayed the best result for the treatment of desquamative gingivitis with hydrocortisone sodium succinate.

Disclosure statement
The authors declare that they have no conflict of interest.

Ethical approval
This article does not contain any studies with human participants or animals performed by any of the authors.

Footnotes
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References


