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Development of bioadhesive polysaccharide-based films for topical release of the immunomodulatory agent imiquimod on oral mucosa lesions

Lucas Garcia Camargo [a], Paula de Freitas Rosa Remiro [a], Gabriela Souza Rezende [a], Stephany Di Carla Santos [b], Michelle Franz-Montan [b], Ângela Maria Moraes [a,*]

[a] Department of Engineering of Materials and of Bioprocesses, School of Chemical Engineering, University of Campinas, Campinas, SP, Brazil
[b] Department of Physiological Sciences, Piracicaba Dental School, University of Campinas, Piracicaba, SP, Brazil

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ABSTRACT

The immunomodulatory agent imiquimod (IQ) was incorporated in chitosan (Ch) and alginate (A) films aiming at developing a biomaterial to topically treat oral mucosal squamous cell carcinomas. IQ was directly added to either suspensions of the isolated polysaccharides or polyelectrolyte complexes formed by their mixture before film casting. The films presented incorporation efficiencies varying from 55 to 100%, and the highest values were attained for those containing chitosan. The addition of IQ generally increased film opacity and reduced fluid absorption by the films. The drug was released predominantly by diffusion in artificial saliva, resulting in releases of 1.49 to 2.76 μg of drug per mg of film, values considered as appropriate for oral use. Films of different formulations showed no significant differences concerning mucoadhesion properties, and thermogravimetric analysis demonstrated the occurrence of interactions between IQ, Ch, and A. The tensile strength of the films ranged from 16.43 to 64.17 MPa for those without IQ, and from 4.75 to 18.25 MPa for those with the drug, while elongation at break for all dried formulations did not exceed 4%. In conclusion, the films may have high potential as oral cancer therapeutic tools, replacing usual topical gels and creams, which are susceptible to leaching.

1. Introduction

Oral cancer is one of the most common types of neoplasia. In the USA, in 2019, it was estimated that about 53.000 people would develop an oral cavity or oropharyngeal cancer, and over 10.000 people would die because of these conditions [1]. Surgical resection of the lesioned tissue is a frequent procedure for the therapy of oral cancer. However, it can be associated with post-operative difficulties such as swallowing difficulties and esthetic sequelae [2]. Therefore, less invasive options should be considered whenever possible. In this sense, local treatment of oral carcinoma could offer some advantages, such as improved accessibility, ease of administration, rapid oral mucosal repair, and the potential to reduce systemic side effects. Besides, local therapy may be combined with other forms of treatment, such as systemic chemotherapy or radiation.

The topical use of immune response modifiers for this purpose, e.g. imiquimod (IQ), is then quite attractive. This drug is typically prescribed for topical therapy of actinic keratoses, squamous and superficial basal cell carcinomas, and for warts on the genital and anal areas. Imiquimod is a Toll-like receptor 7 (TLR7) agonist that up-regulates cytokines and activates cell-mediated immune response [3]. It was approved by the Food and Drug Administration (FDA) of the United States in 1997 [4], and its use in the form of a 5% topical cream (commercialized as Aldara® and Zyclara® proprietary names, for instance) has been successful for the treatment of skin neoplasms. Besides, IQ also induces apoptosis of tumor cells [5], stimulating the patient's immune system, and when used *in situ* for the treatment of squamous cell carcinoma of genital mucosa, imiquimod is active against human papillomavirus (HPV), further improving the overall therapeutic results [6].

Despite imiquimod is not formally indicated for the therapy of oral lesions, Gkoulioni et al. [5] pointed promising results from at least six different studies while discussing their findings on the inhibition of the progress of carcinogenesis of dysplastic mucosal lesions on the left buccal pouch of Wistar rats with the use of IQ cream for 8 weeks. Ahn et al. (2012) have shown that imiquimod can be considered also as an effective therapeutic drug for the treatment of oral squamous cell carcinomas (OSCC). These authors reported that, at concentrations as low as 100 μg/mL, imiquimod inhibits the proliferation of YD-10B cells

* Corresponding author.
E-mail address: ammoraes@unicamp.br (Â.M. Moraes).

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(tongue squamous cell carcinoma) and FaDu cells (pharynx squamous cell carcinoma), as well as induces apoptosis through the caspase-dependent mitochondrial pathway and necrosis as a result of complete disruption of cell membrane. More recently, Wester et al. (2017) [7] reported the resolution, after two weeks, of recurrent oral squamous cell carcinoma in a 81-year-old patient with topical 5% imiquimod cream associated with 10 mg of acitretin daily for pulmonary metastasis, emphasizing that topical imiquimod may consist in an adequate and well-tolerated alternative therapy for patients with comorbidities.

In the skin, the direct use of imiquimod cream can result in local adverse reactions, such as pain, erythema, edema, itching, burning, pain, flaking, scabbing, ulceration, and erosion, among others and even in systemic effects, which can include nausea, headache, fatigue flu-like symptoms and myalgia [8]. Since the affected area should be in contact with the imiquimod cream for 6 to 10 h [5], facing these side-effects for long periods may reduce patient adherence to the treatment. However, given that the permeability of buccal mucosa is higher than that of the skin [9], a dose considerably smaller than that currently employed to treat skin lesions and exposure times significantly shorter could lead to effective clinical results for oral targets. There is a clear gap, however, in the development of biomaterials for this purpose, what opens opportunities for other possible drug release vehicles in addition to cream formulations, such as mucoadhesive polysaccharide films, which are particularly attractive for lesions in wet soft tissues as the oral mucosa.

In this study, we aimed then to develop degradable mucoadhesive films composed of the biopolymers chitosan (Ch) and alginate (A), isolated or combined, loaded with imiquimod (IQ), as alternatives to circumvent potential problems associated with surgical resection. This approach could also offer an additional option to the limited choices of adjuvant local therapies for lesions in the oral mucosa. The final purpose was to promote effective and preferential *in situ* release of IQ in oral neoplastic lesions while simultaneously avoiding harmful side effects and systemic action of the drug due to high dosage.

Chitosan (Ch) is a polysaccharide obtained from the alkaline deacetylation of chitin, found in the exoskeleton of crustaceans, insects and in the cell wall of some fungi. It is a copolymer formed by randomly distributed N-acetyl-glucosamine and N-glycosamine units [10] which can be positively charged at pH conditions below the pKa of its amino group (from around 6.5 to 7.0) [11].

Alginate (A), on the other hand, is usually extracted from brown algae. It is a copolymer formed by (1,4) β-D-mannuronate (M) and α-L-guluronate (G) groups (pKa values of 3.38 and 3.65 for M and G groups, respectively) [12]. Above pH 3.4, alginate can then be negatively charged, being able to interact electrostatically with positively charged compounds such as chitosan. A number of bioactive agents from different classes has been already incorporated to stable, non-cytotoxic Ch:A polyelectrolyte complexes (PECs), from antimicrobial compounds to plant extract components, antibiotics and growth factors, among others. Caridade et al. (2013) [13], for instance, developed films composed of chitosan and alginate using the layer-by-layer technique and concluded that it is possible to release model drugs using these devices. The authors also used these devices to assess the adhesion of myoblasts. Kashiwagi et al. (2013) [14], developed polymeric devices based on chitosan and alginate for the loading of anti-glaucoma drugs. The authors concluded that a single application of the biomaterial containing the drug lantosperol reduced, in one week, the eye pressure of rats without any side effects. In this regard, the authors concluded that this device shows potential as a new drug delivery system for glaucoma therapy. Criado-Gonzalez et al. (2019) [15], also using a layer-by-layer technique, developed a chitosan and alginate biomaterial, but for local release of tamoxifen, an antitumor agent widely used for the treatment of breast cancer, showing that sustained drug release over time modulated by the number of deposited bilayers could be achieved. In this case, the release mechanism was controlled by diffusion, and the Ritger-Peppas model was the most accurate to describe the system behavior.

According to Wittaya-areekul et al. (2006) [16], high flexibility of polymer backbone structure and presence of polar functional groups are important molecular characteristics to ensure mucoadhesiveness of a given formulation. Because of showing many polar groups in their composition, chitosan and alginate may interact with glycoproteins of the buccal mucosa [17]. The production of chitosan and chitosan-alginate mucoadhesive films loaded with miconazole nitrate by casting/solvent evaporation has been reported for the treatment of oral candidiasis [18], and mucoadhesive chitosan/alginate polyelectrolyte complex films incorporating clindamycin phosphate for periodontal therapy have been described by Kilicarslan et al. (2018) [19].

Thus, the hypothesis behind the present was that films produced with polysaccharides incorporating antineoplastic drugs could consist of an attractive alternative to enhance *in situ* results in the treatment of cancer lesions, both improving and prolonging local contact of the bioactive agent with the tumor.

The type and proportion of polysaccharide, the concentration of drug incorporated, the number of excipients, the formulation production technique used, and the thickness of the films produced are some of the factors that can influence the phenomena involved in controlled drug release. Some of these phenomena include drug dissolution, partition, and diffusion in the matrix, as well as swelling, erosion, and degradation of the release device, among others [20].

Since the use of imiquimod in the form of a cream is consolidated in the medical literature for the therapy of neoplastic skin diseases and its off-label use to treat oral lesions shows promising results, the novelty of this work comes from the alteration of the release vehicle to mucoadhesive polysaccharide-based films. Through this approach, it could be theoretically possible to reduce side effects resulting from high doses of imiquimod applied topically for long periods. In addition, other limitations related to the use of drug creams in the buccal region could be avoided by this strategy, such as unpleasant taste of both the drug and its vehicle, enzymatic degradation, no availability of a drug reservoir for longer treatment periods, constant drug leaching due to saliva production and increased intake of a significant fraction of the drug by swallowing the saliva in which the bioactive agent was prematurely released instead of being absorbed by the lesion [21]. In fact, in this sense, the limited solubility of the drug in aqueous solutions is quite advantageous to prevent premature release (in water, IQ solubility is only of about 0.60 μg mL^{-1}, [22].

Despite recently the development of imiquimod-loaded chitosan films for topical delivery to the skin was successfully reported in the literature[23], as well as mucoadhesive polyvinylpyrrolidone and carboxymethylcellulose-based matrices containing imiquimod [24–26], to the best of our knowledge, the use of chitosan and alginate, isolated or combined, as components of alternative mucoadhesive matrices incorporating imiquimod designed for this purpose has not been yet described. In this way, this problem is addressed in detail in this work, with the goal to develop devices able to meet critical parameters concerning film morphology, imiquimod loading efficiency and distribution in the matrix, film swelling, stability, mucoadhesivity, mechanical properties, transparency and drug release profile.

2. Materials and methods

2.1. Materials

The films were prepared using chitosan from shrimp shells (Sigma-Aldrich, C3646, lot number SLBL3564V, with deacetylation degree of 82% and average molar mass – MM – equal to 1.26×10^6 g/mol), medium viscosity sodium alginate from brown algae (Sigma-Aldrich, A2033, lot number 058 K0126, MM of 9.11×10^4 g/mol), glacial acetic acid, calcium chloride dihydrate and sodium hydroxide (Merck), sodium chloride and sodium dibasic anhydrous phosphate (Synth), potassium chloride, calcium chloride dihydrate, magnesium chloride hexahydrate and 1-octanol (Sigma-Aldrich). Deionized water was obtained in a Milli-

Q system from Millipore. Imiquimod was kindly donated by the pharmaceutical company EMS S/A Hortolândia Division (Brazil).

2.2. Films preparation

The films were prepared using different mass proportions of the polysaccharides suspended in aqueous solutions. Ch:A mass ratios of 1:0 and 0:1 were used for the isolated polymers and 1:1, 1:3 and 3:1 for their combinations, basically as described by Bierhalz and Moraes (2016) [27] and Pires et al. (2018) [28].

Briefly, the chitosan films were prepared using 400 mL of 1% (w/v) solution in acetic acid (1% v/v), under stirring, at 25 °C, to which the drug was also added (0.22 g). The suspension was then cast into four 15 cm films in polystyrene Petri dishes at 37 °C in a convection oven (410D, Nova Ética) for 24 h, and afterward, they were neutralized by exposure to 150 mL of 1 mol/L of aqueous NaOH solution, washed with 200 mL of deionized water and again dried at room temperature.

In the case of alginate films, 400 mL of 1% (w/v) aqueous solution of this polysaccharide were mixed (under stirring, 25 °C) with 0.22 g of the drug and 40 mL of 0.5% $CaCl_2$ aqueous solution (m/v) under constant stirring of 500 rpm, for primary crosslinking. The suspension was transferred to four 15 cm polystyrene Petri dishes, dried at 37 °C for 24 h, and secondarily cross-linked by immersion in 150 mL of 2% (w/v) aqueous $CaCl_2$ solution for 30 min, washed and dried again at room temperature.

Films containing simultaneously chitosan and alginate were prepared by mixing from 100 to 300 mL of 1% alginate solution, at a flow rate of 6 mL/min, to respectively from 300 to 100 mL of 1% chitosan solution in a tank mechanically stirred at 500 rpm. After the addition of the chitosan solution, the stirring conditions were changed to 1000 rpm for 10 min, and then the pH was corrected to 7.0 by slowly adding aqueous NaOH solution at 2 mol/L. Following, 0.22 g of imiquimod were added to the suspension, while stirring at 1000 rpm. Then, a primary cross-linking procedure was performed by adding 20 to 30 mL of $CaCl_2$ aqueous solution at 0.5% (m/v) under stirring for another 10 min, to reticulate carboxyl groups from alginate which were not bound to chitosan amino groups. Afterward, the suspension was deaerated for two hours, aliquoted in four Petri dishes, dried at 37 °C for 24 h, and additionally crosslinked, washed and dried as described for alginate films.

In all cases, control films free of imiquimod were also produced using equivalent procedures for comparison purposes.

2.3. Morphological analysis

The films were evaluated by visual inspection and their aspect was recorded by means of digital photography (iPhone 8 Plus). Scanning electron microscopy (LEO 440i microscope, Leica) was employed to evaluate the microstructure of the biomaterials after surface metallization with gold (mini Sputter coater, SC 7620).

2.4. Color and opacity evaluation

The color and opacity of the films were analyzed using a Hunterlab colorimeter (Colorquest II, Fairfax, USA) and CIELab standards. This system separates the total color difference (ΔE*) into three components: luminosity (L*), chroma, and Hue angle.

The total color difference between the films of different polysaccharide compositions with and without drug was calculated using Eq. (1), while the Hue angle was determined by Eq. (2) and the chroma parameter, by Eq. (3):

$$\Delta E^* = \sqrt{\left(L_I^* - L_p^*\right)^2 + \left(a_I^* - a_p^*\right)^2 + \left(b_I^* - b_p^*\right)^2} \quad (1)$$

$$Hue = \tan^{-1}\left(\frac{b^*}{a^*}\right) \quad (2)$$

$$Croma = \sqrt{(a^*)^2 + (b^*)^2} \quad (3)$$

where a and b describe CIELab Hue sequence and Hue angle orientation parameters, L_p^*, a_p^* and b_p^* are the color parameters of films without imiquimod and L_I^*, a_I^* and b_I^* refer to color parameters of films containing the pharmaceutical compound.

The net opacity of the specimens was calculated as the ratio between the opacity of the film placed determined over black and white patterns.

2.5. Imiquimod incorporation efficiency

The efficiency of imiquimod incorporation was determined by spectrophotometry. Film samples were exposed to different dissolution media, according to the formulation composition. Chitosan films were dissolved using 100 mmol/L acetate buffer at pH 4.0 for 5 h. Alginate films were exposed to citrate buffer (2% w/v aqueous sodium citrate solution) for 2 h and then to an equal volume of acetate buffer. The Ch:A films were exposed for 5 h to phosphate-buffered saline solution (PBS, 2.38 g/L Na_2HPO_4, 0.19 g/L KH_2HPO_4 and 8.0 g/L NaCl, pH 7.0) and afterward, to an equal volume of acetate buffer. The addition of acetate buffer was required to improve the solubility of the drug in all solutions.

All formulations were then sonicated (3510DTH, Branson) for one hour at room temperature, to assure drug solubilization. Solutions with Ch:A specimens were filtered (14 μm pore size filter paper, Qualy) to minimize interference from PEC fragments, and analyzed by light absorption spectrophotometry (Beckman DU 640) at 243 nm. The incorporation efficiency (ε) was calculated using Eq. (4):

$$\varepsilon = \frac{\frac{m_{IQ}}{m_{sample} - m_{IQ}}}{R_{IQ/film}} * 100\% \quad (4)$$

where m_{IQ} is the drug mass determined in the final material, m_{sample} is the mass of polysaccharides in the film, $R_{IQ/film}$ is the mass ratio between the amount of drug added and the mass of polysaccharides initially present in the formulation, equal to 0.055 g/g for all types of films.

2.6. Thermogravimetric analysis

Thermogravimetric analysis (TGA) was performed using a TGA/DSC1 equipment (Mettler Toledo). The mass variation of samples weighing approximately 8 mg placed in 70 μL alumina pans and heated from 25 °C to 600 °C at a rate of 10 °C/min under nitrogen atmosphere (flow rate of 100 mL/min) was analyzed.

2.7. Fourier transform infrared spectroscopy (FTIR)

FTIR spectroscopy analyses were performed for both the films and the isolated drug to identify the possible interactions between the polysaccharides and imiquimod. The samples (1 cm × 2 cm) were previously stored (for at least 24 h) in a desiccator with lithium chloride at 11% relative humidity.

The analysis was performed using a spectrophotometer (Nicolet 6700, ThermoScientific) with wavenumbers ranging from 4000 to 675 cm^{-1} for the films and from 4000 to 400 cm^{-1} for the free drug, 4 cm^{-1} resolution and 32 accumulated scans.

2.8. Mechanical properties and thickness

Tensile strength and elongation at break of the different film formulations were determined based on the D-882 standard [29], using a TA.XT2 texture analyzer equipment (Stable Micro System). A minimum of eight 10 cm long and 2.5 cm wide films with average thickness values measured with a micrometer (Digimess, 110200) in at least 10 random positions were used for each formulation. All formulations were previously stored in a desiccator containing LiCl (11% RH) for at least 48 h

prior to the analysis.

The initial grip distance was 5 cm, the crosshead speed was equal to 0.1 cm/s and the cell load was of 5.098 kgf. The tensile strength (T_S) and the elongation (E_L) of the films at the rupture moment were determined using Eqs. (5) and (6), respectively:

$$T_S = \frac{F_m}{A_T} \tag{5}$$

$$E_L = \frac{(d_f - d_i)}{d_i} x100 \tag{6}$$

in which F_m is the maximum breaking force, A_T is the cross-sectional area and d_f and d_i are respectively the final and the initial grip distance.

2.9. Mucoadhesiveness

The mucoadhesion of the films was analyzed *in vitro* based on the work of Abruzzo et al. (2012) [30] in a TA.XT plus texture analyzer (Stable Micro Systems SMD) in texture profile analysis mode. Due to its similarity to human oral tissue, porcine buccal mucosa was used [31]. The mucosa of freshly slaughtered pigs was collected in a local slaughterhouse and immediately immersed in phosphate buffer at pH 7.4. Then, buccal mucosa flaps were cut with areas greater than 1 cm^2 and hydrated for 5 min in saline solution prior to the assay. Each film sample (approximately 1 cm in diameter) was attached to the analytical probe (10 mm diameter) using double-sided adhesive tape. The buccal mucosa was fixed at the inferior accessory of an A/MUC probe.

A force of 0.50 N was then applied during 30 s to lightly press the mucosa to the surface of the film, and then the sample was moved vertically at a rate of 1 mm/s until it separated from the mucosa flap. The detachment force and work of mucoadhesion required for the separation of the film from the mucosa were calculated from the force by distance curve by using the Exponent software (Stable Micro Systems, UK). All data were obtained at (21 ± 1) °C.

2.10. Absorption of artificial saliva

The behavior of the films regarding fluid absorption was tested in the presence of artificial saliva solution (0.9 g/L NaCl, 1.2 g/L KCl, 0.19 g/L $CaCl_2{\cdot}2H_2O$, 0,11 g/L $MgCl_2{\cdot}6H_2O$, and 0.35 g/L K_2HPO_4, pH 6.8) formulated according to Desai et al. (2011) [2]. Samples of 6 cm × 1 cm with initial mass ($m_{initial}$) were weighed (analytical balance model TE214S, Quimis) and stored in the presence of LiCl to achieve films with standardized initial relative humidity (RH) of 11%. The films were then immersed in 10 mL of artificial saliva for 24 h at 37 °C and periodically, their weight was determined (m_{humid}) after blotting the excess solvent with filter paper. The solution absorption capacity (A) was calculated by Eq. (7), in grams of solution per gram of dry film.

$$A = \frac{m_{humid} - 0.89 m_{initial}}{0.89 m_{initial}} \tag{7}$$

2.11. Imiquimod release kinetics

The determination of drug release kinetics was performed using 2 cm × 2 cm samples of known initial mass. All films were exposed to 15 mL of artificial saliva at 37 °C and periodically, aliquots of 1.0 mL were collected and analyzed at 243 nm to determine drug concentration. The release solution volume was maintained constant by replenishing the spent aliquots with fresh artificial saliva. The total time length was selected as 8 h to be equivalent to the potential maximum application period (overnight), since the patient would possibly remove the film during the daily meals.

The drug release mechanism was determined by fitting the data to zero order and first order mathematical models, as well as to the Korsmeyer-Peppas model [32], according respectively to Eqs. (8)–(10):

$$M_t = M_0 + k_0 t \tag{8}$$

$$\ln M_t = \ln M_0 + k_1 t \tag{9}$$

$$\frac{M_t}{M_\infty} = k t^n \tag{10}$$

where M_0, M_t and M_∞ are the total accumulated imiquimod mass release respectively at time zero, t and infinite (taken as 8 h); k_0, k_1 and k are the respective kinetic release rate constants in each model and n is the release exponent, indicating the type of release mechanism in the Korsmeyer-Peppas model. These equations were used to fit data only in the range from the initial time to the moment at which $M_t/M_\infty < 0.60$. For $M_t/M_\infty > 0.60$, the primary release mechanism could probably be affected by additional phenomena, related to the destabilization and dissolution of the polysaccharide matrices.

2.12. Determination of imiquimod partition coefficient in 1-octanol/water and distribution coefficient in 1-octanol/artificial saliva systems

The partition coefficient of the drug in 1-octanol/water was determined to access the distribution of imiquimod between hydrophilic and hydrophobic environments. To consider the effect of drug ionization, the coefficient of distribution in 1-octanol/artificial saliva was also analyzed.

For that, 0.5 mg aliquots of imiquimod were mixed with 5 mL of aqueous phase (deionized water or artificial saliva). The tubes were mixed to ensure full drug dissolution and then, 5 mL of 1-octanol were added followed by a new stirring step. This mixture was then equilibrated for 24 h at 25 °C. Aliquots of each phase (top and bottom) were collected and filtered through 0.45 μm syringe filters, diluted and subsequently quantified by HPLC using a Thermo Scientific equipment (Dionex Utimate 300), with a Thermo C18 column (Acclaim 120–4,6 × 250 mm) at room temperature. The mobile phase consisted of a 30:69,85:0,15 v/v acetonitrile:sodium acetate buffer (pH 4.0, 0.05 mol/L):triethylamine solution, at a flow rate of 1 mL/min and the detection wavelength was set in 242 nm. Imiquimod solutions prepared in 99.8% acetic acid in the 0.5 to 100 μg/mL range were used to prepare the calibration curve.

Both the partition and the distribution coefficients were calculated considering the ratio between imiquimod concentration in the hydrophobic and in hydrophybic media in equilibrium conditions, being expressed in logarithmic form.

2.13. Statistical analysis

The Statistica 7® software was used to perform the statistical analysis, employing the Tukey multiple comparison test. Differences among averages were considered significant for p-values below 0.05. All experiments were performed in, at least, triplicate, with exception of the analysis of mucoadhesive and mechanical properties, which were performed using at least 5 and 8 replicates of each film formulation, respectively.

3. Results and discussion

3.1. Morphological and color analysis

All tested formulations were capable to yield coherent films with the exception of the one produced using the 3:1 Ch:A proportion, which was no further analyzed in this work. The visual aspect of the remaining film formulations can be observed in Fig. 1.

The films obtained with isolated polymers are transparent, shiny and smooth (Fig. 1a and b), whereas those produced with combinations of the polysaccharides were less translucent (Fig. 1c and d). The addition of

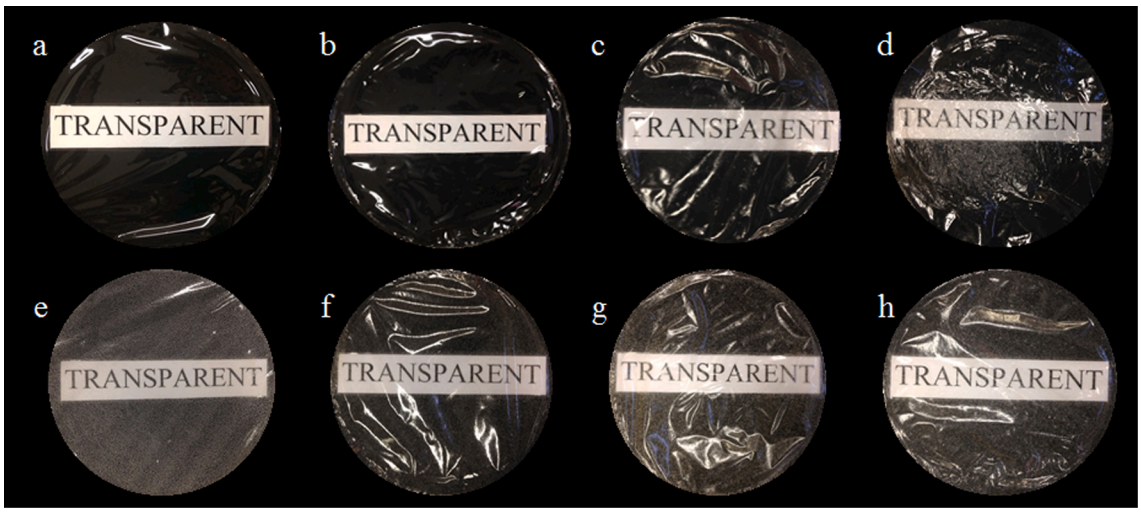

Fig. 1. Visual aspect of the films prepared with different proportions of chitosan and alginate in the absence or presence of the drug formulations free of imiquimod: chitosan (a), alginate (b), Ch:A 1:1 (c) and Ch:A 1:3 (d). Formulations incorporating imiquimod: chitosan (e), alginate (f), Ch:A 1:1 (g) and Ch:A 1:3 (h).

imiquimod clearly affects the structure of the film, turning the matrix whitish and less transparent, as observed in Fig. 1e to h. However, all formulations can be considered as partially transparent. This attribute is rather desirable since it allows lesion monitoring without film removal.

Loss of transparency is associated with the decreasing solubility of imiquimod with solvent evaporation during the casting process. In addition, significant shrinkage was observed in the films to which the drug was added, which may be the result of the interaction of imiquimod primary amines with alginate free carboxyl groups and also of hydrogen bond formation between the drug and the polysaccharides, leading to a greater approximation of the chains and in changes in molecular packing.

When observed in the microscopic scale (Fig. 2), it is possible to notice that in the three formulations containing alginate, imiquimod is distributed throughout the matrix structure roughly as aggregates, while in films consisting of only chitosan, the drug is dispersed in the polysaccharide matrix as long fibers, possibly as the result of the higher concentration of acetic acid in this formulation, which improved drug

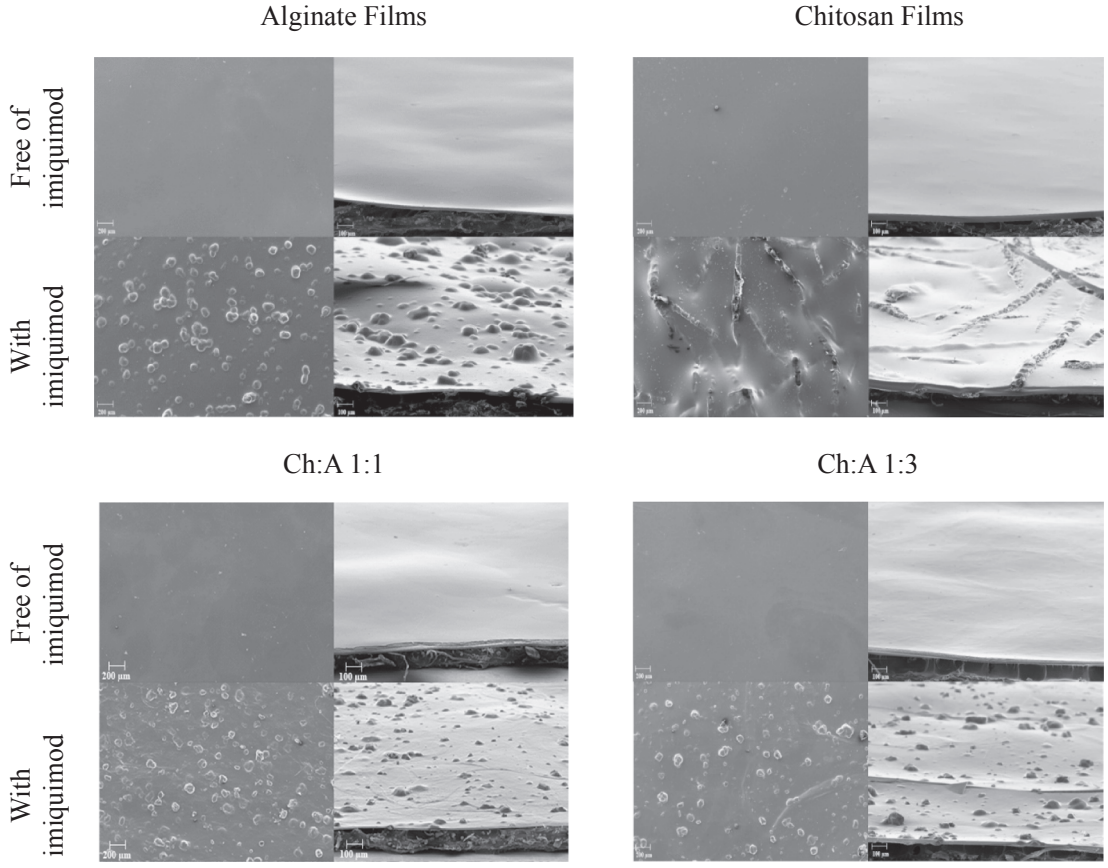

Fig. 2. Scanning electron microscopy of the surface (images on the left for each formulation) and cross-section (images on the right) of films with different compositions containing or not imiquimod.

dissolution prior to film casting.

By analyzing the data shown in Table 1, it is possible to observe that the addition of imiquimod resulted in an increase of up to 5 times the value found for the opacity of formulations without drug. Even though, all formulations have low opacity values, <6%, and can be considered as sufficiently transparent, mostly after wet, to allow the inspection of the wound bed without the need to remove the films. The addition of the drug also resulted in a reduction of the HUE angle and in an increase in the chroma parameter of all formulations. The highest color difference was observed for the chitosan formulation, while for formulations containing both alginate and chitosan, a more significant variation in the total color difference is noticed for the formulation with the higher proportion of alginate.

3.2. Imiquimod incorporation efficiency

Films prepared with chitosan showed high drug incorporation efficiencies, while alginate films were the least effective regarding imiquimod retention, as shown in Table 2.

This result can be explained by the higher drug solubility in acidic conditions, such as those employed for chitosan solubilization. In fact, the only formulation that was able to fully solubilize imiquimod, and that showed no visible drug crystals after the addition of imiquimod do the polysaccharide suspension, was the one consisting solely of chitosan, for which a larger volumetric proportion of acetic acid was used. During the casting process, however, the presence of precipitated drug became evident also in this formulation, as thin imiquimod fibers were formed while solvent evaporation occurred, contrasting with the large drug aggregates observed in the remaining formulations for which solvents with higher pH values were used.

Less efficient retention of imiquimod in the alginate film can be attributed to more extensive loss of drug during the washing steps, since it was not as effectively distributed as small aggregates in the matrix, but dispersed in large aggregates that were not firmly trapped in the polymer matrix, mostly the ones closer to the film surfaces. Films containing simultaneously Ch and A were able to efficiently retain the drug due to the PEC formation, which contributed to entrap the drug aggregates in the polysaccharide matrix.

3.3. Thermogravimetric behavior

The thermogravimetric behavior of the formulations produced and of the drug alone was analyzed, and the results achieved are expressed in Fig. 3, both in terms of mass loss and of its derivative. The values of peaks referring to the four major thermal events detected are summarized in Table S1, in the Supporting Information section.

Isolated chitosan and alginate formulations presented similar behavior, with two thermal events. The first occurred at a temperature below 100 °C and can be attributed to the loss of residual water. The second event, associated with polymer degradation, occurred at 249.3 for alginate and 293.6 °C for chitosan. Similar data were reported for alginate by Sarmento et al. (2006) [33] (equal to 247.8 °C) and Siddaramaiah et al. (2008) [34] (235 °C) and for chitosan by Ferfera-Harrar et al. (2014) [35] (298 °C) and Neto et al. (2005) [36] (equal to 297.3 °C). The high residual mass of the films containing alginate at the higher temperature limit may be attributed to the degradation of this polysaccharide to Na_2CO_3 and a carbonized material that decomposes slowly at higher temperatures (from 600 to 750 °C in N_2, as pointed by Soares et al. (2004) [37]. In the films containing chitosan, the high residual mass may be a result of incomplete polysaccharide decomposition, given that this process occurs in three phases as the temperature is increased, *i.e.* volatilization of low-molecular-weight components, thermal degradation of chitosan main chains, and finally, decomposition of residual carbon, which is probably still present at high amounts at the higher temperature conditions [38].

Imiquimod alone presents only one thermal event, related to its fusion, at 333.4 °C, higher than that reported in the literature, in the range of 292 to 294 °C [39], possibly due to differences in the analysis methodology or the halogenated drug form.

The addition of imiquimod to all film formulations generated small shifts in relation to the events detected in the films not containing it. For both formulations consisting of the isolated polymers, the temperature peak related to water loss decreased with the incorporation of imiquimod, but while for the chitosan formulation the polysaccharide degradation temperature was also reduced, for the alginate film, this temperature increased. In both cases, the peak related to imiquimod fusion is not visible, and these changes probably result from alterations in hydrogen bonding and to electrostatic interactions between imiquimod and alginate.

In the case of both PEC formulations, amines and carboxyl groups interact with each other, and the PEC can co-exist with isolated polysaccharide molecules not involved in complex formation as well as with imiquimod both free and bond to alginate, explaining the occurrence of four different peaks.

3.4. Infrared spectroscopy

The occurrence of possible interactions between the polysaccharides and the drug in the different formulations was analyzed using FTIR spectroscopy. The results are shown in Fig. 4, and the main peaks detected in each formulation are summarized in Table S2, in the Supporting Information section.

The films without imiquimod showed a significant band in the 3600–3000 cm^{-1} region. According to Pavia et al. (2015) [40], this band can be attributed to stretching of –OH groups from chitosan, alginate and their PECs. The peaks characteristic of amino groups in chitosan films (1540 and 1570 cm^{-1}) and in powdered imiquimod (1640 a 1550 cm^{-1}) as well as the peaks referring to carboxyl groups in alginate films (1420 and 1595 cm^{-1}) can be observed. However, these same peaks are not easily identified for the Ch:A PEC formulations due to overlapping.

Table 1
Color and opacity parameters of the films produced.

Formulation	HUE (degrees)	Croma	Opacity (%)	ΔE*
Alginate	94.41 ± 0.52[a]	1.54 ± 0.17[a]	0.95 ± 0.58[a]	–
Alginate + IQ	91.27 ± 0.30[b]	1.89 ± 0.10[a,b]	4.27 ± 0.39[b,c]	1.38 ± 0.09[a]
Chitosan	98.36 ± 0.71[c]	0.78 ± 0.06[c]	1.02 ± 0.42[a]	–
Chitosan + IQ	88.89 ± 0.55[d]	1.72 ± 0.31[d]	5.02 ± 0.57[b]	4.68 ± 0.51[b]
Ch:A 1:1	98.53 ± 0.37[c]	1.67 ± 0.18[a]	2.04 ± 1.53[a,c,d]	–
Ch:A 1:1 + IQ	94.37 ± 0.19[a]	2.23 ± 0.11[b,d]	5.10 ± 0.47[b]	1.45 ± 0.15[a]
Ch:A 1:3	96.43 ± 0.61[e]	1.77 ± 0.24[a]	4.24 ± 3.01[b,d]	–
Ch:A 1:3 + IQ	93.71 ± 0.25[a]	2.87 ± 0.24[e]	5.14 ± 0.84[b]	2.44 ± 0.06[c]

Mean ± standard deviation of the replicates. Means with equal letters indicate that there is no significant difference ($p < 0.05$) according to the Tukey test.

Table 2
Imiquimod incorporation efficiency (%) in all film formulations and final drug to polysaccharide mass ratio in each formulation.

Formulations	Efficiency (%)	Final imiquimod to polysaccharide mass ratio (mg/g)
Chitosan	99.88 ± 1.79[a]	54.9 ± 1.0
Alginate	55.41 ± 0.63[b]	30.5 ± 0.3
Ch:A 1:1	99.46 ± 0.03[a]	54.7 ± 0.0
Ch:A 1:3	99.61 ± 0.10[a]	54.8 ± 0.1

Mean ± standard deviation of the replicates. Means with equal lowercase letters indicate that there is no significant difference ($p < 0.05$) according to the Tukey test.

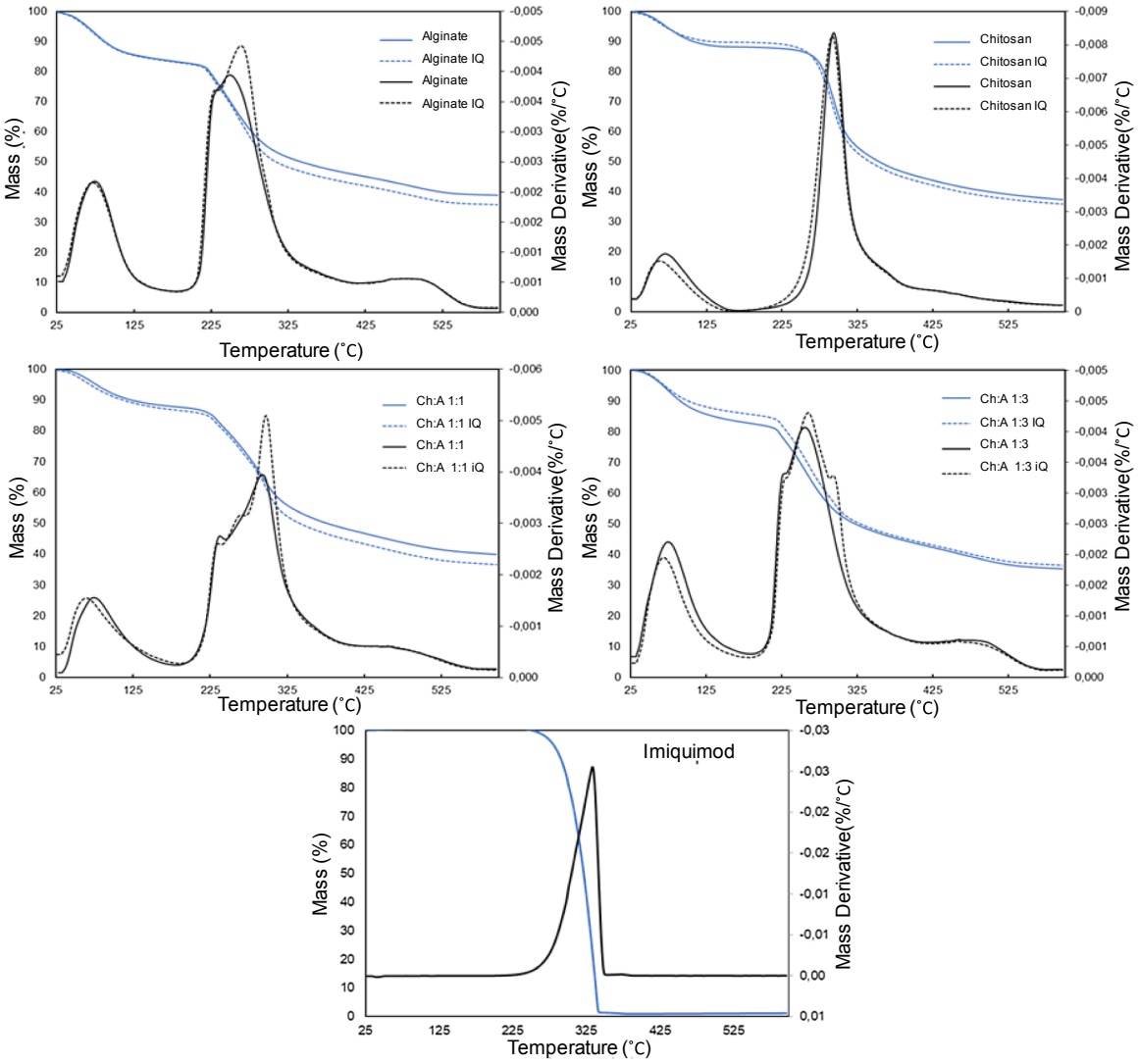

Fig. 3. Thermograms of alginate, chitosan, Ch:A 1:1 and Ch: A 1:3 films containing or not imiquimod (IQ), as well as of isolated imiquimod. Mass loss is indicated in blue, while the mass loss derivative is shown in black.

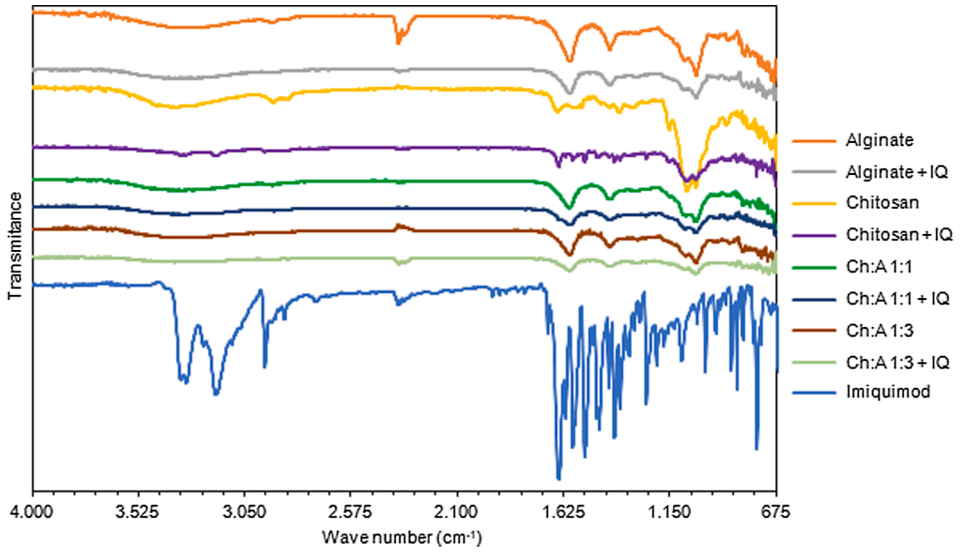

Fig. 4. FTIR spectra of the films incorporating or not the drug and also of imiquimod in powder form.

In the case of chitosan films containing imiquimod, mild interactions in the 3200–3600 cm^{-1} range could be attributed to hydrogen bonding between – NH_2 of imiquimod and –OH groups of chitosan, as reported by Layek et al. (2019) [23].

3.5. Mechanical properties and thickness

The mean values of the films' thickness, tensile strength, and elongation at break are shown in Table 3. Alginate and chitosan films without the drug are significantly thinner than the remaining formulations, but after imiquimod incorporation, no statistically differences are observed. The increase of film thickness after drug incorporation can be explained by two reasons. First, a significant part of the drug is not effectively solubilized in the polysaccharide matrix after drying, but it is instead dispersed in it as aggregates of different sizes. Differences among films of different formulations are probably not observed since the standard deviations in this analysis were significant because of size heterogeneity of the drug aggregates. Second, the presence of imiquimod can hinder the interaction between adjacent polymer chains (either in the formation of the PEC, or in the interaction between two chains of the same polymer), causing vacancies to arise between them, increasing their distance in some regions, and consequently increasing film thickness.

Given that all formulations with the drug, despite showing a significant increase in thickness after imiquimod incorporation can still be considered as very thin, with thickness values not exceeding 132 μm, their use would probably not bring discomfort to the patient. In addition, according to Rajaram and Laxman [41], mucoadhesive films with thickness from 5 to 200 μm can provide accurate drug dosage and good absorption.

The mechanical properties data show that the films produced with the isolated polysaccharides are significantly more resistant to stress than the PECs and that imiquimod addition reduces significantly this property, with exception of the Ch:A formulation. The reduction of tensile strength is probably a consequence of stiffening and shrinkage of the polysaccharide matrix structure, and also of low adhesivity of the drug agglomerates to the continuous phase.

For all formulations, containing or not imiquimod, the elongation at break is very low, not exceeding 3.62%, due to their semicrystalline behavior. However, since aqueous fluids as saliva can exert a plasticizer effect on polysaccharide membranes[42], probably, *in vivo*, film elongation would be significantly higher.

Regarding oral application, according to Goktas et al. (2011) [43], the rupture tension of the buccal mucosa and other regions of the interior of the mouth is in the order of 1.06 to 3.94 MPa. Therefore, for the intended application, all formulations would be suitable if only the mechanical properties were considered.

3.6. Mucoadhesion

The effectiveness of a dosage form applied to mucosa tissues can be significantly increased if the drug delivery system is mucoadhesive. Therefore, to assess the potential of the films produced to be used in oral mucosa lesions, the detachment force and work of mucoadhesion in the presence of artificial saliva were analyzed. The results are shown in Table 4.

The average detachment force values varied from 0.0185 to 0.0333 N, but only the formulations consisting solely of alginate or produced with chitosan and alginate at a mass ratio of 1:3 and incorporating the drug showed values significantly different from each other. Statistically significant differences were not observed for the work of mucoadhesion, despite the variations on the average values from 0.816 to 1.022 N.mm. Therefore, minor effects on mucoadhesive properties were noticed when the composition of the polysaccharide matrix was varied and when imiquimod was incorporated in the films.

Mucoadhesiveness of biomaterials consisting of chitosan and alginate has been the focus of several studies and good mucoadhesive properties have been reported for films, particles and sponges produced with these polysaccharides. Maestrelli et al. (2018) [44], for instance, showed that chitosan-alginate microspheres developed for cefixime vaginal administration have *in situ* permanence longer than two hours. Darwesh et al. (2018) [45] have observed that freeze-dried PECS of chitosan and alginate produced for vaginal delivery of fluconazole show higher maximum detachment force than PECs of chitosan and xanthan gum or chitosan and carbopol 971. In the same line, Abruzzo et al. (2013) [46] have prepared vaginal inserts by freeze-drying chitosan-alginate complexes aiming at chlorhexidine digluconate local delivery in genital infections. These authors demonstrated that the detachment force of a formulation consisting of equal parts of both polysaccharides is equal to 27 ± 2 μN, a value significantly lower than that reported herein. However, values above that were detected for constructs produced only with alginate or chitosan, probably because these biomaterials were not reticulated and the analysis was performed at pH 4.5. Analogously to the present study, Abruzzo et al. (2013) [46] also verified that drug (propranolol hydrochloride) incorporation in chitosan/gelatin films does not significantly affect the mucoadhesion results. Discs of pectin, another type of polysaccharide, analyzed in a similar way, showed maximum detachment strength similar to the values observed in the present work, but significantly lower adhesion work [47].

3.7. Artificial saliva absorption

It is possible to observe in Fig. 5 that all film formulations were capable of absorbing artificial saliva, and that, as expected, increasing the proportion of alginate resulted in higher absorption rates, mainly in the initial exposure periods. After 12 h of exposure of the films to the artificial saliva, a plateau was observed in nearly all cases, with exception of alginate films, which continued to slightly absorb the fluid.

Table 3
Thickness, tensile strength, and elongation at break of the films with different formulations incorporation or not imiquimod.

Formulation	Thickness (μm)	Tensile strength (MPa)	Elongation (%)
Alginate	29.0 ± 6.3^{a}	50.16 ± 18.45^{a}	3.62 ± 1.49^{a}
Alginate + IQ	131.6 ± 76.5^{b}	$11.91 \pm 7.46^{b,c}$	$3.39 \pm 1.45^{a,b}$
Chitosan	29.1 ± 2.4^{a}	64.17 ± 18.36^{a}	1.65 ± 0.93^{b}
Chitosan + IQ	105.1 ± 31.6^{b}	4.75 ± 2.42^{c}	$2.32 \pm 0.68^{b,c}$
Ch:A 1:1	$73.9 \pm 13.8^{b,c}$	16.43 ± 6.62^{b}	$1.77 \pm 0.49^{a,c}$
Ch:A 1:1 + IQ	101.3 ± 10.3^{b}	18.25 ± 5.71^{b}	$2.38 \pm 0.66^{a,b}$
Ch:A 1:3	$63.6 \pm 19.1^{a,c}$	19.38 ± 6.47^{b}	$2.97 \pm 0.77^{a,b}$
Ch:A 1:3 + IQ	$96.0 \pm 10.5^{b,c}$	$9.12 \pm 5.09^{b,c}$	$2.27 \pm 0.94^{a,b}$

Mean ± standard deviation of the replicates. Means with equal letters indicate that there is no significant difference ($p < 0.05$) in the property studied according to the Tukey test.

Table 4
Detachment force and mucoadhesive work of the films.

Formulation	Detachment force (N)	Work of mucoadhesion (N.mm)
Alginate	0.0185 ± 0.0034^{a}	1.022 ± 0.203^{a}
Alginate + IQ	$0.0235 \pm 0.0050^{a,b}$	0.858 ± 0.197^{a}
Chitosan	$0.0217 \pm 0.0050^{a,b}$	0.816 ± 0.152^{a}
Chitosan + IQ	$0.0246 \pm 0.0079^{a,b}$	0.976 ± 0.369^{a}
Ch:A 1:1	$0.0318 \pm 0.0120^{a,b}$	0.930 ± 0.132^{a}
Ch:A 1:1 + IQ	$0.0309 \pm 0.0099^{a,b}$	0.992 ± 0.350^{a}
Ch:A 1:3	$0.0276 \pm 0.0031^{a,b}$	0.855 ± 0.165^{a}
Ch: A 1:3 + IQ	0.0333 ± 0.0080^{b}	0.892 ± 0.132^{a}

Mean ± standard deviation of the replicates. Means with equal letters indicate that there is no significant difference ($p < 0.05$) in the property studied according to the Tukey test. Each parameter was analyzed separately.

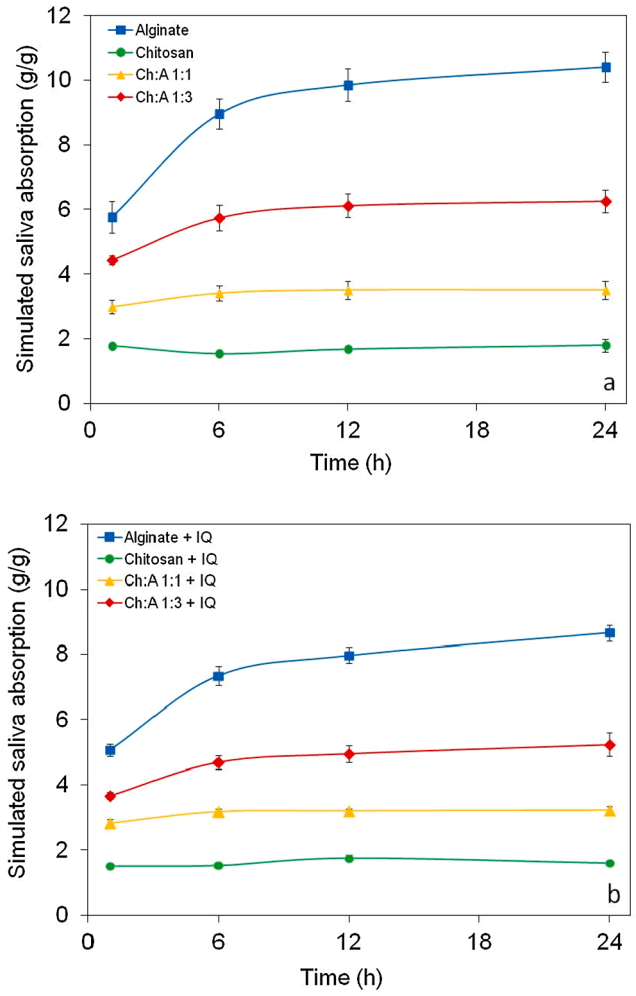

Fig. 5. Capacity of fluid absorption by films exposed to artificial saliva at 37 °C monitored during 24 h: films free of imiquimod (a) or incorporating the drug (b).

The absorption of fluids by the films can be influenced by the amount of free charges inside the film. Since the pH of the artificial saliva fluid is equal to 6.8 and the pKa of chitosan amino groups varies from around 6.5 to 7.0 [11], free amino groups of polysaccharide can be neutral in artificial saliva, but not free carboxyl groups from alginate, which can be negatively charged above pH 3.4, absorbing then more aqueous fluid. Despite the swelling state of the polysaccharide contributes to improve its bioadhesive behavior, this is not reflected in the analysis of detachment force and mucoadhesive work of the films, possibly because the swelling period was short for all films. However, mucoadhesiveness would probably improve with time after the films were applied to the patient mucosa due to gradual fluid absorption, mostly for alginate-rich films.

The addition of imiquimod reduced the proportion of fluid absorbed, mainly for the films rich in alginate. The pKa of imiquimod is 5.4 [23], therefore, in the artificial saliva solution, the molecules not previously bound to alginate would possibly be neutral, affecting the number of free sites for the formation of hydrogen bonds with the aqueous solvent.

According to Mallepally et al. (2013) [48], ionic polymers such as alginate, when in saline solutions, show a typical salting-in behavior, i.e., the presence of salts improve the polysaccharide interactions with water, resulting in an increase in fluid absorption and ultimately, to matrix destabilization in longer exposure periods. In this sense, the 1:0 and 1:1 Ch:A formulations would probably not be as prone to disruption as the two other matrices, due to their slower fluid absorption, but they would also show lower mucoadhesiveness improvement with time.

3.8. Release of imiquimod in artificial saliva

The behavior of all formulations was analyzed regarding the release kinetics of imiquimod in artificial saliva, as shown in Fig. 6 with regard to the cumulative concentrations reached, while the results of fitting the imiquimod release data using the three different mathematical models are shown Table 5.

Despite the films richer in alginate could more intensively absorb the simulated saliva, the chitosan and chitosan:alginate 1:1 formulations were capable to release higher concentrations of the drug. As a whole, the kinetic behavior of imiquimod release from all formulations was well described by the Korsmeyer-Peppas model (Fig. 7), even though the zero order model was effective to fit the results attained with chitosan and Ch:A 1:1 formulations. The zero order model is indeed appropriate to describe systems incorporating drugs showing low solubility, in which the properties of the release rate controlling barrier remain practically constant throughout the release period, e.g. film thickness and permeability to the drug. The first order model, which presupposes that the amount of drug released is proportional to the quantity remaining in the device, being generally useful for describing drug dissolution from porous matrices incorporating water-soluble drugs, was not able to effectively predict the behavior of any of the tested formulations. The results attained herein are then consistent with the limited solubility of imiquimod both in the polysaccharide matrices and in the simulated saliva solution.

The analysis of the results obtained by fitting the Korsmeyer-Peppas model to the release data shows that for the chitosan and Ch:A 1: 1 formulations, since $0.5 < n < 1.0$, the release mechanism is described as anomalous transport, potentially involving different phenomena (e.g. drug diffusion and polymer swelling). Given that these two formulations and also the membrane produced only with alginate show values close to 0.5, diffusion is probably the dominant mechanism for drug release. In the case of the Ch:A 1:3 formulation, for which n is significantly lower than 0.5, the quasi-Fickian transport mechanism is observed. For this film formulation, diffusion potentially occurs partially through the matrix and partially through water-filled pores. The presence of more freely moving chains in the Ch:A 1:3 formulation, as compared to more fully calcium crosslinked alginate chains with limited mobility in the case of films composed only of alginate likely promotes faster drug release than would be expected for the pure Fickian diffusion.

With respect to the rate constant (k) of the Korsmeyer-Peppas model, the higher the value obtained, the shorter the time required for releasing imiquimod. Formulation Ch:A 1: 3 and, to a lesser extent, the one consisting solely of alginate, are the ones that release the drug faster in the initial periods, but a larger amount of released drug was attained for the matrix consisting of chitosan alone for longer exposure periods.

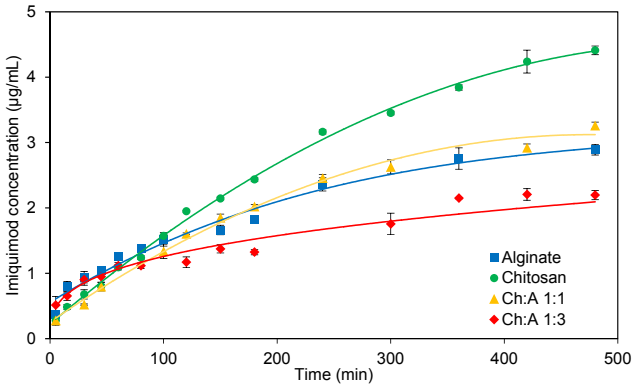

Fig. 6. Cumulative release of imiquimod from films of different compositions exposed to artificial saliva at 37 °C.

Table 5
Values of the parameters calculated for the zero order, first order and Korsmeyer-Peppas mathematical models, together with the respective coefficient of determination (R^2) values, for Imiquimod release data from all tested matrices. When required, the M_∞ values were assumed as those attained after 8 h of exposure to simulated saliva solution.

Mathematical model	Alginate	Chitosan	Ch:A 1:1	Ch:A 1:3
Zero order				
M_0	0.6236	0.2844	0.2346	0.6523
k_0	0.0082	0.0125	0.011	0.0051
R^2	0.8639	0.9895	0.9949	0.8466
First order				
M_0	0.6175	0.4317	0.3755	0.6865
k_1	0.0084	0.0113	0.0108	0.0045
R^2	0.6829	0.8846	0.9024	0.7229
Korsmeyer-Peppas				
n	0.4326	0.6188	0.5772	0.2771
k	0.0716	0.0205	0.0291	0.1504
R^2	0.9654	0.9804	0.9664	0.9727

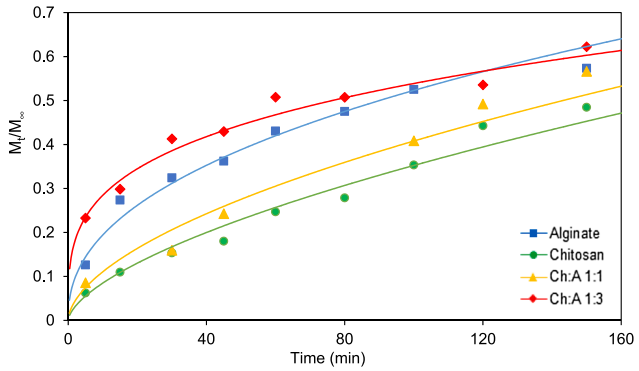

Fig. 7. Imiquimod release of all matrices fitted by the Korsmeyer-Peppas model.

Therefore, the overall analysis of the release kinetics data shows that mass transfer issues related to the nature of the polymer matrices were considerable and also that two other factors contributed to the low accumulation of the drug in artificial saliva. The first one involves the large size of the drug aggregates formed, mostly in the formulations rich in alginate. These aggregates would first need to be dissolved to be able to release the drug by diffusion. The second reason, already discussed above, refers to the very low solubility of imiquimod in aqueous solutions and also in the polysaccharidic matrices produced.

The total amount of imiquimod released from the films was significantly lower than that reported for the commercial cream Aldara®, equal to 200 μg/mL [49]. This can be attributed to different factors, such as cream composition (hydrophobic formulation) and higher solubility of the drug in the acetate buffer used by Donnelly et al. (2006) [49] as receptor fluid in comparison to artificial saliva. Drug interaction with the polysaccharide matrices, in addition to mass transfer limitations associated with drug dissolution and diffusion through the films, is also probably relevant in this sense, as already pointed. A positive outcome deriving from these factors is that no burst release of imiquimod is observed for any of the film formulations developed.

Although the films containing only alginate showed a greater fluid absorption capacity, the formulation consisting of only chitosan was the one that showed the greatest drug release. We believe that this fact results from the pattern of drug packing and distribution in this polysaccharide matrix, which was clearly different in the matrix consisting of chitosan in comparison to all others. Also, the chitosan formulation is produced in an acidic environment, which favors the solubility and dispersion of imiquimod. As a consequence, no direct correlation between the content of liquid absorbed and the amount of drug released was observed.

Since studies on the penetration of imiquimod through murine skin showed that only 11.5% of the drug is released *in vitro* by the Aldara cream and after 3 h of exposure, a total of only 11 μg of imiquimod is transferred per cm^2 of skin [50], the total amount of drug released during the entire 8 h contact period analyzed herein would probably be enough to achieve significant therapeutic effects, mostly for the film consisting solely of chitosan. Besides that, the permeability of many drugs through the buccal mucosa is much higher than through the skin [51]. Therefore even lower imiquimod concentrations could be required to achieve clinically relevant results.

Considering also that the buccal mucosa consists of a multilayered squamous epithelium containing around 50% of polar lipids in the intracellular space [52], drugs delivered to the oral mucosa may permeate the lesion by lipoidal and/or aqueous pathways. Given that the analysis of imiquimod partition coefficient in 1-octanol/water and distribution coefficient in 1-octanol/artificial saliva resulted respectively in log P and log D values equal to respectively 1.56 ± 0.06 and 2.15 ± 0.01, the lipophilic character of imiquimod would possibly exert a positive effect in its ability to reach even deep lesions.

Finally, it is also worth mentioning that if a fraction of the imiquimod released was swallowed by the patient, this event would possibly not bring severe adverse effects. Savage et al. [53], in a clinical study, investigated the tolerability, toxicity, and biological effects caused by daily oral imiquimod administered on 21 patients with non-responding refractory tumors, showing that only dosages ranging from 50 to 200 mg resulted in significant toxicity. In the present work, the quantities observed are well below the toxicity limit, since even in the case in which the highest accumulation of drug was attained (in simulate saliva after 8 h), for the chitosan formulation, no more than a total of 130 mg of imiquimod was released.

4. Conclusions

The results achieved showed that several of the films produced presented many of the expected attributes for the aimed goal. The films were thin, transparent, mucoadhesive, had moderate (alginate) or very high (all other formulations) imiquimod incorporation efficiencies, absorbed appropriate amounts of artificial saliva at relatively high rates, were mechanically resistant and stable at a wide temperature range. In addition, the films released the drug in a sustained manner, with no burst effect. Despite the rate of drug released from the films was not as high as expected, results in terms of drug accumulation in the cells would probably be very attractive due to the high partition of this drug in cell membranes. Thus, the developed films can be considered as a relevant alternative for the treatment of oral mucosal cancer, since they can control drug release, minimizing drug loss by leaching and swallowing, being a viable option for adjuvant/palliative treatment of patients with multiple comorbidities. As future perspectives, further improvements in film design can certainly be performed, for instance to ensure unidirectional drug release towards the mucosal tissue, and additional studies to confirm the effectiveness of the developed systems, e.g. *ex-vivo* tests with porcine mucosa models and *in-vivo* tests.

CRediT authorship contribution statement

Lucas Garcia Camargo: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Visualization, Writing - review & editing. **Paula de Freitas Rosa Remiro:** Investigation, Validation, Writing - original draft, Visualization, Writing - review & editing. **Gabriela Souza Rezende:** Investigation, Methodology, Visualization. **Stephany Di Carla Santos:** Methodology, Investigation, Visualization, Writing - original draft, Writing - review & editing. **Michelle Franz-Montan:** Conceptualization, Methodology, Validation, Formal analysis, Visualization, Writing - review & editing. **Ângela Maria Moraes:** Conceptualization, Methodology, Formal analysis,

Resources, Visualization, Writing - review & editing, Writing - original draft, Supervision, Project administration.

Declaration of Competing Interest

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

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eurpolymj.2021.110422.

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