Comparison of the Properties of Compacted and Porous Lamellar Chitosan–Xanthan Membranes as Dressings and Scaffolds for the Treatment of Skin Lesions

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Received 11 October 2011; Revised 19 December 2011; accepted 24 December 2011
DOI 10.1002/app.36693
Published online in Wiley Online Library (wileyonlinelibrary.com).

ABSTRACT: Compacted and porous lamellar membranes of xanthan (Xn) and chitosan (Ch) at mass ratios of 1 : 1 and 1.2 : 0.8 were prepared and tested to verify possible applications in the treatment of skin lesions. All membranes were prepared by complexation of the polysaccharides in solution and subsequent casting. The porous membranes were obtained by adding either Tween 80 or Pluronic F68 to the polysaccharide complexes before casting. Membranes prepared in the absence of surfactants at a mass ratio of Xn to Ch of 1 : 1 proved ideal for use as wound dressings, as they were thin (around 0.10 mm in thickness) and transparent and had low *in vitro* cytotoxicity to L929 cells, a tensile strength at break of 25 MPa, water absorption after 24 h of around 86 g/g and simulated body fluid absorption of 16% and adequate stability in the presence of the same solutions. Membranes prepared at the mass ratio of Xn to Ch of 1 : 1 in the presence of Pluronic F68 showed the most favorable characteristics for application as scaffolds for tissue engineering. These membranes consisted of a matrix with interconnected pores which were distributed homogeneously throughout the structure and had a thickness of 1.84 mm, high capacity for FBS uptake (around 18 g/g) and cell culture medium uptake (8.6 g/g), a loss of mass in the culture medium of 33% after 144 h, and low *in vitro* toxicity to L929 cells. In conclusion, membranes of Ch and Xn produced in the presence or absence of the surfactant Pluronic F68 have a high potential for use as scaffolds in tissue engineering or as dermal dressings, respectively, whereas in contrast, membranes prepared in the presence of Tween 80, regardless of the mass ratio of Xn to Ch, were very cytotoxic to L929 cells and therefore were not appropriate for any of the proposed applications. © 2012 Wiley Periodicals, Inc. J Appl Polym Sci 000: 000–000, 2012

Key words: chitosan; xanthan; membrane; wound dressing; scaffold; tissue engineering

INTRODUCTION

The largest organ of the human body, the skin, constitutes the first barrier of defense, playing an important role in homeostasis and regulation of body temperature and providing protection from dehydration and support to blood vessels and nerves.[1,2] Extensive and deep skin lesions can cause the destruction of the dermis and dermal elements, resulting in a slow repair process prone to complications.[3,4]

A current solution to this problem consists in the transplantation of human skin grafts, whether or not autologous, which accommodate the connective tissue and stimulate the development of blood vessels.[5,6] However, this solution is limited by the scarcity of donors and always involves a considerable risk of rejection. Because skin grafts are, in general, degraded prematurely,[6] they normally work only as temporary replacements, but while covering the lesion, these devices are capable of reducing fluid loss and the occurrence of infections, allowing for improved patient outcomes and reduced length of stay in hospitals.

Currently, the dressings used in this type of injury primarily seek to protect the injured region. However, an ideal dressing should have a set of relevant properties,[7] such as the ability to prevent secondary infections, provide a moist microenvironment for wound regeneration and provide thermal insulation. In addition, it should be able to absorb the exudate present in the lesion, be free of particles and toxins and stimulate tissue reconstruction. In the case that its removal is necessary, this should be done without causing damage to the recovering tissue.

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Contract grant sponsors: Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)

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Different types of polymeric materials of natural origin or synthetic have been used in the production of dressings and scaffolds useful as structural templates to direct cell growth.[7] Properties such as the biocompatibility of the material, its mechanical strength, its fluid absorption capacity, and the size and shape of its pores are of great importance in the selection of the raw material used for the production of scaffolds or dressings.[8] Compounds such as poly (methyl methacrylate), polyurethane,[2] cellulose and its derivatives,[7,9] dextran,[10] and alginate[10–13] are currently used in clinical medicine, and the polysaccharide chitosan (Ch) has been increasingly evaluated for the purpose of skin tissue regeneration.[14,15]

Ch is a polysaccharide derived from chitin which is composed of two monomers, D-glucosamine and N-acetyl-D-glucosamine.[15] Features such as high biocompatibility, biodegradability, and properties of absorption and adsorption,[16] coupled with its ability to speed healing and its antimicrobial activity[1,3] turns possible its use in the production of dermal dressings and scaffolds. The polycationic nature of Ch when in acid solutions allows its use in the preparation of polyelectrolyte complexes (PECs) with polyanions such as alginate.[1,10–12] dextran,[17] carboxymethyldextran,[18] heparin,[19] xanthan (Xn),[20,21] carrageenan, and pectin.[21] In the presence of Xn gum, as an example, Ch forms a complex insoluble in water through interactions between the protonated amino groups of Ch and the Xn deprotonated carboxyl groups, creating a microsystem that favors the stabilization of molecules such as proteins.[20]

Xn gum is a high-molecular weight branched heteropolysaccharide containing the sugars D-glucose, D-mannose and D-glucuronic acid. It is produced by the bacterium *Xanthomonas campestris* with frequent application in food and pharmaceutical industries as suspension emulsifier and stabilizer and can be processed in the form of gels, films, and membranes.[22]

The Ch–Xn complex is already used for the immobilization of enzymes[23] and as a component of microspheres,[20] tablets,[24] capsules,[25] and membranes[21]; however, no reports were found on obtaining porous devices from xanthan or on the evaluation of the cytotoxicity of membranes prepared from it in combination with the biocompatible and biodegradable polymer Ch.

Porous biomaterials are usually produced by freeze-drying methods, an expensive, laborious and not usually easily up-scalable technique[26] or by adding porogenic agents such as glucose or NaCl.[27] Other methods such as phase inversion, high-pressure gasification, and electrospinning processes are also used.[28] Alternatively, it is possible to obtain porous polysaccharide membranes by incorporation of surfactants such as Tween 80 and Pluronic F68[26] into the polymer mixture. The addition of these surfactants to PECs promotes the formation of pores and also facilitates polymer dispersion, improving the system homogeneity, as described by Bueno and Moraes[26] for Ch–alginate membranes. Because both surfactants belong to the class of nonionic surfactants, which are considered less harmful to the skin,[29] they are currently used as additives in cosmetics and in the preparation of emulsions for topical application.

Taking into account all the above considerations, the purpose of this study was to obtain membranes by complexing Ch and Xn at different mass ratios in the presence or absence of the surfactants Pluronic F68 and Tween 80 and to analyze their potential use as dressings and scaffolds for tissue engineering, respectively, with application in the treatment of skin lesions. Further, the fact that Xn is a branched polysaccharide might enable the formation of membranes with a structure different from those obtained by combining alginate, a linear compound, with Ch.

MATERIALS AND METHODS

Materials

Ch–Xn membranes were produced using 96% deacetylated Ch (lot number 9012-76-4, Sigma–Aldrich, St. Louis, MO), Xn gum (lot number 056K0007) and Pluronic F68 (Sigma Chemical, St. Louis, MO), Tween 80 (Synth-São Paulo, Brazil), and acetic acid (Merck-São Paulo, Brazil). The water used was distilled and deionized in a Millipore MilliQ system. All other reagents used were of analytical grade.

Membrane preparation

The Xn and Ch complex was prepared based on procedures described by Veiga and Moraes[21] and Bueno and Moraes.[26] Two different mass ratios of Xn to Ch, 1 : 1 and 1.2 : 0.8, were used varying the volume ratio of aqueous solutions of Ch at 1% (w/w) in 2% (v/v) acetic acid and Xn at 1% (w/w), totaling 200 mL final volume of mixture with 2 g of polysaccharides in the presence or absence of the surfactants Pluronic F68 (at 0.75%, w/w) or Tween 80 (at 0.75%, v/w). The Ch solution was added using a peristaltic pump (model TE 184, Tecnal) at a 10 mL/min flow rate to the Xn solution with the presence or absence of surfactant, under a constant agitation of 1000 rpm using a mechanical stirrer (model TE 038, Tecnal). The temperature was maintained at 25°C in a jacketed glass reactor with an internal diameter of 11 cm and a height of 12 cm during all preparation steps. The suspension obtained was degassed for 2 h using a vacuum pump (model TE 058, Tecnal) to eliminate air bubbles, transferred to a polystyrene Petri dish (15 cm in diameter) and dried in an oven with air

circulation (model TE 394/1, Tecnal) at 37°C for about 24 h. The degassing step was not used for production of membranes in the presence of surfactants and drying time was about 48 h.

For removal of the residual acetic acid, the dried membranes were immersed in 500 mL of deionized water for 30 min, and this process was repeated three times. Then the samples were immersed in 200 mL of 10 mM Hepes buffer for 30 min and again in 500 mL of water for 30 min. Finally the membranes were dried in an oven with air circulation at 37°C for 24 h.

The membranes were cut into appropriate sizes and sterilized with ethylene oxide (EO) by exposure to Oxyfume-30 (30% EO and 70% carbon dioxide) for 8 h at 40°C and a relative humidity of 30–80% at Acecil Central de Esterilização Comércio e Indústria (Campinas, SP, Brazil). The residual EO was removed by keeping the samples under aeration for 48 h.

Membrane characterization

The samples were characterized according to morphology, thickness, mechanical strength, absorption of aqueous solutions and stability in the same solutions, and cytotoxicity *in vitro* as described below.

Morphology

The morphology of the surface and of the cross section of the membranes was examined using a scanning electron microscope (Leo 440i model, Leica). Samples of 2 cm × 1 cm were lyophilized, coupled in suitable holders and coated with gold (Sputter coater mini, SC 7620) before the analysis.

Thickness

The thickness of the membranes was measured using a micrometer (Digimess) at seven different positions near the edge of the membrane. The results were expressed as means.

Solution uptake and stability in physiological media

The degree of absorption of different aqueous solutions by the membranes was evaluated based on the method proposed by Rodrigues et al.[1] Dried samples (1 cm × 6 cm) of known weight (W_{dry}) were immersed in 7 mL of water, 0.9% NaCl (w/v) saline solution (SS), simulated body fluid (SBF) prepared according to Kokubo et al.[30] and RPMI culture medium supplemented with 0.3 g/L L-glutamine, 2 g/L D-glucose, 2 g/L $NaHCO_3$, 10,000 IU/L penicillin, 0.05 g/L streptomycin, 5.958 g/L Hepes, and 10% (v/v) fetal bovine serum (Nutricell, Brazil). The weight of the hydrated samples (W_{wet}) in water, SS, and SBF was determined after 24 h and in supplemented RPMI medium, after 144 h, and the amount of absorbed solution (S) was calculated using eq. (1).

$$S = \frac{W_{wet} - W_{dry}}{W_{dry}} \quad (1)$$

Each wet sample was dried at 37°C for 10 h and its final weight (W_{final}) was measured to determine sample stability in each solution (L), described with respect to its loss of mass according to eq. (2):

$$L = \frac{(W_{dry} + W_{SOL}) - W_{final}}{W_{dry}} \times 100 \quad (2)$$

where W_{SOL} refers to the equivalent mass of solids in the solutions incorporated by the samples, considering that the concentration of solids in SS, SBF, and supplemented RPMI medium was 9.0 g/L, 15.3 g/L, and 19.3 g/L, respectively. All experiments were performed in at least duplicate.

Mechanical properties of membranes

The tensile mechanical properties of the membranes were evaluated based on ASTM D-882[31] employing eight independent test samples for each formulation (2.54 cm × 10 cm) in a texturometer (TA.XT2 model, Stable Micro System SMD, England), using a cell load of 5 kgf, a gauge length of 5 cm and a crosshead speed of 1 cm/s. The tension (T) and the elongation (E) at break were calculated using eqs. (3) and (4), respectively:

$$T_{(MPa)} = \frac{F_m}{A_S} \quad (3)$$

$$E = \frac{d_{final}}{d_{initial}} \quad (4)$$

where F_m is the maximum force at breaking in N, A_S is the cross-sectional area of the sample in mm^2 (thickness × width), and d is the gauge length at the beginning and end of analysis.

In vitro cytotoxicity

The indirect and direct toxicity of the membranes to cells in culture was evaluated using nearly confluent monolayer L929 fibroblasts cultivated in supplemented RPMI medium.

The indirect *in vitro* cytotoxicity was tested using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, from Sigma Chemical) assay, which is based on analysis of the mitochondrial

activity of cells exposed to extracts of the samples. The extracts of the membranes were obtained by incubating the samples in supplemented RPMI culture medium at a concentration of 0.05 g of dry material per milliliter of medium for 48 h at 37°C and a 5% CO_2 atmosphere. The cells were initially seeded (100 μL) in 96-well flat-bottom plates (TPP) at a concentration of 1×10^5 cells/mL in supplemented RPMI medium and maintained at 37°C and 5% CO_2 for 24 h. Then the medium was removed, the membrane extracts were added (100 μL), and the cells were incubated under the same conditions for a further 24-h period. After that, the extracts were removed, the wells were washed twice with 100 μL of phosphate buffered saline (PBS)/ethylenediaminetetraacetic acid (EDTA) and 100 μL of supplemented RPMI medium, followed by 10 μL of MTT at 5mg/mL in PBS/EDTA buffer, were added to each well. After 4 h of incubation at 37°C, 100 μL of sodium dodecyl sulfate (Sigma Chemical) solution at 100 g/L dissolved in dimethyl sulfoxide (Sigma Chemical) containing 0.6% acetic acid (Merck) were added to each well. The samples were gently homogenized and the cultures returned to incubation at 37°C for 1 h. After this period, the absorbance of the samples at 620 nm was analyzed in a microplate photometer (Thermo Scientific Multiskan FC). Extracts of culture plate fragments and latex (in the form of yellowish tourniquets regularly used for blood collection) were used as negative and positive controls, respectively, and the culture medium without cells was used as a blank. The experiments were performed in at least quintuplicate.

The direct *in vitro* cytotoxicity to L929 cells in culture was analyzed by directly exposing the cells to hydrated membranes and afterwards assessing cell presence by the use of crystal violet (Vetec) solution. Cells in supplemented RPMI medium were then initially seeded (2mL) in flat-bottom 6-well plates of (TPP) at a concentration of 1×10^5 cells/mL and incubated at 37°C and 5% CO_2 for 24 h to form a cell monolayer. After this period, the culture medium was removed and circular membrane samples 1.5 cm in diameter and equilibrated overnight with the culture medium at 37°C and 5% CO_2 were placed on top of the cell layers together with 1.5 mL of fresh supplemented culture medium. After 24 h at 37°C and 5% CO_2, the medium was removed, and the membrane samples were gently taken off the top of the monolayers. The wells were then washed with 1 mL of PBS/EDTA, and five drops of crystal violet at 2% in 20% ethanol were added. After 1–2 min, the dye solution was aspirated, the cell monolayer was washed twice with 1 mL of deionized water, and the plates were dried at room temperature for at least 10 min. Then the aspect of the monolayers was examined under an inverted microscope (Nikon eclipse TS100) by assessing the area that had been in contact with the material and the surrounding area, determining the percentage of affected cells. Cells cultured in the absence of membranes were used as the negative cytotoxicity control, whereas cells cultivated in the presence of latex were considered the positive control. All experiments were performed in at least triplicate.

Estimation of cell proliferation

In this test, the fibroblasts were inoculated directly into the membranes, and cell growth was monitored for 72 h. Aliquots of 50 μL of a suspension containing 1×10^5 cells/mL were seeded on the surface of membranes 1.5 cm in diameter, previously swollen in medium as described above, and arranged on 24-well flat-bottom plates (TPP). Cells inoculated directly onto the wells without samples were defined as positive growth controls, and conversely, cells cultured over 1.5 cm in diameter latex samples were considered as negative. The cells were incubated at 37°C and 5% CO_2 for 2 h to assure cell adhesion and after this period, 950 μL of supplemented RPMI medium were added. The cells were further incubated for 72 h, and every 24 h samples were taken in triplicate and analyzed to determine cell concentration and viability using trypan blue dye (Sigma Chemical) after trypsinization.

RESULTS AND DISCUSSION

Aspect and morphology of the membranes

The Ch–Xn PEC suspension prepared without the addition of surfactants and degassed was relatively homogeneous and translucent and had a high viscosity. The PEC suspension obtained in the presence of Tween 80 and Pluronic F68, on the other hand, had the aspect of a white, opaque, dense, and homogeneous foam with small air bubbles uniformly distributed throughout the mixture. After drying, the formulations became lamellar and porous membranes, whose typical feature can be seen in Figure 1.

The membranes obtained in the absence of surfactant and employing vacuum degassing had no apparent pores on their surfaces [Fig. 1(a,d)] and showed good transparency in both wet and dry states, as observed by Rodrigues et al.[1] for alginate and Ch membranes and by Veiga and Moraes[21] for Xn and Ch membranes. Transparency is a feature of great importance for their application as a dermal dressing, because such a material may permit the observation of the wound bed during recovery of the skin without dressing removal.

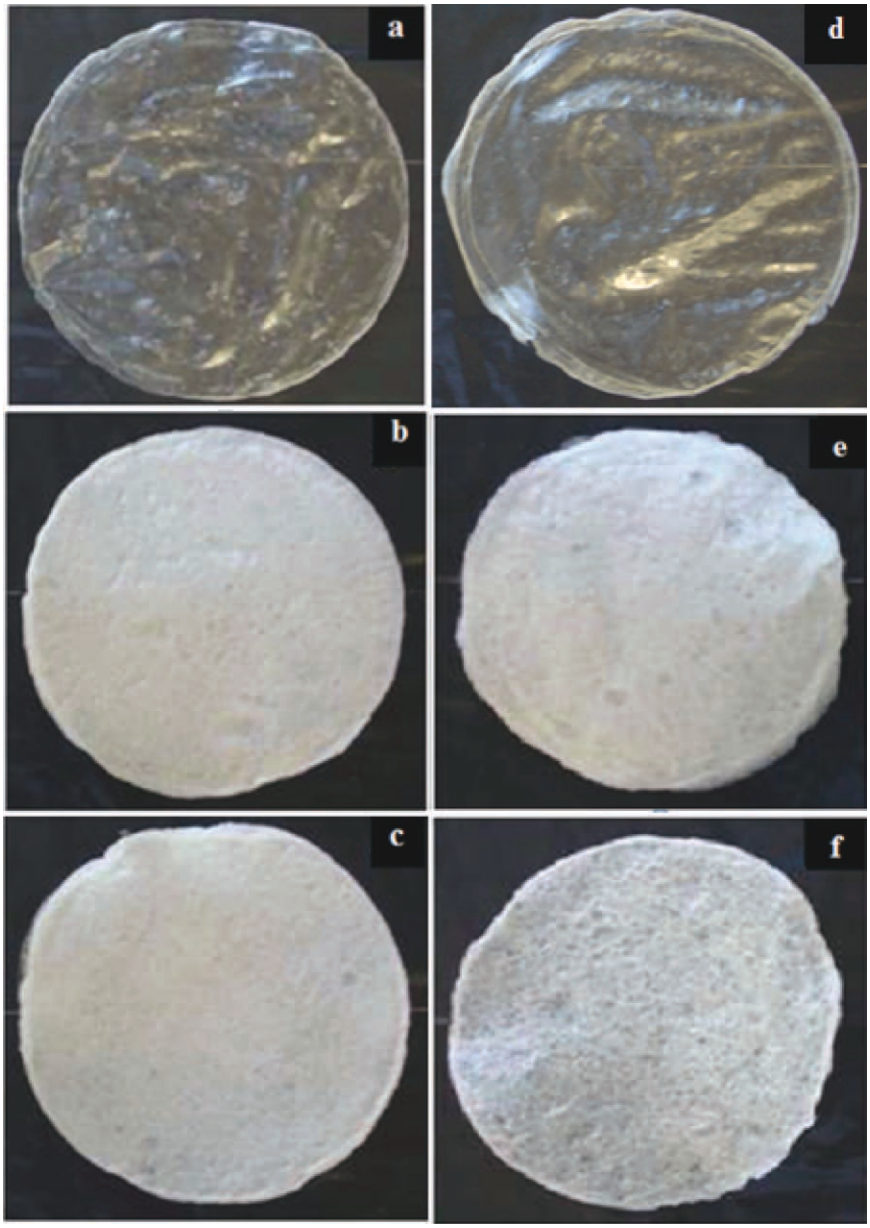

Figure 1 Visual aspect of membranes prepared at (a) mass ratio of Xn to Ch of 1 : 1 in the absence of surfactants; (b) mass ratio of Xn to Ch of 1 : 1 in the presence of Tween 80; (c) mass ratio of Xn to Ch of 1 : 1 in the presence of Pluronic F68; (d) mass ratio of Xn to Ch of 1.2 : 0.8 in the absence of surfactants; (e) mass ratio of Xn to Ch of 1.2 : 0.8 in the presence of Tween 80; and (f) mass ratio of Xn to Ch of 1.2 : 0.8 in the presence of Pluronic F68 [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com].

The membranes prepared in the presence of surfactants, on the other hand, were highly opaque, with a large number of uniformly distributed pores [Fig. 1(b,c,e,f)], an important characteristic for their application as scaffolds for tissue engineering.

Typical scanning electron microscopy analyses showed the presence of polysaccharide fibers in all samples, as shown in Figure 2. Samples prepared without the addition of surfactants [Fig. 2(a,d)] showed a lamellar aspect, compact structure and no pores on the surface even at high magnification, in accordance with the description of Veiga and Moraes.[21] The attribute of integrity of the surface layer is of fundamental importance in materials intended for use as barriers to microbial penetration in skin lesions.

A difference in polymeric matrix compaction is clearly observed in the membranes prepared in the presence of the surfactants. The presence of interconnecting pores with diameters between 90 and 300 μm distributed in the lamellar structure is also observed [Fig. 2(b,c,e,f)], similarly to what was reported by Bueno and Moraes[26] for alginate and Ch membranes, despite the fact that Xn has a branched

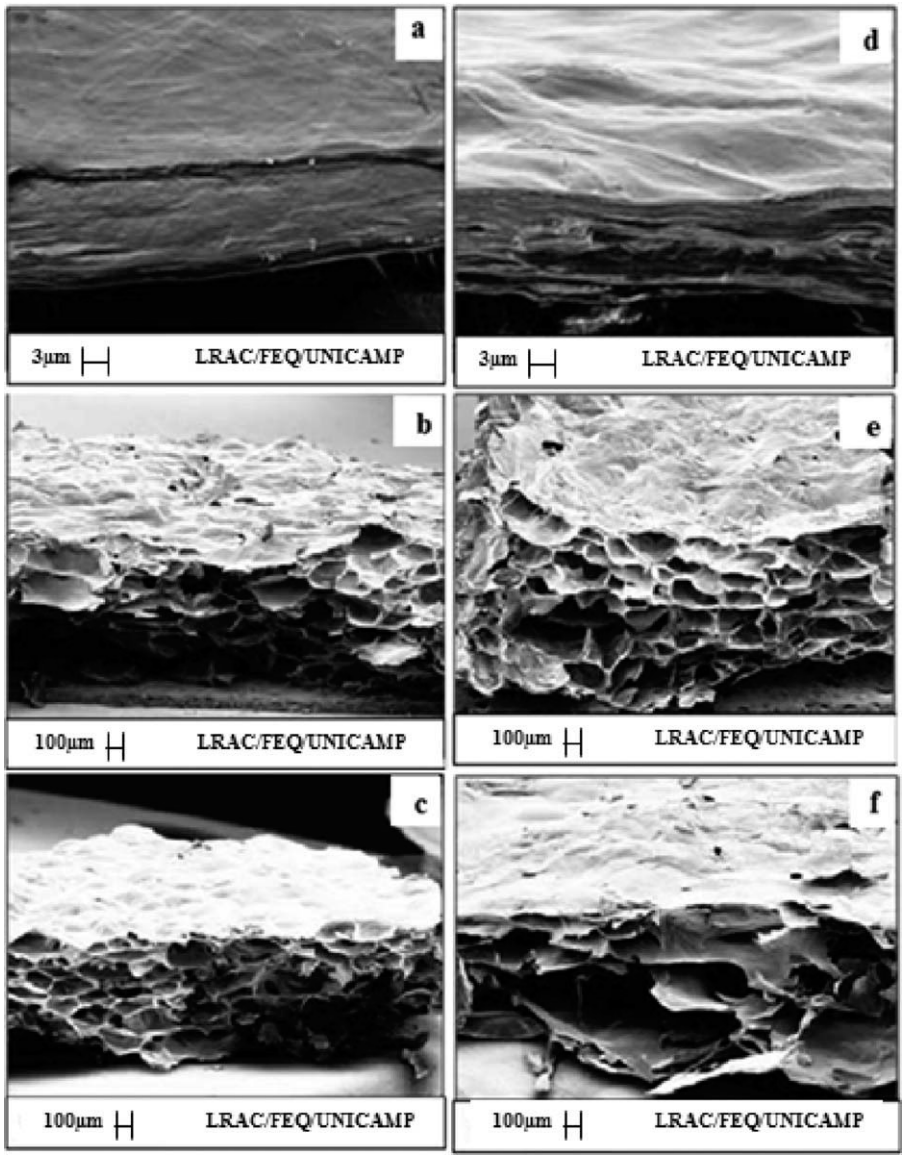

Figure 2 Electron scanning micrographs of membranes prepared at (a) mass ratio of Xn to Ch of 1 : 1 in the absence of surfactants; (b) mass ratio of Xn to Ch of 1 : 1 in the presence of Tween 80; (c) mass ratio of Xn to Ch of 1 : 1 in the presence of Pluronic F68; (d) mass ratio of Xn to Ch of 1.2 : 0.8 in the absence of surfactants; (e) mass ratio of Xn to Ch of 1.2 : 0.8 in the presence of Tween 80; and (f) mass ratio of Xn to Ch of 1.2 : 0.8 in the presence of Pluronic F68.

molecular structure and alginate has a linear one. An extensive network of interconnected pores in the structure of a scaffold is an ideal characteristic for cell migration, adhesion, and proliferation, facilitating the transport of oxygen and nutrients to the innermost portions of the support.[32,33] Given that Xn is a polysaccharide obtained by bacterial fermentation, and alginate is a product extracted from algae, the use of the former could be advantageous in tissue engineering, allowing improved quality control of the materials employed to obtain the scaffold.

No significant differences in the morphology of membranes prepared using different proportions of Xn and Ch or varying the type of surfactant used for the formation of pores were detected.

Membrane thickness

The average thickness of dry membranes was determined, and the values are presented in Table I. Membranes prepared in the absence of surfactants and including the degassing step were shown to be thinner, with thicknesses ranging between 0.10 and 0.13 mm. No statistically significant difference was observed for the membranes prepared in different polysaccharide proportions. Biomaterials with applications as dermal substitutes should ideally have a thickness lower than the human dermis,[27] which varies from 0.5 to 2.0 mm, depending on age and sex as well as the region of the body. Thus, the materials obtained in this work could potentially be used as dermal dressings.

TABLE I
Thickness of Dry Membranes Prepared at Different Mass Ratios of Xanthan to Chitosan in the Presence or Absence of Either of the Surfactants, Tween 80, or Pluronic F68

Ratio of Xn to Ch	Type of surfactant	Thickness (mm)
1 : 1	—	0.10 ± 0.04[a]
1 : 1	Tween 80	2.49 ± 0.24[b]
1 : 1	Pluronic F68	1.84 ± 0.23[c]
1.2 : 0.8	—	0.13 ± 0.03[a]
1.2 : 0.8	Tween 80	3.08 ± 0.37[d]
1.2 : 0.8	Pluronic F68	1.86 ± 0.15[c]

Different letters in the same column indicate significant difference at 95% confidence intervals (Tukey's test).

Membranes obtained in the presence of surfactants were thicker, ranging from 1.84 to 3.08 mm in thickness. These values are higher than those reported by Bueno and Moraes[26] for Ch–alginate membranes produced with the same surfactant (between 0.38 and 0.41 mm when dry), which can be explained by the lower concentration of polysaccharides and surfactants used by these authors. The values presented herein are comparable to those reported for other porous materials with applications in the tissue engineering area, such as collagen scaffolds (thickness of 1 mm[34]), Ch–alginate membranes obtained by freeze-drying[35] (average thickness of 2.5 mm) and porous polylactide–Ch membranes[36] (thickness of 4 mm). Therefore, the porous membranes produced in this work by the use of the tested surfactants are thick enough for application as supports for skin cell regeneration.

Mechanical properties of the membranes

Mechanical properties compatible with the injured tissue, such as tensile strength and elongation, are significant requirements for materials intended for tissue regeneration. The results obtained for the mechanical properties of the membranes prepared are shown in Table II.

Samples prepared in the absence of surfactants and including the degassing step had higher tensile strength, and the increase in the mass ratio of Xn to Ch from 1 : 1 to 1.2 : 0.8 significantly reduced this property from 25.12 MPa to 8.73 MPa. The increase in Xn concentration may have reduced the number of bonds between the polymers and thus the organization of crystalline areas, which favored the formation of amorphous areas, thus reducing the material's resistance to traction.

The tensile strength of Xn–Ch lamellar samples in comparison to that of normal skin, ranging between 2.1 and 16 MPa,[10] indicates their potential application as wound dressings. However, low values of elongation of between 1% and 2% were observed for these membranes, whereas the values for the skin were around 70%.[37] Thus, application of these materials in body regions with high mechanical requirements, such as knees and elbows, is not recommended.

The values of elongation at rupture of the membranes prepared in the presence of the surfactants Tween 80 and Pluronic F68 did not differ significantly. However, a large drop in tensile strength was noted for membranes prepared in the presence of any of the surfactants. The porous samples showed tensile strengths between 0.41 and 1.25 MPa, which was expected due to the introduction of air bubbles in to the material structure. Bueno and Moraes[26] also observed low mechanical resistance in Ch–alginate membranes prepared with the addition of the same surfactants, reporting tensile strength values in the same range (1.54 MPa for membranes prepared in the presence of Tween 80 and 0.98 MPa for those produced with Pluronic F68). Similarly, values ranging from 0.11 to 0.40 MPa were reported for Ch and silk fiber supports also applicable in the tissue engineering area.[38]

Membrane swelling and stability in physiological media

Values obtained for the uptake of water, 0.9% NaCl aqueous solution and SBF at 37°C for 24 h and also the mass loss of the membranes when exposed to these media are shown in Tables III and IV, respectively.

Membranes prepared with only Ch and Xn showed higher water uptake capacities (from 49 to 86 g H_2O/g dry membrane). These values are higher than those described in the literature for Ch–alginate[1] membranes, which range from 11 to 19 g H_2O/g dry membrane, and also for Ch–Xn[21] membranes (between 24 and 61 g H_2O/g dry membrane)

TABLE II
Mechanical Properties of the Membranes Prepared at Different Mass Ratios of Xanthan to Chitosan, in the Presence or Absence of Either of the Surfactants, Tween 80, or Pluronic F68

		Property	
Ratio of Xn to Ch	Type of surfactant	Tensile strength (Mpa)	Elongation at break (%)
1 : 1	—	25.12 ± 7.8[a]	2.02 ± 0.72[d]
1 : 1	Tween 80	0.53 ± 0.05[b]	2.10 ± 0.40[d]
1 : 1	Pluronic F68	1.25 ± 0.17[b]	2.13 ± 0.63[d]
1.2 : 0.8	—	8.73 ± 3.01[c]	1.05 ± 0.31[e]
1.2 : 0.8	Tween 80	0.41 ± 0.05[b]	1.82 ± 0.28[d]
1.2 : 0.8	Pluronic F68	1.22 ± 0.12[b]	1.71 ± 0.12[d,e]

Different letters in the same column indicate significant difference at 95% confidence intervals (Tukey's test).

TABLE III
Liquid Uptake After 24 h at 37°C in Physiological Solutions of Membranes Prepared at Different Mass Ratios of Xanthan to Chitosan in the Presence or Absence of Either of the Surfactants, Tween 80, or Pluronic F68

Ratio of Xn to Ch	Type of surfactant	Solution uptake (g/g)		
		Water	0.9% NaCl	SBF
1 : 1	—	$85.6 \pm 3.7^{a,A}$	$24.6 \pm 2.4^{d,B}$	$15.8 \pm 0.1^{f,C}$
1 : 1	Tween 80	$34.1 \pm 1.7^{b,E}$	$15.8 \pm 0.9^{e,F}$	$14.5 \pm 0.8^{f,F}$
1 : 1	Pluronic F68	$29.8 \pm 1.8^{b,H}$	$17.1 \pm 1.2^{e,I}$	$18.0 \pm 0.3^{g,I}$
1.2 : 0.8	—	$48.9 \pm 1.1^{c,K}$	$16.1 \pm 0.5^{e,L}$	$11.9 \pm 0.0^{h,M}$
1.2 : 0.8	Tween 80	$30.4 \pm 1.3^{b,O}$	$14.6 \pm 0.7^{e,P}$	$14.0 \pm 0.8^{f,P}$
1.2 : 0.8	Pluronic F68	$29.5 \pm 1.2^{b,R}$	$16.6 \pm 1.2^{e,S}$	$15.4 \pm 0.2^{f,S}$

Different lower case letters in the same column indicate significant difference at 95% confidence intervals (Tukey's test). Different capital letters in the same line indicate significant difference at 95% confidence intervals (Tukey's test).

prepared under similar conditions, but with a lower total polysaccharide content.

The higher water uptake observed for the membranes with a mass ratio of Xn to Ch of 1 : 1 can be explained by suboptimal bonding between the polymers, probably caused by an excess of amino groups of Ch with respect to the Xn carboxyl radicals, resulting in looser polymer networks capable of absorbing more water. Similar results were also observed by Macleod et al.[39] for membranes of pectin and Ch in different polymer proportions.

Still regarding water absorption, there was no significant variation among the results for solution uptake capacity of the membranes prepared in the presence of the different surfactants tested; however, significant lower absorption was noticed in 0.9% SS and in SBF for all formulations. According to Bueno and Moraes,[26] solutions with increased ionic strength would cause higher polysaccharide molecular packing, hindering water from penetrating the membrane structure and resulting in lower liquid uptake capacity.

Considering that the prevention of the buildup of exudates in the injury and also of its excessive dehydration are important characteristics required of biomaterials designed for skin regeneration,[40] adequate fluid absorption is important as a mean to control water loss through the skin. In addition, by estimating the potential tridimensional expansion of the samples based on the amount of liquid absorbed and on the fact that membrane length and width practically do not change (significant variation is observed only in membrane thickness), it could be inferred that these devices would be able to support a high number of cells in their structure.

When the results achieved are compared with others previously reported for porous materials, it is possible to observe that the absorption values found are higher than those described for Ch–alginate membranes[26] (up to 13.8 g/g in water, 12 g/g in 0.9% NaCl SS, and 7.7 g/g in SFB), for Ch/alginate spongy structures obtained by lyophilization[41] (about 12g/g in phosphate buffer at pH 7.4 after 10 min) and dressing sponges made of Ch–alginate[35] (17.5 g/g in water after 180 min immersion).

All membranes prepared in the absence of surfactants and the ones obtained in the presence of Tween 80 remained fairly stable when in contact with the aqueous solutions tested, with a maximum mass loss of 20% (Table IV). The lower mass losses were detected for the formulations free of surfactants, with no statistically significant differences resulting from the increase in proportion of Xn. The least stable formulations were the ones involving Pluronic F68 in the production process, losing up to 51% of their original mass in SBF. Although the

TABLE IV
Membrane Stability Referring to Variation in Mass After Exposure to Different Physiological Solutions for 24 h at 37°C

Ratio of Xn to Ch	Type of surfactant	Loss of mass (%)		
		Water	0.9% NaCl	SBF
1 : 1	—	$14.0 \pm 0.9^{a,c,A}$	$12.7 \pm 2.2^{e,g,A}$	$10.3 \pm 1.4^{i,A}$
1 : 1	Tween 80	$20.4 \pm 2.5^{b,d,B}$	$19.6 \pm 1.7^{f,B}$	$16.8 \pm 1.6^{j,B}$
1 : 1	Pluronic F68	$16.9 \pm 0.4^{b,c,C}$	$24.9 \pm 1.6^{f,C}$	$34.7 \pm 7.5^{k,C}$
1.2 : 0.8	—	$9.8 \pm 0.2^{a,D}$	$7.1 \pm 1.2^{e,D}$	$6.7 \pm 2.5^{i,D}$
1.2 : 0.8	Tween 80	$10.7 \pm 2.9^{a,E}$	$13.2 \pm 2.1^{f,g,E}$	$13.0 \pm 1.9^{j,E}$
1.2 : 0.8	Pluronic F68	$25.2 \pm 0.2^{d,F}$	$33.9 \pm 0.5^{h,G}$	$51.4 \pm 2.9^{l,H}$

Different lower case letters in the same column indicate significant difference at 95% confidence intervals (Tukey's test). Different capital letters in the same line indicate significant difference at 95% confidence intervals (Tukey's test).

TABLE V
Solution Uptake and Membrane Stability After Exposure to Supplemented RPMI Medium for 72 h at 37°C

Ratio of Xn to Ch	Type of surfactant	Solution uptake (g/g)	Loss of mass (%)
1 : 1	—	6.3 ± 1.8[a]	17.1 ± 3.2[d]
1 : 1	Tween 80	7.2 ± 0.4[a]	24.4 ± 0.4[e]
1 : 1	Pluronic F68	8.6 ± 0.4[b]	33.2 ± 1.3[f]
1.2 : 0.8	—	7.5 ± 0.3[a]	23.3 ± 2.7[e,g]
1.2 : 0.8	Tween 80	9.3 ± 0.6[b]	19.7 ± 1.5[d,g]
1.2 : 0.8	Pluronic F68	12.4 ± 1.2[c]	34.6 ± 0.6[f]

Different letters in the same column indicate significant difference at 95% confidence intervals (Tukey's test).

membranes prepared in the presence of Pluronic F68 were less stable when the mass of Xn used was higher than that of Ch, the opposite is observed for formulations obtained in the presence of Tween 80. Interestingly, the individual formulations showed the same behavior in the same solutions, except for that prepared at the mass ratio of Xn to Ch of 1.2 : 0.8 in the presence of Pluronic F68, which showed significantly higher mass loss in salt-containing solutions.

The behavior of membranes exposed to cell culture medium for longer periods (144 h) was evaluated aiming at analyzing their applicability as scaffolds, and the results are shown in Table V.

The membranes showed relatively high levels of absorption of cell culture medium, ranging between 6.3 and 12.4 g/g; however, these values are significantly lower than those verified for the absorption of water, 0.9% NaCl solution and SBF. Except for the 1.2 : 0.8 Xn–Ch formulation prepared in the presence of Pluronic F68, all other preparations showed mass losses higher in supplemented RPMI than in the other solutions; however, considering that a weight loss of up to 35% in 6 days of incubation could be of relevance if absorbable biomaterials were desired, all formulations could be considered stable enough to withstand application in tissue engineering. If the degradation rates remained constant, the membranes would be completely degraded along the tissue regeneration process, i.e. during the adaptation of the inoculated cells to the new environment and the neovascularization process, leading to the successful formation of new tissue at the injury site.

Characterization of *in vitro* cytotoxicity of the membranes

In Figure 3, the results obtained in the evaluation of the cytotoxicity of the membrane extracts to L929 cells are shown. Samples prepared with Xn and Ch in the presence or absence of Pluronic F68 had low cytotoxic effects, mostly the ones obtained with the mass ratio of Xn to Ch of 1 : 1. Membranes prepared in the presence of Tween 80, on the other hand, resulted in very high cytotoxicity, above 99%. Similar results were observed when directly exposing the cell monolayers to the membranes for 24 h, as shown in Figure 4.

Samples prepared with the addition of Tween 80 showed pronounced cytotoxic effects, resulting in areas of cell lysis under the samples greater than 80%, which extended to adjacent areas, as seen in Figure 4. The remaining membranes showed low direct toxicity, with areas of lysis less than 20% limited to the region directly below the samples. These results indicate that it is possible that not all the surfactant added is effectively removed during the wash step in sample preparation, and because Tween 80 has a lower hydrophilic–lipophilic balance than Pluronic F68, it is easier to remove the latter surfactant from the membrane structure. Probably a reduction in the Tween 80 proportion in the mixture could substantially reduce the cytotoxicity of membranes prepared in its presence.

Sakai et al.[42] analyzed the effect of these same surfactants on CHO cells grown in serum-free medium when used as a means to disperse phosphatidic acid in the medium, noting that whereas Tween 80 should be used at concentrations lower than 20 mg/L, Pluronic F68 even at a concentration of 500 mg/L did not cause a reduction in cell population. Other studies confirm the beneficial action of Pluronic F68 in cell cultures, such as the work developed by Kerleta et al.[43] and Clincke et al.[44], and all of these observations support the hypothesis that even if Pluronic F68 is not fully removed from the Xn–Ch matrix, its residual effects are not harmful. However, both cytotoxicity analyses showed that membranes with a higher proportion of Xn gum than of Ch

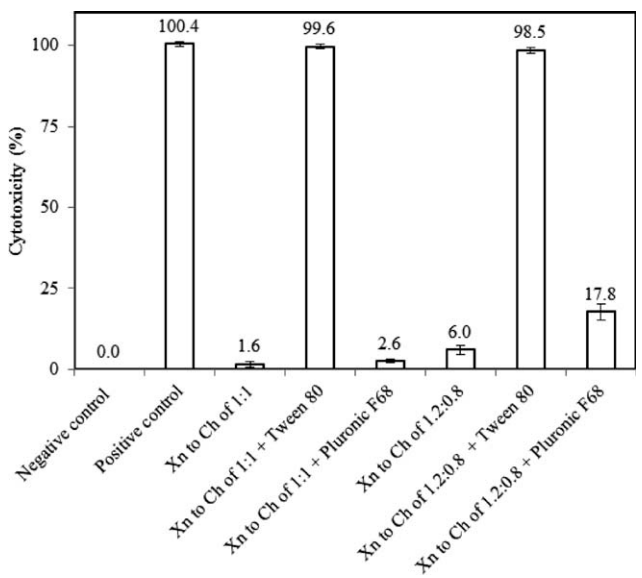

Figure 3 Cytotoxicity of membrane extracts to L929 cells.

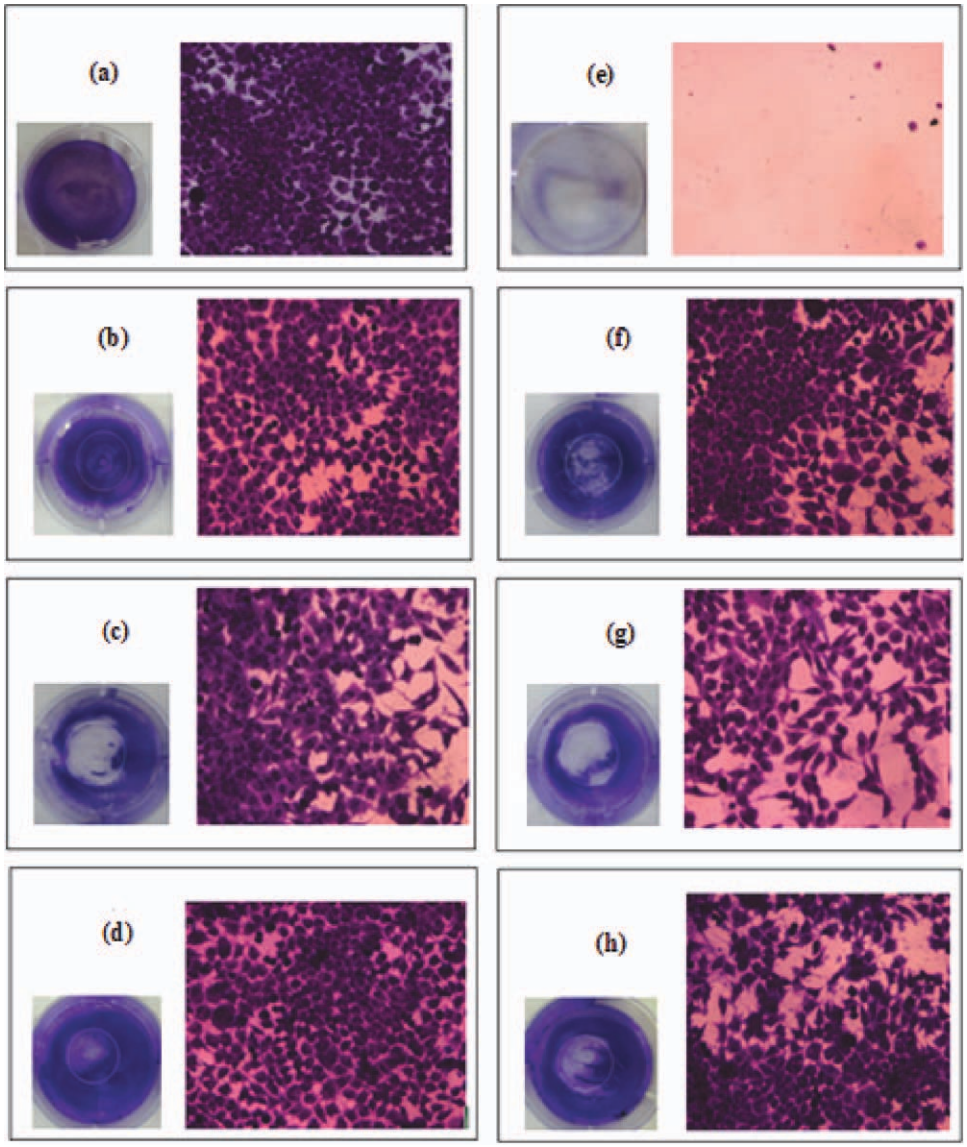

Figure 4 Typical aspect of the L929 cell cultures exposed directly to: (a) supplemented culture medium; (e) latex; membranes prepared at: (b) mass ratio of Xn to Chof 1 : 1 in the absence of surfactants; (c) mass ratio of Xn to Chof 1 : 1 in the presence of Tween 80; (d) mass ratio of Xn to Chof 1 : 1 in the presence of Pluronic F68; (f) mass ratio of Xn to Chof 1.2 : 0.8 in the absence of surfactants; (g) mass ratio of Xn to Chof 1.2 : 0.8 in the presence of Tween 80; and (h) mass ratio of Xn to Chof 1.2 : 0.8 in the presence of Pluronic F68 [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.].

prepared in the presence of Pluronic F68 are more cytotoxic, perhaps because this formulation retains more surfactant in its structure.

Because the formulations consisting of Xn and Ch in equal parts whether or not mixed with Pluronic F68 caused the lowest cytotoxic effects, the proliferation of L929 cells was evaluated for these membrane types only, and the data on cell population recovered from samples 1.5 cm in diameter after 72 h are shown in Figure 5.

Cells have grown in all materials tested, but the cell concentration values obtained for the control group proved to be higher than those in the results achieved for the two membrane formulations tested. Comparisons of the specific cell growth rates ($\mu_{máx}$) for the three groups considering that practically no lag phase was observed in any of the cultivations show that the behavior of the cells cultured only over the membranes prepared in the presence of Pluronic F68 is statistically different from that of the control group at a confidence level of 95%. Although the cells cultured on the polysterene plates showed a specific growth rate of $0.0487 \pm 0.002\ h^{-1}$, the cells cultivated on the membranes prepared in the absence of the surfactant achieved a $\mu_{máx}$ of $0.0464 \pm 0.003\ h^{-1}$ and the cultures developed on the samples obtained in the presence of Pluronic F68 reached $0.0404 \pm 0.001\ h^{-1}$. Because cell recovery from

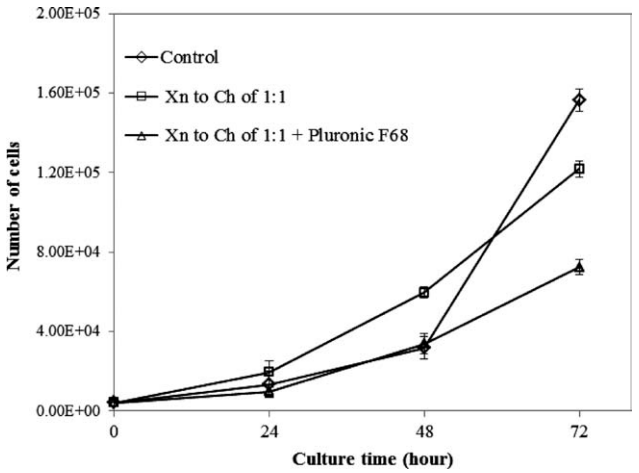

Figure 5 Growth curve for L929 cells inoculated on the culture plate (control) and on membranes prepared at a mass ratio of Xn to Chof 1 : 1 in the presence or absence of Pluronic F68.

porous devices is not a straightforward procedure, the possibility that the cell population in the membranes might be underestimated should be taken into account. With this in mind, the application of membranes prepared at a mass ratio of Xn to Ch of 1 : 1 in the presence or absence of Pluronic F68 can be considered as viable for use as dressings and scaffolds, respectively, in the treatment of skin lesions.

CONCLUSIONS

In this work, porous lamellar membranes of Ch and Xn were tested to verify their applicability as dermal dressings and supports for tissue engineering. The results showed that membranes prepared with the same mass ratios of Xn and Ch in the presence or absence of Pluronic F68 have low cytotoxicity *in vitro*, tensile strength compatible with that of human skin, a high capacity to absorb large quantities of physiological fluids and adequate stability in their presence. Membranes prepared in the presence of Tween 80, despite having adequate characteristics of mechanical resistance and behavior in physiologic solutions, caused massive cell death.

References

1. Rodrigues, A. P.; Sanchez, E. M. S.; Costa, A. C.; Moraes, A. M. J Appl Polym Sci 2008, 109, 2703.
2. Ratner, B. D.; Hoffman, A. S.; Schoen, F. J.; Lemons, J. E. Biomaterials Science, An Introduction to Materials in Medicine. Academic Press: San Diego, 1996.
3. Mi, F. L.; Shyu, S. S.; Wu, Y. B.; Lee, S. T.; Shyong, J. Y.; Huang, R. N. Biomaterials 2001, 22, 165.
4. Dallan, P. R. M.; Moreira, P. L.; Petinari, L.; Malmonge, S. M.; Beppu, M. M.; Genari, S. C.; Moraes, A. M. J Biomed Mater Res B 2007, 80, 394.
5. Souto, L. R. M.; Rehder, J.; Vassalo, J.; Cintra, M. L.; Kraemer, M. H. S.; Puzzi, M. B. Sao Paulo Med J 2006, 124, 71.
6. Souto, L. R. M.; Vassallo, J.; Rehder, J.; Pinto, G. A.; Puzzi, M. B. Sao Paulo Med J 2009, 127, 28.
7. Wittaya-Areekul, S.; Prahsarn, C. Int J Pharm 2006, 313, 123.
8. George, J.; Onodera, J.; Miyata, T. J Biomed Mater Res A 2008, 87, 1103.
9. Hutmacher, D. W. Biomaterial 2000, 21, 2529.
10. Wang, L.; Khor, E.; Wee, A.; Lim, L. Y. J Biomed Mater Res 2002, 63, 610.
11. George, M.; Abraham, T. E. J Control Release 2006, 114, 1.
12. Lai, H. L.; Abu'khalil, A.; Craig, D. Q. M. Int J Pharm 2003, 251, 175.
13. Ikada, Y. In Polysaccharides in Medical Applications; Dumitriu, S., Ed.; Marcel Dekker: New York, 1996.
14. Dumitriu, S. In Polymeric Biomaterials; Dumitriu, S., Ed.; Marcel Dekker: New York, 2002.
15. Meng, X.; Tian, F.; Yang, J.; Chun-Nian, H.; Xing, N.; Li, F. J Mater Sci Mater Med 2010, 21, 1751.
16. Campos, M. G. N.; Rawls, H. R.; Innocentini-Mei, L. H.; Satsangi, N. J Mater Sci Mater Med 2009, 20, 537.
17. Kikuchi, Y.; Fukuda, H. Makromol Chem 1974, 175, 3593.
18. Fukuda, H.; Kikuchi, Y. Bull Chemical Soc Japan 1978, 51, 1142.
19. Kikuchi, Y.; Noda, A. J Appl Polym Sci 1976, 20, 2561.
20. Chellat, F.; Tabrizian, M.; Dumitriu, S.; Chornet, E.; Magny, P.; Rivard, C. H.; Yahia, L. J Biomed Mater Res 2000, 51, 107.
21. Veiga, I. G.; Moraes, A. M. J Appl Polym Sci 2011, DOI: 10.1002/app.35526.
22. Bejenariu, A.; Popa, M.; Cerf, D. L.; Picton, L. Polymer Bull 2008, 61, 631.
23. Dumitriu, S.; Magny, P.; Montane, D.; Vidal, P. F.; Chornet, E. J Bioact Compat Polym 1994, 9, 184.
24. Eftaiha, A. F.; El-Barghouthi, M. I.; Rashid, I. S.; Al-Remawi, M. M. J Mater Sci 2009, 44, 1054.
25. Argin-Soysal, S.; Kofinas, P.; Lo, Y. M. Food Hydrocolloids 2009, 23, 202.
26. Bueno, C.; Moraes, A. M. J Appl Polym Sci 2011, 122, 624.
27. Ma, J.; Wang, H.; He, B.; Chen, J. Biomaterials 2001, 22, 331.
28. Shoichet, M. S. Macromolecules 2010, 43, 581.
29. Rhein, L. D.; Schlossman, M.; O'lenick, A.; Somasundaran, P. Surfactants in Personal Care Products and Decorative Cosmetics; Taylor and Francis: New York, 2007.
30. Kokubo, T.; Kushitani, H.; Sakka, S.; Kitsugi, T.; Yamamuro, T. J Biomed Mater Res 1990, 24, 721.
31. American Society for Testing and Materials ASTM D882–95a: Standard Test Methods for Tensile Properties of Thin Plastic Sheeting, 1995.
32. Chen, Q. Z. Bubble Sci Eng Technol 2011, 3, 34.
33. Liu, C.; Xia, Z.; Czernuska, J. T. Chem Eng Res Des 2007, 85, 1051.
34. George, J.; Onodera, J.; Miyata, T. J Biomed Mater Res A 2008, 87, 1103.
35. Kucharska, M.; Niekraszewicz, A.; Wišniewska-Wrona, M.; Brzoza-Malczewska, K. Fibres Tex East Eur 2008, 16, 109.
36. Wan, Y.; Wu, Q.; Wang, S.; Zhang, S.; Hu, Z. Macromol Mater Eng 2007, 292, 598.
37. Hansen, B.; Jemec, G. B. Arch Dermatol 2002, 138, 909.
38. She, Z.; Jin, C.; Huang, Z.; Zhang, B.; Feng, Q.; Xu, Y. J Mater Sci 2008, 19, 3545.
39. Macleod, G. S.; Collett, J. H.; Fell, J. T. J Control Release 1999, 58, 303.
40. Adekogbe, I.; Ghanem, A. Biomaterials 2005, 26, 7241.
41. Öztürk, E.; Ağalar, C.; Keçeci, K.; Denkbaş, E. B. J Appl Polym Sci 2006, 101, 1602.
42. Sakai, K.; Hayashi, C.; Yamajl, H.; Fukuda, H. J Biosci Bioeng 2001, 92, 256.
43. Kerleta, V.; Andrlik, I.; Braunmüller, S.; Franke, T.; Wirth, M.; Gabor, F. Altex 2010, 27, 191.
44. Clincke, M.; Guedon, E.; Yen, F. T.; Ogier, V.; Roitel, O.; Goergen, J. Biotechnol Prog 2011, 27, 181.