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Tuning the properties of alginate–chitosan membranes by varying the viscosity and the proportions of polymers

Andréa C. K. Bierhalz, Ângela Maria Moraes
Department of Engineering of Materials and of Bioprocesses, School of Chemical Engineering, University of Campinas, Campinas, Brazil
Correspondence to: Â. M. Moraes (E-mail: ammoraes@feq.unicamp.br)

ABSTRACT: In this study, alginate (A) and chitosan (C)-based membranes designed for skin tissue engineering applications were prepared using three different A to C mass ratios (3:1, 1:1, and 1:3). Each formulation was produced with alginate of two different viscosities (low and medium). Porous membranes were obtained through foaming by adding the surfactant Poloxamer 188 to the formulations at the concentrations of 1%, 5%, and 10% (w/w) in excess of the biopolymers mass. The physicochemical properties of the membranes were evaluated, showing that the formation of more stable, resistant, and porous structures with Poloxamer 188 was favored in membranes prepared with medium-viscosity alginate. The surfactant also exerted the most pronounced porogenic effect on the formulation with alginate:chitosan mass ratio equal to 3:1. These membranes consequently had greater thickness, roughness, opacity, water vapor transmission rate, and lower mechanical resistance than 1:1 and 1:3 membranes. Taken together, the results indicated that it is possible to improve and tune the properties of alginate–chitosan polyelectrolyte complexes by varying the viscosity of alginate and proportions of biopolymers and surfactant. © 2016 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2016**, *133*, 44216.

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INTRODUCTION

The limitations associated with conventional treatments of chronic skin wounds have intensified researches in the field of tissue engineering.[1–4] In one approach, tissues can be obtained *in vitro* from cells seeded and cultured on biodegradable scaffolds to be subsequently transplanted to the patient.[5,6] The scaffold must then be designed to conform to a specific set of requirements comprising biocompatibility, controlled degradation kinetics, mechanical properties, appropriate surface chemistry, and permeability.[7–11] In the case of skin tissue engineering, the scaffolds may also cover the lesion, providing the additional benefits of mechanical protection, prevention of dehydration and physical barrier against external infection in the form of wound dressings.[12] Potential materials for the development of devices with these characteristics include natural polymers, such as chitosan and alginate.

Chitosan is a derivative of chitin, being a cationic polysaccharide composed of D-glucosamine and *N*-acetyl-D-glucosamine residues with β(1,4) linkages at pH conditions above approximately 6.5.[13] Among the properties of chitosan are the gradual biodegradability, the stability for long periods, and the bacteriostatic activity.[14] Several studies report specific advantages of chitosan for wound healing such as hemostasis, ability to accelerate tissue regeneration and to stimulate collagen synthesis in fibroblasts.[8,15]

Alginates are anionic linear copolymers of (1,4) D-mannuronic acid (M) and L-guluronic acid (G) residues arranged in a nonregular blockwise pattern. This polymer, typically obtained from brown seaweeds, is biocompatible, relatively inert, bioresorbable, and has good mucoadhesive properties, being capable to form resistant gels and membranes through ionic interactions between the G units of alginate and divalent cations.[16,17] Due to these properties, alginate has been extensively studied in the production of scaffolds and vehicles to deliver proteins, drugs, and cells that can direct the regeneration or engineering of various tissues and organs in the body.[17,18]

The association of these two oppositely charged polysaccharides at an adequate pH level via ionic interaction between protonated amines and carboxylate groups leads to the formation of polyelectrolyte complexes (PECs) that have demonstrated potential for biomedical applications.[14,19,20] Evaluations of PEC membranes as wound dressings indicated that these materials are nontoxic toward mouse and human fibroblast cells and are able both to increase cell viability and to accelerate the healing of incisional and excisional wounds in animal models.[20,21]

Compared to alginate or chitosan alone, alginate–chitosan PEC membranes and scaffolds have better mechanical stability, resistance to pH variations, and effectiveness as controlled-release systems.[22–24]

The scaffold morphology plays an important role in uniform cell distribution, in the transport of nutrients, and consequently, in the quality of the tissue developed.[4,25] Therefore, a large number of fabrication techniques have been developed to obtain porous biodegradable scaffolds with reproducible pore size and interconnectivity. Recently, Bueno *et al.*[26] optimized a simple and cost/time effective approach developed formerly[27] by obtaining porous alginate–chitosan (at a 1:1 mass ratio) membranes through the addition of the biocompatible non-ionic surfactant Pluronic F68 to the polymeric solution. Pluronic F68, also known as Poloxamer 188, Kolliphor P188, Flocor, and RheothRx, is a water-soluble, amphiphilic, and uncharged triblock copolymer containing a hydrophobic core (polypropylene oxide) and two hydrophilic tails (polyethylene oxide). This surfactant is frequently used as a porogenic agent in the production of polymeric spheres and particles for enhancing the delivery of encapsulated compounds and in pharmaceutical formulations due to its compatibility with various drugs and excipients, being approved by the FDA as a component of skin products.[27–29] Although the results obtained by the aforementioned method are quite promising, dense membranes prepared in the absence of surfactant have considerably better stability and mechanical properties [e.g., tensile strength (TS) and elongation at break] than similar porous alginate–chitosan membranes[26,27]

The TS of the material should be compatible with that of the damaged tissue, which is around 21 MPa for skin.[30] However, the values reported for alginate- and chitosan-based porous matrices ranges from 1 to 3 MPa.[26,27] To overcome these limitations and obtain a suitable biomaterial for skin tissue engineering applications, the present study aimed to investigate the effect of the proportion of the biopolymers alginate and chitosan as well as of the alginate viscosity on the physicochemical properties of membranes prepared with different quantities of Poloxamer 188.

EXPERIMENTAL

Materials

Low-viscosity sodium alginate (A-0682, molar mass of 2.49×10^4 g/mol, intrinsic viscosity of 197 mL/g at 25 °C), medium-viscosity sodium alginate (A-2033, molar mass of 9.14×10^4 g/mol, intrinsic viscosity of 690 mL/g at 25 °C), and chitosan from shrimp shells (deacetylation degree of 95%, molar mass of 1.26×10^6 g/mol, intrinsic viscosity of 848 mL/g at 25 °C), obtained from Sigma-Aldrich (St. Louis, Missouri, USA), were used as biopolymer to produce the membranes. The intrinsic viscosities were determined by viscosimetry at 25 °C (capillary viscometer Ostwald-Cannon-Fenske; size 200), and information regarding the deacetylation degree of chitosan was provided by the manufacturer.

Poloxamer 188 and phosphate-buffered saline (PBS) were also obtained from Sigma-Aldrich. Fetal bovine serum (FBS) and α-MEM cell culture medium were obtained from Nutricell (Campinas, Brazil). Calcium chloride dihydrate and sodium hydroxide were obtained from Merck (Darmstadt, Germany), and acetic acid was obtained from Synth (Diadema, Brazil). All reagents were of at least analytical grade quality and suitable for cell culture.

Membrane Preparation

Alginate–chitosan membranes obtained using low- and medium-viscosity alginate (A_L and A_M, respectively) and chitosan (C) at three different A_i:C proportions (3:1, 1:1, and 1:3), in relation to total mass of polysaccharides. The subscript "i" in A denotes low or medium viscosity, and the membranes were prepared based on modifications of the process previously reported by Bueno and Moraes.[27] The alterations of the procedures allowed the increase in the number of membranes prepared per batch, the reduction of the amount of $CaCl_2$ required for membrane ionic reticulation and improved the membrane washing steps.

Chitosan solution (1%, w/v) was prepared by dissolving the biopolymer in 1% (v/v) acetic acid aqueous solution, followed by vacuum filtration to remove impurities. The alginate solution (1%, w/v) was prepared in deionized water containing the surfactant Poloxamer 188 (P) at a concentration enough to exceed in 1, 5, or 10% (w/w) in relation to the total mass of biopolymers. All polysaccharides solutions were prepared using the polymers in equilibrium with the environment moisture (around 50%).

With a peristaltic pump (Model Minipuls 3; Gilson, Middleton, USA), the chitosan solution (100, 200, or 300 mL) was added at a flow rate of 200 mL/h to the appropriate amount of aqueous alginate-Poloxamer 188 solution to obtain a final volume of 400 mL. The mixture was prepared in a jacketed stainless steel tank with an internal diameter of 10 cm and a height of 20 cm. The system was maintained under mechanical stirring of 500 rpm (Q-251D; Quimis, Diadema, Brazil), and the temperature was controlled at 25 °C (thermostatic bath Q-214M2, Quimis). Following, the stirring rate of the final mixture was increased to 1000 rpm for 10 min. At the end of this step, the pH of the suspension was elevated to 7.0 by the addition of 1 *M* NaOH, and the same stirring rate was maintained for 10 additional minutes. Later, a calcium chloride solution (1%) was added slowly, until a total amount of 0.05 g $CaCl_2$/g alginate was transferred. The stirring rate and temperature were kept constant for another 10 min. Aliquots (85 g) of the membrane-forming solution were poured into polystyrene petri dishes ($d = 15$ cm) and dried in a convection oven (420D; Nova Ética, Vargem Grande Paulista, Brazil) at 37 °C for 24 h. After releasing the resulting membrane from the support, the crosslinking was complemented by total immersion of the membranes in 150 mL of an aqueous calcium chloride solution (2%, w/v) for 30 min. The membranes were then washed twice in 200 mL of deionized water for 30 min. The excess surface liquid was removed, and the membranes were dried at room temperature (25 °C) for 24 h with the membrane borders fixed by Teflon (DuPont, Wilmington, USA) rings to avoid wrinkling of the edges.

The preparation of control membranes (without Poloxamer 188) required the deaeration of the polymeric mixture under vacuum for 2 h before drying. The remaining steps were performed as previously described for the porous membranes.

Analysis of Membrane Characteristics

Aspect. The aspects of the dense and porous membranes were evaluated with respect to morphology, thickness, roughness, and opacity.

The morphology of the samples was evaluated macroscopically with a photographic camera (model DMC-F2; Panasonic) and microscopically with a scanning electron microscope (SEM; model LEO440i; LEO Electron Microscopy, Cambridge, UK) operating at 15 kV and 100 pA. Before the microscopy analysis, the samples were fixed on a stub and then coated with an ultrathin layer of gold (92 Å) in a sputter coater (K450, Emitech, Kent, UK) to enhance surface conductivity.

A digital micrometer (MDC-25S; Mitutoyo, Suzano, Brazil) was used to measure the thickness of the membranes at 10 random positions.

The roughness (Ra) of the membrane surface was determined in triplicates, using a portable rugosimeter (SJ-210; Mitutoyo) with cutoff length set at 0.8 mm and total length at 5 mm. Measurements were performed on both surfaces of the membranes (10×10 cm^2 samples) in at least ten positions for each membrane.

The opacity of the membranes was measured with a colorimeter (Coloquest II; Hunterlab, Reston, USA), operating in the transmittance mode according to the Hunterlab method.[31]

Mechanical Properties. TS, elongation at break (E), and Young's modulus (YM) were determined at 25 °C (±1 °C) using a TA.XT2 (Stable Microsystems SMD, Godalming, UK), according to the ASTM standard method D882.[32] Membranes strips (10×2.54 cm^2) were preconditioned at 52% relative humidity for 48 h before the test. The cross-head speed and the initial grip spacing were set at 0.1 cm/s and 5 cm, respectively. Measurements were repeated at least 10 times. The TS was expressed as the maximum force at break per initial cross-sectional area of the membrane and the elongation as a percentage of the original length.

Water Vapor Transmission Rate and Water Vapor Permeability. The water vapor transmission rate (WVTR) and the water vapor permeability (WVP) of the membranes were determined gravimetrically, in triplicates, at 25 °C (±1 °C), according to the ASTM method E96-95.[33] Samples were sealed over a circular opening of a Plexiglas permeation cell filled with anhydrous calcium chloride. These cells were individually kept in chambers closed hermetically (capacity around 500 mL) containing a saturated solution of sodium chloride to maintain a relative humidity difference of 75%. The salt solution in the bottom was occasionally agitated using a magnetic stirrer. After the system reached steady-state conditions (approximately 2 h), the cell was weighed for 5 days every 12 h. The WVTR and WVP were calculated using the following equations, respectively:

$$\mathrm{WVTR} = \frac{G}{A} \quad (1)$$

$$\mathrm{WVP} = \frac{\mathrm{WVTR} \cdot \delta}{\Delta\mathrm{RH} \cdot P_w} \quad (2)$$

where G is the permeation rate (g/day) calculated by linear regression of the mass gain versus time, A is the permeation area (15.21 cm^2), δ is the membrane average thickness (mm), ΔRH is the difference in relative humidity (0.75), and P_w is the partial water vapor pressure at the test temperature (3.167 kPa).

Liquid Uptake and Mass Loss. Liquid uptake and mass loss were determined in deionized water, PBS, and the culture medium α-MEM supplemented with 10% FBS following the same experimental protocol adopted by Bueno *et al.*[26]

Thermogravimetric Analysis. The water content and degradation temperature of the membranes were determined by thermogravimetric analysis (TGA) in a TGA Q50-M (Shimadzu, Japan). Samples with about 6 mg were processed over a temperature range of 25 to 600 °C at a scan rate of 10 °C/min. Tests were performed in triplicates, under nitrogen gas purge (100 mL/min).

Statistical Analysis. Analysis of variance and the Tukey test were used to determine statistically significant differences ($p < 0.05$) among averages, using the SAS Software version V8.2.

RESULTS AND DISCUSSION

Membrane Aspect and Morphology

The aspect of the dense and porous membranes prepared with different mass ratios of chitosan (C) and alginate of low and medium viscosity (A_L and A_M, respectively) was analyzed and compared in terms of morphology, thickness, roughness, and opacity. The macroscopic aspect and the micrographs of the cross-section of the membranes are shown in Figure 1.

The micrographs of the samples without the surfactant Poloxamer 188 (P) indicate that the membranes have dense structures with proper structural cohesion, without evidence of phase separation. At the macroscopic level, the 3:1 membrane prepared with low viscosity alginate (A_L:C) had greater heterogeneity and surface roughness than the other samples.

The addition of the surfactant to the formulations prepared with low-viscosity alginate did not promote the formation of porous structures. The foam formed in the biopolymeric solution was not stable, collapsing completely during the casting step. Therefore, only the membranes prepared with medium-viscosity alginate were capable of forming porous matrices in the presence of the surfactant Poloxamer 188. In these membranes, a substantial change in the structure could be observed with the increase in Poloxamer 188 concentration. The number and size of pores increased with the presence of surfactant and the membranes tended to be whitish.

The change in morphology of the membranes with the inclusion of Poloxamer 188 can be attributed to the high hydrophilic–lipophilic balance of this surfactant, which implies good foam-formation ability in aqueous media.[34] The increase in the amount of surfactant used in the preparation of the membranes promotes greater retention of air during mixing, with consequent increase in the porosity of the material.

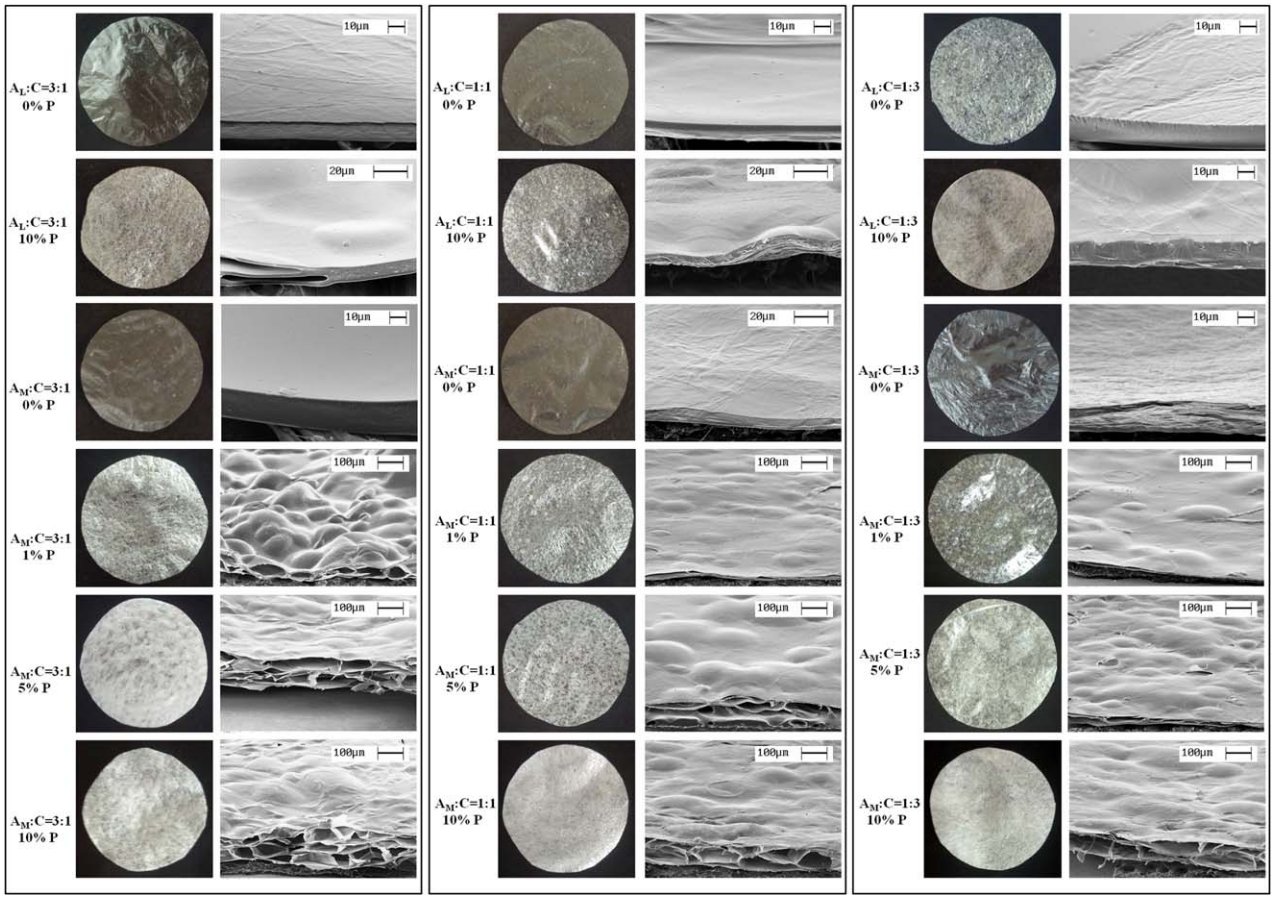

Figure 1. Macroscopic aspect and morphology of the cross-sections of different formulations of A_L:C (low-viscosity alginate:chitosan) and A_M:C (medium-viscosity alginate:chitosan) membranes in the absence and presence of Poloxamer 188. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Among the different polymeric formulations, the highest porosity was observed in the membranes containing the proportion of alginate 3:1, followed by formulations 1:1 and 1:3. Poloxamer 188 interacts with chitosan and alginate mainly through hydrophilic interactions, because of the high –OH group content of the polysaccharides. Since alginate is relatively more hydrophilic than chitosan, it is assumed that there may have been a greater interaction of the former with the surfactant, enabling the formation of a larger quantity of foam and, consequently, of pores. In addition, the membranes containing more alginate have the highest crosslinking degree with calcium ions, which made the polymeric solution more viscous and increased the stability of the foam. The SEM micrographs of the membranes revealed that the pores were interconnected in the lamellar structure, but did not go across the entire thickness of the membrane. Bueno and Moraes[27] observed a similar conformation and noted that this characteristic could be favorable for applications such as wound dressings, since the direct penetration of microorganisms in lesions would be prevented. However, for application of the membranes as scaffolds in the area of tissue engineering, high pore interconnectivity would be desirable. This could be achieved for instance by coupling to the used foaming approach solid leaching techniques or even by mechanical perforation of the biomaterial.

The physicochemical characterization of membranes produced with the combination of chitosan, low-viscosity alginate and Poloxamer 188 was not performed because porous membranes were not obtained, even when using 10% of surfactant.

The results attained regarding thickness, roughness, and opacity are summarized in Table I.

It is possible to observe that the thickness of the membranes increased considerably with the increase in the proportion of Poloxamer 188 used in the formulation. This behavior was expected since using high amounts of surfactant results in larger cavities being formed and more air being stably trapped within them. The increase in thickness was more expressive in the formulation 3:1, due to the higher porosity of the structure and the increased spacing of the layers. In formulations A_M:C 1:1 and 1:3, significant ($p < 0.05$) increases in thickness values were obtained only after the addition of 5% and 10% Poloxamer 188, respectively. These results are in agreement with those noted in the micrographs in Figure 1, showing that greater concentrations of the surfactant in formulations alginate:chitosan 1:1 and 3:1 resulted in more pronounced morphological changes.

The dense membrane prepared with low-viscosity alginate (at A_L:C equal to 3:1) had a significantly higher thickness value than the dense membrane prepared with medium-viscosity

Table I. Thickness, Roughness and Opacity of the Membranes

Membrane formulation					
Alginate type	A:C mass proportion	Poloxamer (%)	Thickness (μm)	Roughness Ra (μm)	Opacity (%)
A_L	3:1	0	118 ± 12^{ef}	9.97 ± 1.63^{b}	5.43 ± 0.84^{g}
A_M	3:1	0	54 ± 5^{hi}	3.37 ± 0.31^{ef}	8.13 ± 0.77^{ef}
	3:1	1	144 ± 20^{de}	5.71 ± 1.08^{cd}	21.15 ± 1.08^{b}
	3:1	5	345 ± 62^{b}	10.88 ± 1.12^{b}	34.87 ± 1.20^{a}
	3:1	10	477 ± 44^{a}	17.71 ± 1.47^{a}	35.68 ± 0.32^{a}
A_L	1:1	0	48 ± 4^{i}	3.18 ± 0.47^{ef}	7.47 ± 0.97^{f}
A_M	1:1	0	47 ± 6^{hi}	1.06 ± 0.17^{g}	8.68 ± 0.25^{ef}
	1:1	1	65 ± 9^{h}	4.54 ± 0.42^{de}	18.33 ± 0.65^{cd}
	1:1	5	100 ± 14^{fg}	6.28 ± 0.94^{cd}	20.12 ± 0.83^{b}
	1:1	10	254 ± 23^{c}	9.20 ± 1.08^{b}	21.48 ± 0.38^{b}
A_L	1:3	0	52 ± 2^{ih}	0.94 ± 0.12^{g}	12.55 ± 0.96^{d}
A_M	1:3	0	64 ± 3^{h}	1.99 ± 0.52^{fg}	7.73 ± 0.86^{f}
	1:3	1	72 ± 6^{gh}	2.52 ± 0.82^{efg}	15.42 ± 1.93^{d}
	1:3	5	77 ± 7^{fgh}	3.18 ± 0.75^{eg}	21.50 ± 1.14^{b}
	1:3	10	165 ± 15^{d}	6.97 ± 0.28^{c}	22.47 ± 0.37^{b}

Average ± standard deviation of experimental determinations. Averages with the same letter, in the same column, indicate no significant differences ($p < 0.05$) by the Tukey test.

alginate (A_M:C of 3:1). This result is also in agreement with the macroscopic observations, which indicated a rough and heterogeneous surface. Among the remaining dense samples, no differences were observed between the thickness values.

The literature does not indicate an optimal thickness for the porous membranes, since this property depends on the region of the body to be treated. According to Ma *et al.*[15], the polymer substitutes of the dermis should be thinner than human skin, whose thickness varies from 0.5 to 2 mm, depending on age, gender, and body region. The thickness of the membranes produced in this study was less than 500 μm for all formulations evaluated, which indicates that the membranes can be considered appropriate.

A trend similar to that of thickness was observed for the surface roughness results. Addition of the surfactant Poloxamer 188 promoted increase in the surface roughness of membranes, which can be attributed to the presence of ripples on the surface due to the air bubbles trapped within the samples. This behavior was also observed by Bueno and Moraes[27] for chitosan and alginate membranes, in which roughness increased from 1.3 to 21 μm after the addition of 10% surfactant.

Several studies have suggested that the surface roughness of the scaffolds influences cell adhesion, proliferation, and differentiation, as well as the shape assumed by the cells on the surface.[35,36] According to Milleret *et al.*[37], the rougher the surface, the greater the adherence of platelets and the formation of thrombin, which are favorable conditions to accelerate the healing of skin lesions. Roughness values of up to 200 μm have been reported in the literature, being recommended for the treatment of lesions in the early stages of healing.[38]

The opacity of the membranes was evaluated to quantitatively verify the effect of the formulation on the material aspect. Considering the possibility of direct observation of the wound bed during treatment, membrane transparency is a desirable factor. The data in Table I indicate that the addition of the surfactant promoted increase in the opacity of the membranes, what can be attributed to the increase in the spacing between the lamellae, representing resistance to the passage of light. Therefore, regarding this property, dense membranes are more attractive due to their high transparency.

WVP and WVTR

The results of WVP and WVTR measured at 25 °C are presented in Table II. This property is important for materials that have contact with skin lesions, since a favorable environment should be ensured for the wound-healing process, by adequate moisture and gas exchange rates. The evaporative water loss from a wound should be controlled at an optimal rate to prevent excessive dehydration as well as buildup of exudates around the wound.[39]

Analysis of the data indicated that the presence of pores favored the permeation of water vapor and that the membranes containing 10% Poloxamer showed the highest value for this property. In addition, among the different formulations, the membranes with a higher proportion of alginate (3:1), which had more interaction with the surfactant and greater porosity, showed a higher WVTR.

The viscosity of the alginate used had a significant effect on the vapor-transmission capacity of the dense membranes (prepared in the absence of Poloxamer 188) produced with the three A:C formulations (3:1, 1:1, and 1:3). The WVP of the dense membranes prepared with low-viscosity alginate (A_L:C at 3:1, 1:1, and 1:3 mass ratios) was approximately two times greater than

Table II. Water Vapor Permeability (WVP) and Water Vapor Transmission Rate (WVTR)

Membrane formulation				
Alginate type	A_i:C mass proportion	Poloxamer (%)	WVP (g mm/m^2 d kPa)	WVTR (g/m^2 d)
A_L	3:1	0	30.26 ± 3.47^{d}	642.11 ± 32.52^{d}
A_M	3:1	0	13.50 ± 1.23^{fg}	523.68 ± 40.14^{ef}
	3:1	1	32.45 ± 0.63^{d}	696.84 ± 26.62^{c}
	3:1	5	117.78 ± 9.83^{b}	782.63 ± 20.61^{a}
	3:1	10	135.71 ± 7.79^{a}	778.57 ± 7.79^{a}
A_L	1:1	0	13.84 ± 1.27^{fg}	649.47 ± 25.72^{d}
A_M	1:1	0	7.62 ± 0.42^{h}	495.79 ± 35.73^{f}
	1:1	1	16.95 ± 2.33^{ef}	640.53 ± 7.81^{d}
	1:1	5	27.55 ± 1.58^{de}	651.05 ± 28.08^{d}
	1:1	10	73.18 ± 5.46^{c}	743.68 ± 5.23^{b}
A_L	1:3	0	11.90 ± 2.54^{g}	513.16 ± 41.02^{ef}
A_M	1:3	0	6.18 ± 0.69^{h}	484.28 ± 13.72^{f}
	1:3	1	18.27 ± 2.47^{ef}	646.32 ± 9.52^{d}
	1:3	5	23.35 ± 0.65^{de}	644.74 ± 8.10^{d}
	1:3	10	82.39 ± 5.25^{c}	737.37 ± 10.02^{b}

Average ± standard deviation of experimental determinations. Averages with the same letter, in the same column, indicate no significant differences ($p < 0.05$) by the Tukey test.

their respective formulations obtained with medium viscosity alginate (A_M:C at 3:1, 1:1, and 1:3 mass ratios).

According to George and Abraham,[40] several factors can affect chitosan–alginate PEC properties, such as the composition of alginate, the molar mass of the polysaccharides, and the degree of deacetylation of chitosan. The closer the molar mass of the chitosan is to the alginate, the more crystalline and compact the PEC formed is.[41,42] As the medium-viscosity alginate had a molar mass more similar to that of chitosan, the membranes made with this polysaccharide probably achieved a more compact molecular packing (also roughly confirmed by the analysis of membrane thickness) and, consequently, showed lower WVP.

Lamke *et al.*[43] reported that the WVTRs of normal skin and injured skin, with first, second, and third degree burns, are 204, 278, 4274, and 3436 g/m^2/day, respectively. Therefore, the membranes obtained in this study have a relatively low WVTR, being only suitable for application in wounds with low amount of exudates or in injuries at a relatively advanced stage of healing.

Similar results were observed for alginate and chitosan (1:1) membranes crosslinked with calcium by Wang *et al.*[24] These authors obtained permeability rates between 566 and 765 g/m^2/day.

Mechanical Properties

The adequacy of mechanical properties of a membrane is an important requirement in tissue engineering[44] for the prevention of matrix collapse during the routine activities of the patient.

Analysis of the TS and YM of the dense membranes indicated (Table III) the significant effect of alginate viscosity and molar mass on these properties.

The membranes made with medium-viscosity alginate were more rigid and had higher TS than membranes prepared with low-viscosity alginate. For the formulation A_M:C at proportion 3:1, the TS increased by almost six times when compared to that of A_L:C membranes prepared using the same proportions. For the other formulations, the increase was less pronounced and the TS approximately doubled. Draget *et al.*[45] evaluated the effect of molar mass of different alginate samples on properties of alginic acid gels and also observed an increase in YM values with an increase in molar mass.

Among the porous membranes, on the other hand, there was a drastic reduction in TS and YM after the addition of the surfactant in all samples. The greatest reduction was obtained for the membrane with the higher proportion of alginate (3:1 prepared in the presence of 5 and 10% of Poloxamer 188) due to the higher porosity. Despite the observed reduction, all membrane formulations analyzed should be suitable for application in skin, in which resistance can vary from 2.1 to 21 MPa.[30,44]

This reduction behavior was observed by Bueno and Moraes[27] for membranes of alginate and chitosan (1:1) made with alginate of a viscosimetric molar mass of $4.69 \cdot 10^4$ g/mol. For dense and porous (10% Pluronic F68) membranes, these authors obtained TS values of 31 and 1.1 MPa, respectively. Modifications in this work regarding to the formulation of these authors also included increasing of concentration of the alginate solution from 0.5 to 1.0% (w/v) and reducing the concentration of the $CaCl_2$ solution used in the first step of crosslinking from 2 to 1% (w/v). This reduction was required to avoid local $CaCl_2$ supersaturation due to increased viscosity and consequent localized gelification of the polymeric solution. The gentle addition of the crosslinking agent, the use of alginate with medium viscosity and the increase of the concentration of alginate solution contributed to the formation of a more compact and homogeneous structure, allowing obtaining dense and porous membranes of improved mechanical resistance.

Table III. Tensile Strength (TS), Elongation at Break (%), and Young's Modulus (YM) of the Membranes

	Membrane formulation				
Alginate type	A_i:C mass proportion	Poloxamer (%)	TS (MPa)	E (%)	YM (MPa)
A_L	3:1	0	10.93 ± 4.14^{ef}	5.68 ± 1.19^{a}	7.61 ± 2.01^{f}
A_M	3:1	0	61.83 ± 5.27^{b}	5.22 ± 0.87^{a}	34.30 ± 3.76^{bc}
	3:1	1	14.53 ± 5.13^{e}	3.08 ± 0.56^{cd}	16.54 ± 4.42^{e}
	3:1	5	3.28 ± 0.73^{g}	2.35 ± 0.16^{d}	3.51 ± 0.96^{g}
	3:1	10	3.87 ± 0.59^{g}	2.39 ± 0.33^{d}	5.03 ± 1.22^{fg}
A_L	1:1	0	37.27 ± 7.48^{c}	5.03 ± 1.08^{ab}	24.40 ± 5.92^{d}
A_M	1:1	0	67.43 ± 9.36^{ab}	4.38 ± 0.54^{abc}	46.11 ± 7.49^{ab}
	1:1	1	29.17 ± 2.85^{cd}	3.59 ± 0.61^{bcd}	29.07 ± 2.38^{c}
	1:1	5	15.63 ± 3.44^{e}	2.83 ± 0.74^{d}	17.99 ± 0.95^{e}
	1:1	10	11.51 ± 1.71^{ef}	3.65 ± 0.66^{bcd}	8.76 ± 1.24^{f}
A_L	1:3	0	30.81 ± 2.97^{cd}	5.38 ± 1.39^{a}	28.09 ± 2.60^{cd}
A_M	1:3	0	74.09 ± 6.40^{a}	3.23 ± 0.42^{cd}	51.92 ± 7.37^{a}
	1:3	1	34.34 ± 3.68^{cd}	3.06 ± 0.87^{cd}	24.28 ± 5.10^{de}
	1:3	5	27.06 ± 1.78^{d}	2.49 ± 0.30^{d}	25.81 ± 3.07^{d}
	1:3	10	7.82 ± 0.77^{f}	3.76 ± 0.62^{bcd}	5.61 ± 0.76^{fg}

Average ± standard deviation of experimental determinations. Averages with the same letter, in the same column, indicate no significant differences ($p < 0.05$) by the Tukey test.

In general, the elongation at break also decreased in porous membranes. The results obtained here are considered low, especially for applications in regions of the body that require greater elasticity, such as joints. Bellini *et al.*[46] also observed low mechanical resistance in chitosan–xanthan (1:1) membranes prepared with Pluronic F68 (0.75%, w/w), reporting values of elongation at break of about 1.25 MPa and 2%, respectively. To overcome this drawback, studies on the incorporation of biocompatible plasticizers and, also, on the formation of blends with polymers that have high elongation properties should be intensified.

Behavior in Aqueous Media

The results obtained for absorption and mass loss of the membranes in water, PBS, and culture medium are shown in Figure 2.

When comparing the dense membranes prepared with alginate of different viscosities, it is possible to notice that membranes prepared with medium-viscosity alginate (A_M-0%P) showed lower absorption and greater stability in water compared to membranes prepared with low viscosity alginate (A_L-0%P). The membranes containing Poloxamer 188 showed higher absorption capacity than the dense membranes because the pores facilitate the liquid penetration process in the polymeric matrix.

It was not possible to determine the absorption capacity in PBS for the membranes prepared with alginate:chitosan proportions equal to 3:1 (Figure 2A) because they disintegrated after 24 h of contact with the medium as a result of the chelation process of the calcium ions. In membranes containing higher levels of alginate, the PECs are probably embedded in free alginate chains crosslinked with calcium ions, resulting in a greater exchange through chelation. On evaluating the absorption kinetics in PBS for chitosan and alginate membranes prepared with different proportions of the biopolymers, Verma *et al.*[23] observed that the membranes containing 70 and 65% of alginate absorbed significantly larger amounts of PBS than the membranes with higher proportions of chitosan. After 6 h of immersion, there was a reduction in mass gain, which the authors attributed to the dissolution of the membranes in the medium.

Although the culture medium α-MEM also contains sodium salts, the 3:1 membranes remained more stable in it, with absorption ranging from 6.58 to 18.68 g/g and mass loss ranging from 26.4 to 59%. The increased stability in the culture medium is related to its low amount of sodium salts, about 1%, which is probably insufficient to promote the membrane disintegration as occurs with PBS (about 10% of sodium salts). The amount of salts in culture media is similar to that found in simulated body fluid. Therefore, it can be assumed that A_i:C membrane formulations at 3:1 would maintain adequate stability when in contact with wound exudates, for example.

The absorption of PBS and culture medium by the membranes and the loss of mass in these media decreased with increase in the proportion of chitosan in the formulation. It is assumed, therefore, that the intensity of the interaction of the alginate carboxyl groups with the chitosan amines and with the calcium ions introduced in the reticulation steps was strong enough to stabilize the structure of the matrix on exposure to saline aqueous solutions.

Given that calcium ions were provided in quantities possibly more than enough to bind the carboxyl groups of alginate not bound to chitosan during the two reticulation steps, it may be considered that the observed differences in behavior are majorly determined by the distinct membranes formulations.

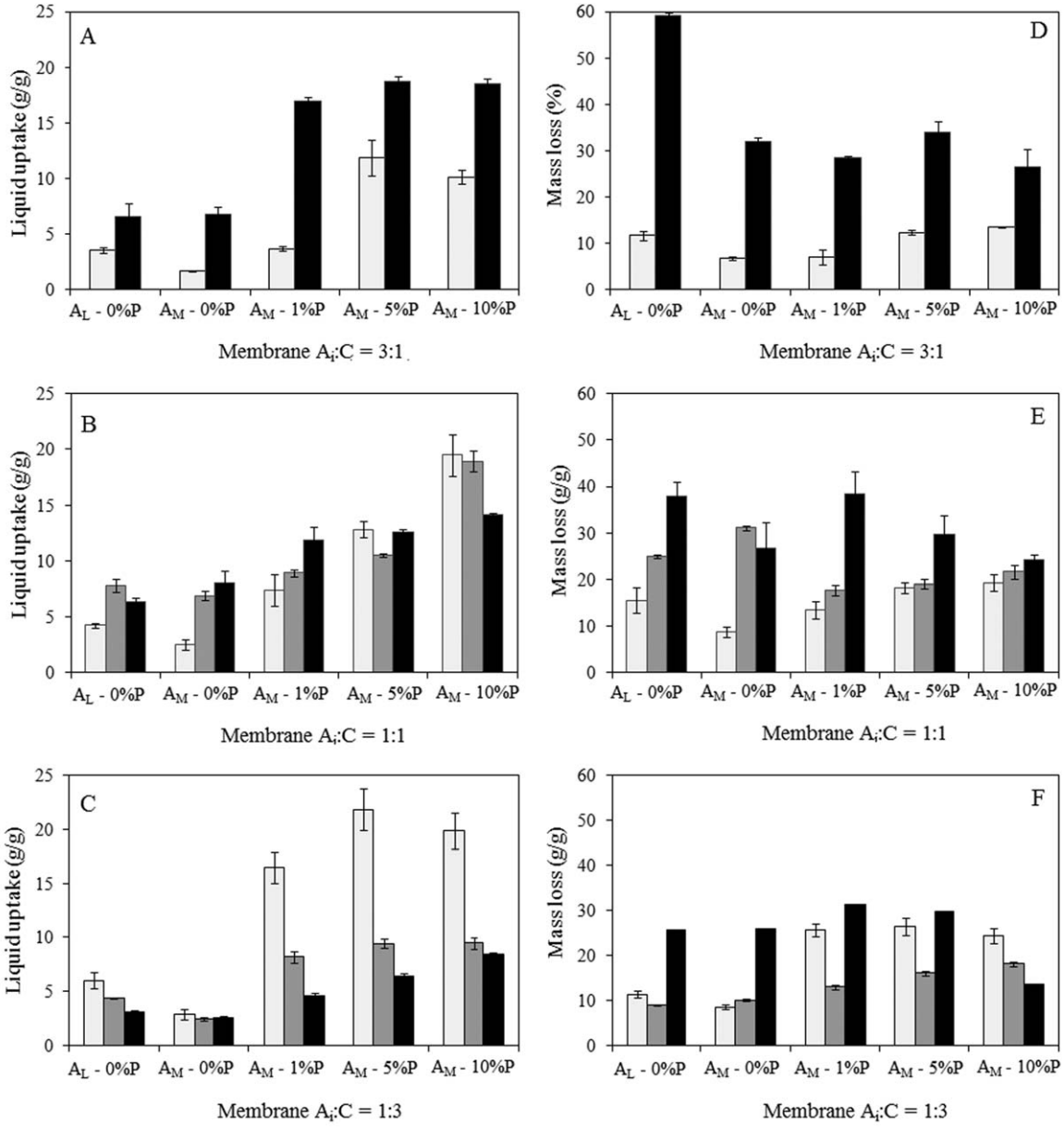

Figure 2. Liquid uptake of the alginate:chitosan membranes prepared at the mass proportions of 3:1 (A), 1:1 (B), and 1:3 (C) and mass loss of the membranes at A_i:C mass ratios equal to 3:1 (D), 1:1 (E), and 1:3 (F) analyzed in deionized water (□), phosphate-buffered saline (■), and α-MEM culture medium containing 10% fetal bovine serum (■).

Thermogravimetric Analysis

The thermal degradation of the membranes obtained in the absence and presence of different amounts of Poloxamer 188 was evaluated by TGA. Figure 3 shows the evolution profiles of the derivative weight loss as a function of temperature for pristine reagents and for membranes. The results regarding the main thermal events observed are summarized in Tables IV and V, respectively.

For pristine chitosan and alginate (Figure 3A), two steps of mass loss can be noticed: the first (DTG_1), at temperatures below 100 °C, is related to water loss, while the following thermal event (DTG_2), above 240 °C, is associated with the degradation of the polymers. The degradation of the chitosan powder occurred at 329.5 °C (Table IV), a temperature related to the depolymerization and pyrolitic decomposition of the polysaccharide. Similar data were observed by Ferfera-Harrar *et al.*[47] (298 °C) and by Neto *et al.*[48] (297.3 °C). For low- and medium-viscosity alginate, the degradation peak occurred at 246 °C (Figure 3A), a temperature associated with degradation by dehydration of the saccharide rings, disruption of the C–H bonds, and breakage of the glycosidic C–O–C bonds in the main chain of alginate.[49] Several authors have reported similar results for thermal degradation of alginate, e.g., Sarmento *et al.*[50] (247.8 °C) and Siddaramaiah *et al.*[51] (235 °C). The thermogravimetric curve of Poloxamer 188 (Figure 3A) shows only one degradation step, at 400 °C, agreeing with the results reported by Bueno *et al.*[52]

The data in Table V indicate that the degradation temperatures of the A_L:C dense membranes (Figure 3B) and A_M:C dense membranes (Figure 3C–E) are intermediate to the degradation

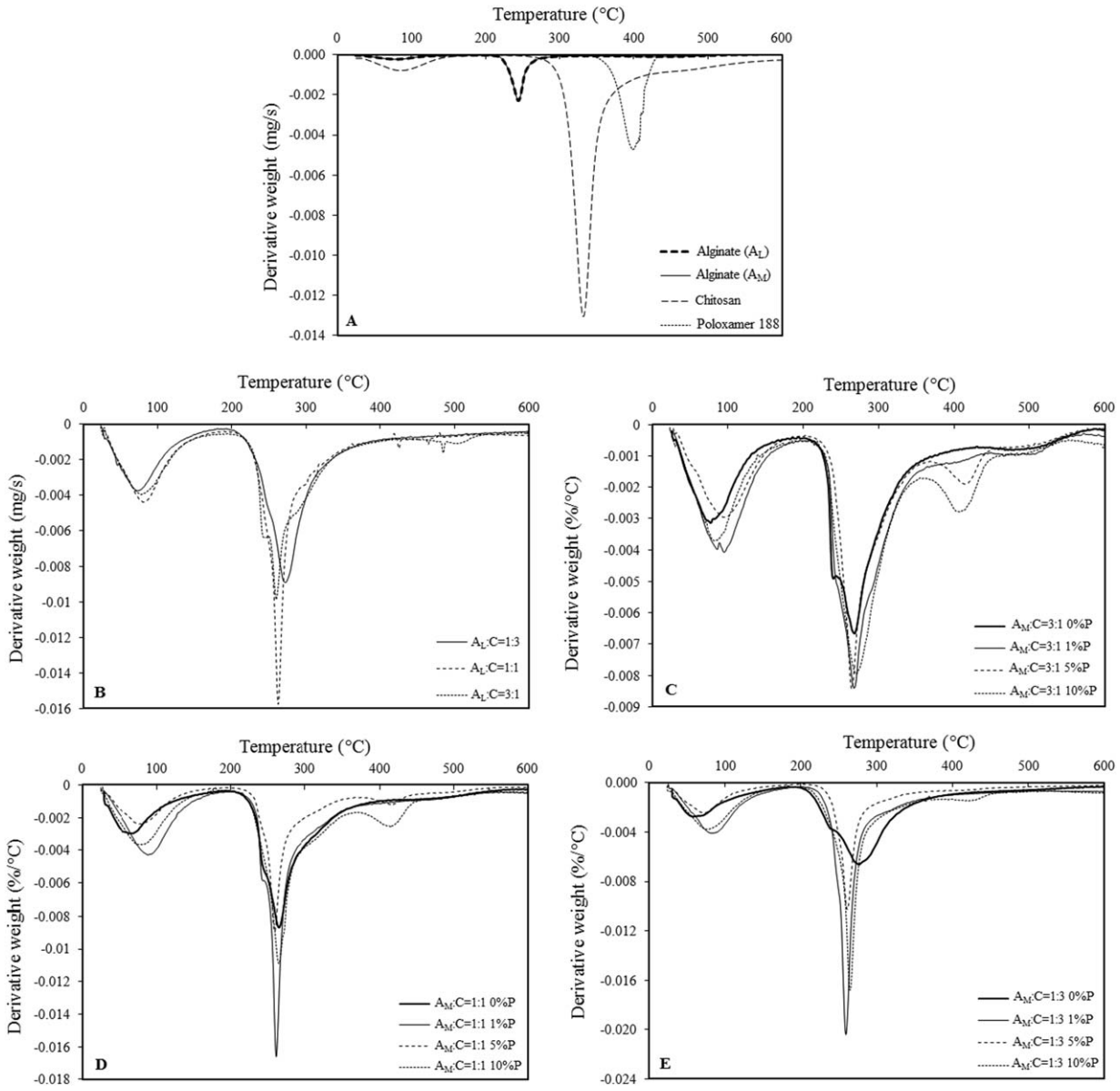

Figure 3. Thermogravimetric curves of pristine biopolymers and Poloxamer 188 (A), A_L:C dense membranes (B), and A_M:C dense and porous membranes prepared at the initial polysaccharide ratios of 3:1 (C), 1:1 (D), and 1:3 (E).

temperatures of pristine polymers (Figure 3A). As a general tendency, the degradation temperature has shifted to lower values for the membranes containing more alginate (A_L:C and A_M:C equal to 3:1) and to higher values for the samples containing the highest proportion of chitosan (A_L:C and A_M:C equal to 1:3).

The presence of a single degradation step in the temperature range from 246 to 300 °C can be considered as evidence of the formation of a PEC, according to Smitha *et al.*[53] These authors observed a single degradation peak around 260 °C for the chitosan–alginate complex. Anbinder *et al.*[54] also reported that alginate capsules coated with chitosan and crosslinked with calcium ions showed a single degradation step at 250 °C.

A third less relevant thermal event (DTG_3) was detected for the 1:1 and 3:1 membranes containing 5 and 10% of Poloxamer 188 (Figure 3C,D), with peaks occurring between 409 and 413 °C, which are associated with the degradation of the surfactant. Therefore, it can be assumed that the formation of thicker and more porous structures favors the retention of Poloxamer

Table IV. Main Thermogravimetric Events of the Pristine Reagents

Reagents	DTG_1 (°C)	DTG_2 (°C)	Weight loss at 100 °C (%)
Chitosan	76.2	329.5	8.95
Alginate A_L	71.5	246.1	8.94
Alginate A_M	70.9	246.0	8.68
Poloxamer 188	—	400	0.40

Table V. Main Thermogravimetric Events of the Alginate–Chitosan Membranes Prepared in the Presence or Absence of Poloxamer 188

Membrane formulation						
Alginate type	A_i:C mass proportion	Poloxamer (%)	DTG_1 (°C)	DTG_2 (°C)	DTG_3 (°C)	Weight loss at 100 °C (%)
A_L	3:1	0	70.9	258.9	—	12.2
A_M	3:1	0	78.3	262.9	—	11.8
	3:1	1	89.2	267.2	—	10.9
	3:1	5	77.4	259.1	412.7	9.1
	3:1	10	80.3	269.8	409.1	10.7
A_L	1:1	0	73.2	262.7	—	12.6
A_M	1:1	0	63.4	264.6	—	11.4
	1:1	1	90.2	260.8	—	11.2
	1:1	5	77.5	259.2	411.8	12.3
	1:1	10	73.6	262.3	413.3	11.6
A_L	1:3	0	66.0	269.3	—	12.4
A_M	1:3	0	58.6	273.1	—	10.8
	1:3	1	81.0	259.9	—	11.5
	1:3	5	67.1	261.4	—	12.7
	1:3	10	70.8	264.6	—	12.1

188 in the polymeric matrix, even after washing. For the 1:3 membranes prepared in the presence of 5 and 10% of Poloxamer 188, in which pore formation was less pronounced, the surfactant may have been removed more efficiently during the washing steps.

CONCLUSIONS

In this study, the characteristics of dense and porous alginate–chitosan membranes, prepared using alginate of two different viscosities and at different A_i to C mass proportions, were evaluated. The dense membranes showed adequate aspect and homogeneous structure. Membranes prepared with medium-viscosity alginate had higher TS, YM, and lower WVP and liquid uptake than membranes with low viscosity.

Membranes prepared with low-viscosity alginate and Poloxamer 188 were not stable in the evaluated conditions. The addition of the surfactant Poloxamer 188 efficiently promoted the formation of porous structures at all tested medium viscosity alginate to chitosan ratios, resulting in an increase in thickness, roughness, and water vapor transmission of the membranes. Compared to the values for the dense membranes, the TS of the porous membranes suffered reductions that ranged from 75% to 94%. The presence of pores was more pronounced in the membrane with an alginate:chitosan mass ratio of 3:1, probably due to the increased interaction with the surfactant and increased reticulation of the chains. These porous membranes showed the highest roughness, opacity, WVP, and the lowest TS and stability in saline media.

In general, formulations containing higher proportions of medium-viscosity alginate enable the fabrication of dense and porous membranes with characteristics that are more suitable for tissue engineering applications.

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