EFFECTS INDUCED BY INITIAL CHITOSAN SOLUTION CONCENTRATION AND CHITIN AND GLYCEROL INCORPORATION ON THE CHARACTERISTICS OF DENSE CHITOSAN MEMBRANES WITH POTENTIAL USE AS BURN DRESSINGS

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Abstract. *The aim of this work was to study the effects induced by chitosan solution concentration (1.0 and 2.5%) and by chitin and glycerol incorporation on dense chitosan membranes with potential use as burn dressings. The membrane properties analyzed were total raw material cost, morphology, cristallinity, swelling ratio, tensile strength, percentage of strain at break, in vitro enzymatic degradation with lysozyme, in vitro cell adhesion and stability in aqueous solutions. The use of the most concentrated chitosan solution reduced the biomaterials cost, cristallinity and swelling ratio in distilled water. The remaining evaluated properties were not affected by chitosan solution concentration. The incorporation of chitin and glycerol produced biomaterials with irregular surface, reduced the membranes cost, cristallinity, swelling ratio, mechanical properties and improved their degradation by lysozyme. When a neutralization stage was included in the chitosan membranes preparation, all formulations showed stability in aqueous solutions. The overall results indicate that most of the prepared membranes meet the performance requirements of temporary non-biodegradable burn dressings.*

Keywords*: chitosan, chitin, glycerol, biomaterial, film, membrane, burn, and burn dressing.*

1. Introduction

Chitin, a poly-β(1→4)-N-acetyl-D-glucosamine, is one of the most abundant polysaccharides found in nature. It is present in crustacean and insect exoskeletons as well as in fungal cell walls and plankton. Chitin derivatives that contain more than 50% of free amino groups in their structure are denominated chitosan. Their potential and existing applications are extensive in the industrial, medical and pharmaceutical sectors. Chitosan has been widely studied as a biomaterial raw material to be used in wound or burn treatment (Paul and Sharma, 2004). In most of the studies, this polysaccharide has been used in the form of films (membranes), colloidal solutions or sponges (Craveiro and Craveiro, 2000).

In order to improve the physical, chemical, mechanical and/or biological biomaterial properties, chitosan can be used alone or associated with other compounds such as polyvinylpirrolidone (Risbud *et al.*, 2000), glycosaminoglycans (Chupa *et al.*, 2000), collagen (Ma *et al.*, 2003), gelatin (Arvanitoyannis *et al.*, 1998; Mu *et al.*, 1999; Mao *et al.*, 2003), alginate (Wang *et al.*, 2002), and chitin (Craveiro and Craveiro, 2000), among others. Improvements in the mechanical properties can be achieved using plasticizers. The most common used plasticizer is glycerol (Arvanitoyannis *et al.*, 1998; Guerreiro-Béltran *et al.*, 1999; Mu *et al.*, 1999; Casariego *et al.*, 2002; Cervera *et al.*, 2004) but sorbitol (Casariego *et al.*, 2002; Cervera *et al.*, 2004), erythritol (Cervera *et al.*, 2004), lauric acid (Guerreiro-Béltran *et al.*, 1999), lactic acid (Khan *et al.*, 2000) and polyethyleneglycol (Caner *et al.*, 1998; Zhang *et al.*, 2002) may also be employed. Chitin, in spite of not implying in a direct improvement of the mechanical characteristics of the biomaterial, can reduce the total raw material cost.

In spite of the intense research using chitosan as wound or burn dressings, there is a lack of detailed information about the conditions for biomaterials production in the form of membranes or films and also about the factors that can affect the physical, chemical, mechanical and/or biological characteristics of the materials. In the consulted literature, it was not observed a standardization concerning to the initial chitosan solution concentration used to produce chitosan membranes. The most commonly used concentration is 1.0%, however, values varying from 0.5 to 6.0% were also detected. No explanations about the influence of this variable on chitosan membrane properties were provided.

In this context, the objective of this work was to study the effects induced by chitosan solution concentration and by chitin and glycerol incorporation on dense chitosan membrane properties, such as total raw material cost, morphology, cristallinity, swelling ratio, tensile strength, percentage of strain at break, *in vitro* enzymatic degradation with lysozyme, *in vitro* cell adhesion and stability in aqueous solutions.

2. Material and Methods

2.1. Material

Chitosan membranes were obtained using 85% deacetylated chitosan, chitin and glycerol (Sigma Chemical Co.), acetic acid (Synth), sodium hydroxide (Ecibra) and deionized water. Physical and mechanical membranes evaluation was performed using lysozyme (Sigma Chemical Co.), phosphate buffered saline (PBS, Nutricell Nutrientes Celulares) and distilled water. Biological membranes characterization was performed using Vero cells (Adolfo Lutz Institute, São Paulo, SP, Brazil), Ham-F10 medium, fetal calf serum (FCS), tripsin-EDTA and PBS (Nutricell Nutrientes Celulares), 3-(4,5-dimethylthiazolyl-2)-2,5 diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), glycine and sodium chloride (Sigma Chemical Co.), ethyl alcohol and sodium hydroxide (Ecibra).

2.2. Methods

The effects induced by initial chitosan solution concentration and by incorporation of chitin and glycerol on dense chitosan membrane properties were evaluated producing three different chitosan solutions. Chitosan, chitin and glycerol mass concentration in the casting solutions as well as the corresponding final mass fractions of each component in the membranes and the total mass of starting solutions are given in Tab. 1.

Table 1. Initial chitosan solutions composition, membranes final composition and chitosan mass solution used for chitosan membranes formulation.

Membrane formulation	Composition of casting solution (% mass concentration)			Total solution mass per membrane (g)	Composition of dried chitosan membrane (% mass concentration)		
	Chitosan	Chitin	Glycerol		Chitosan	Chitin	Glycerol
1	1.00	0.00	0.00	112.50	100	0.00	0.00
2	2.50	0.00	0.00	45.00	100	0.00	0.00
3	1.00	0.50	0.50	56.25	50.00	25.00	25.00

As the final membranes solid mass was kept constant in all experiments (equal to 1.125 g), when chitin and glycerol were employed, part of chitosan was substituted by these components. This study strategy had as objective the reduction of biomaterials final cost without impairing their physical, mechanical and biological characteristics. The membranes were analyzed concerning to their total raw material cost, morphology, cristallinity, swelling ratio, tensile strength, strain percentage at break, *in vitro* degradation, *in vitro* cell adhesion and stability in aqueous solutions. Student's t test was used for statistical evaluation ($p<0.05$).

2.2.1. Membranes preparation and sterilization

Chitosan solutions were prepared dissolving chitosan in 1% (v/v) acetic acid aqueous solution at room temperature. Three different solutions were prepared using chitosan, chitin and glycerol as described in Tab. 1. Each mixture was transferred to glass Petri dishes (11 cm diameter) treated with silicone oil (350 cp, Synth). The Petri dishes containing the solutions were kept in an airtight glass container under vacuum for two hours and then stored for one week in a refrigerator to eliminate air bubbles. The membranes were produced by evaporating the solvent in an oven with air circulation (Nova Ética 410) at 50°C for five hours followed by immersion of the membranes in a 1 M sodium hydroxide aqueous solution at room temperature for 24 hours. The membranes were washed with distilled water (2 liters per membrane) and finally with deionized water. The wet membranes were cut in appropriated dimensions depending on the test they would be used for, dried at room temperature under compression by a stainless steel plate to increase flatness and sterilized at Acecil Central de Esterilização Comércio Indústria Ltda (Campinas, SP, Brazil) by exposure to Oxyfume-30 (30% ethylene oxide and 70% carbon dioxide) for eight hours at 40°C and relative humidity of 40 to 50%. The membranes were aired three times with nitrogen for the removal of residual ethylene oxide and stored at room temperature for at least one week before use.

2.2.2. Membranes characterization

Chitosan membranes were characterized regarding their total raw material cost, morphology, cristallinity, swelling ratio, tensile strength, strain percentage at break, *in vitro* degradation, *in vitro* cell adhesion and stability in aqueous solutions as described below.

Membranes cost was estimated considering only the contribution of the raw materials used for the preparation of membranes with 95 cm^2. Chitosan, chitin, glycerol and acetic acid costs were taken from Sigma Chemical Co. catalog (US$ 455.20/kg, US$ 69.80/kg, US$ 33.04/kg and US$ 32.20/kg, respectively), and deionized water cost was estimated as US$ 0.76/kg. Since neutralization and washing processes were the same for all chitosan membrane formulations, they were not included on the analysis of the membranes cost.

For the evaluation of membranes morphology, the samples were lyophilized, cut in 5 x 5 mm^2, bound to sample stubs with an appropriate adhesive and sputtered-coated with an ultra-thin layer of gold (92 Å) in a coating apparatus (SC 7620 mini-sputter coater). Morphologic analysis was accomplished in the scanning electronic microscope (Leo 440i, Leica) coupled to the Leo UIF series 400 software.

Membranes cristallinity (30 x 30 mm^2) was evaluated by X-ray diffraction in the 2θ range of 5 to 60° at room temperature using CuKα radiation generated at 40 kV and 40 mA (Phillips PW3050). The scan rate used was 3° (2θ)/min. The cristallinity of the samples was estimated measuring the diffraction peak obtained on angle 2θ equal to 20°.

The analysis of the membranes swelling ratio was performed immersing the dried 10 x 60 mm^2 samples with known weights (W_{dry}) in distilled water (pH 3.05) and in PBS (pH 7.4) at 37°C for 24 hours. The chitosan membranes wet weights (W_{wet}) were determined after blotting the samples with filter paper to remove the excess liquid deposited on the membranes surface and the swelling ratios were then calculated according to Eq. (1). Three independent measurements were performed for each membrane formulation, and the average values were taken as the swelling ratios.

$$Swelling\ ratio\ (\%) = [(W_{wet}-W_{dry})/W_{dry}] \times 100 \quad (1)$$

Mechanical properties of the membranes (10 x 60 mm^2) were evaluated after swelling the samples in PBS (pH 7.4) at room temperature for 24 hours. Tensile behavior was evaluated through an Instron 5569 tensile tester employing a cell load of 500 N, gauge length of 25 mm and crosshead speed of 10 mm/min. Five independent test samples were used for each measurement.

For the evaluation of membranes degradation, the materials (10 x 10 mm^2) of known weight (W_0) were immersed in a 5 mg/mL lysozyme solution in 0.1 M PBS (pH 7.4) and kept in a humidified incubator (Microprocessor CO_2 incubator, Lab-Line) at 37°C. After one and two months, the membranes were removed from the incubation medium, rinsed with distillated water, dried at 50°C for 24 hours and weighed (W_f). Samples in 0.1 M PBS (pH 7.4) without the enzyme were used as control tests. The extent of *in vitro* degradation was expressed as the percentage of dried samples weight after lysozyme treatment and calculated according to Eq. (2). Each chitosan membrane formulation was evaluated in triplicate and the average value was taken as the percentage *in vitro* degradation.

$$In\ vitro\ degradation\ (\%) = [(W_0-W_f)/W_0] \times 100 \quad (2)$$

Biological performance of the chitosan membranes was analyzed evaluating Vero cells adhesion on the materials surface using MTT test. Briefly, the different samples were placed on a 96-well plate and covered with 100 μL of Ham-F10 medium without FCS for 24 hours at 37°C. After this incubation period, 100 μL of Vero cell suspension (2.0 x 10^5 cells/mL) in Ham-F10 medium with 10% FCS were inoculated in each of the wells with the different materials. The cells were incubated for two and for 24 hours in Ham-F10 with 10% FCS at 37°C, washed twice with PBS (pH 7.4) at 37°C, and 200 μL of fresh Ham-F10 medium with 10% FCS and 10 mM HEPES (pH 7.4) were added to each well along with 50 μL of a 5 mg/mL MTT solution in PBS. The plate was covered with aluminum foil and after four hours incubation at 37°C, each well was washed three times with PBS and received 200 μL of DMSO followed by 25 μL of Sorensen's glycine buffer. An aliquot of 180 μL of each solution was transferred to a corresponding well in another 96-well plate. The absorbances were determined in the microplate reader (Packard Bioscience Company Fusion with software Fusion Robotics Interface) at 540 nm. The culture plate itself was used as positive control (material that promotes cell adhesion on its surface) and Teflon® disks were used as negative control (material that does not promote cell adhesion on its surface). Eight repetitions were made for each test.

Stability of the membranes in aqueous solutions was performed preparing the chitosan membranes as described in section 2.2.1 and alternatively, submitting the materials to the neutralization procedure with a 1 M NaOH aqueous solution. The neutralized and non-neutralized membranes of known weight were immersed in distillated water (pH 3.05) and in PBS (pH 7.4) at room temperature. After 24 hours, the membranes were removed from the incubation media, rinsed with distilled water, dried at 50°C for 24 hours and weighed. Stability of the membranes in the tested solvents was expressed as the percentage of the remaining mass compared to the initial membrane weight before exposure to the

solvents. Twelve samples were tested for each membrane formulation, half of them were neutralized with NaOH aqueous solution and the remaining were not submitted to the neutralization procedure.

3. Results and Discussion

3.1. Total raw material cost

According to the results shown in Tab. 2, the cost of the membranes composed exclusively by chitosan and obtained using the diluted solution (formulation 1) was 8.2% higher than the cost observed for the membranes obtained using the concentrated solution (formulation 2). This behavior may be attributed to the amount of chitosan solution used on the membranes preparation (Tab. 1). In both formulations, chitosan content is the same, however, the membranes final cost include the costs of all raw materials used, comprising acetic acid and deionized water, which were the responsible for the increase in the final biomaterial cost. The substitution of chitosan by chitin and glycerol (formulation 3) promoted a significant reduction of 42.4% on the membranes total raw material cost, when compared to the membranes composed exclusively by chitosan using the same chitosan solution concentration (1.0%).

Table 2. Dense chitosan membranes total raw materials cost.

Membrane formulation	Cost per 95 cm^2 membrane (US$)
1	0.66
2	0.61
3	0.38

3.2. Morphology

The characteristics of the membranes surface morphology basically do not differ from the ones observed in a previous work (Marreco *et al.*, 2004). The absence of chitin (formulations 1 and 2) resulted in membranes with smooth surfaces and no visible pores. Membranes containing chitin (formulation 3) showed irregular surfaces, although this component seems to be homogenously distributed, and again, no visible pores were detected.

Concerning the color of the materials, samples without chitin on their composition were transparent and, slightly yellow and opaque when chitin was present (Fig. 1).

Figure 1. Dense chitosan membranes visual aspect: (a) formulation 1, (b) formulation 2, (c) formulation 3.

3.3. Cristallinity

X-ray diffraction patterns of the chitosan membranes are shown in Fig. 2 and the estimated chitosan membranes cristallinity are given in Tab. 3.

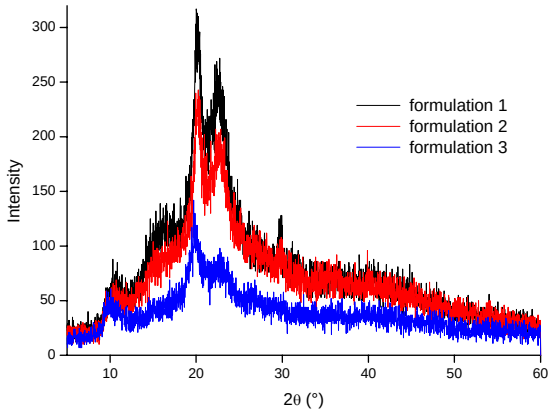

Figure 2. X-ray diffraction of dense chitosan membranes.

Table 3. Dense chitosan membranes cristallinity.

Membrane formulation	Cristallinity
1	294.70
2	239.50
3	118.50

As shown in Fig. 2 and in Tab. 3, both chitosan solution concentration and chitin and glycerol incorporation in the chitosan formulations showed influence on the cristallinity of the samples.

The membranes composed exclusively by chitosan and produced using the diluted solution (formulation 1) showed higher cristallinity than the one observed for the membranes obtained using the concentrated chitosan solution (formulation 2). This behavior may be related to the inter- and intra-molecular interactions formed during the membranes preparation, since to keep constant the mass of chitosan for all conditions, different chitosan solution volumes were used. As shown in Tab. 1, when the diluted chitosan solution was used, the required volume was higher than when the concentrated solution was employed. As a result, during solvent evaporation and, consequently, during membrane formation, because of the higher volume used, the evaporation was slower, the molecules were far apart from each other, and probably, the polymeric chains were more organized, presenting higher cristallinity.

The reduction on the cristallinity observed for the membranes produced by formulation 3 may be due the incorporation of chitin and glycerol in the membranes composition. Glycerol is amorphous and it is able to penetrate between two polymeric chains, separating them and improving chitosan chains motion, which has direct correlation to the final membrane cristallinity (Cervera *et al.*, 2004). Despite being a semi-crystalline polymer (Tomihata and Ikada, 1997; Suh and Matthew, 2000; Senel and Mcclure, 2004), chitin is inserted in chitosan membranes as particles, disturbing the polymeric chain organization. As a result, a reduction on the membranes cristallinity can be observed when chitin is used as a component of the chitosan membranes.

3.4. Swelling ratio

Swelling ratios of the dense chitosan membranes are given in Tab. 4.

Table 4. Dense chitosan membranes swelling ratios.

Membrane formulation	Swelling ratio (%)	
	Distilled water	PBS
1	110.8 ± 0.6	107.6 ± 0.4
2	108.4 ± 1.2	107.3 ± 0.9
3	88.8 ± 0.2	89.9 ± 1.6

While the use of the concentrated chitosan solution concentration (formulation 2) reduced the swelling ratio of the membranes after exposure to distilled water, the same behavior was not observed when PBS was used as solvent. The substitution of part of chitosan by chitin and glycerol promoted a significant reduction on the membranes swelling ratio both in distilled water and in PBS. The reduction on the membranes swelling ratio when chitin was used as biomaterial component can be attributed to the diminished capability of chitin to make hydrogen bonds with water when compared to chitosan. In spite of the negative influence of chitin incorporation in the membranes composition, all the swelling ratio values obtained were higher than 88%.

3.5. Mechanical properties

Chitosan membranes mechanical properties, expressed as averages, for membranes tensile strength and percentage of strain at break are given in Tab. 5.

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Table 5. Dense chitosan membranes mechanical properties.

Membrane formulation	Mechanical properties	
	Tensile strength (MPa)	Percentage of strain at break
1	7.16 ± 2.91	154.84 ± 32.02
2	8.12 ± 2.10	187.50 ± 18.57
3	2.43 ± 0.43	21.33 ± 3.43

While the statistical analysis on both variables did not show influence of chitosan solution concentration, chitin and glycerol incorporation in the membranes formulation promoted a reduction on the mechanical resistance of the materials and on their elasticity. As well as for the membranes cristallinity and swelling ratio, this behavior may be attributed to the presence of chitin in the membranes composition since it is inserted in chitosan membranes as particles, disturbing the polymeric chain organization, which seems to have a fundamental role on the mechanical resistance and elasticity of the dense chitosan membranes.

According to the consulted literature, normal skin shows tensile strength in the 2.5 – 16 MPa range (Silver, 1994) and 70% percentage of strain at break (Hansen e Jemec, 2002). Based on these values, only the formulation obtained when part of chitosan was substituted by chitin and glycerol (formulation 3) can not be considered appropriated to be used as burn dressings, since it showed a tensile strength value under 2.5 MPa and percentage of strain at break under 50%. In spite of the low mechanical properties showed by the membranes containing chitin and glycerol in their composition (formulation 3), chitin has interesting biological characteristics to be used as burn dressings and its use can reduce the biomaterial final cost, therefore, to overcome this mechanical deficiency, these biomaterials could be used in parts of the body where movement is not so frequent.

3.6. *In vitro* degradation

Membranes degradation behavior is given in Tab. 6. The statistical analysis showed that while incorporation of chitin and glycerol in the membranes composition (formulation 3) increased the degradation degree of the materials after the exposure to lysozyme, reducing the resistance of the membranes to lysozyme action, chitosan solution concentration did not show influence on the same variable.

Table 6. Dense chitosan membranes *in vitro* degradation.

Membrane formulation	Chitosan membranes degradation (percentage of mass loss)			
	1 month		2 months	
	PBS	Lysozyme solution	PBS	Lysozyme solution
1	6.30 ± 0.27	6.21 ± 0.96	5.86 ± 0.82	7.97 ± 0.51
2	6.10 ± 0.11	5.19 ± 0.83	5.40 ± 0.35	8.29 ± 0.94
3	5.78 ± 1.98	8.34 ± 0.31	5.72 ± 2.08	14.33 ± 2.19

After one month, no significant differences between the degradation of the membranes in PBS and in lysozyme solution were detected for the different membranes formulation. After two months, all the different chitosan membranes formulation showed higher degradation when incubated in lysozyme solution than in PBS. In spite of this fact, the highest value obtained was 14.33% (formulation 3), a low value considering the long time of the evaluation process.

Despite chitosan is commonly referred to as biodegradable by lysozyme action, the results obtained in this work show high physical and chemical stability of the chitosan membranes when exposed to this enzyme, mostly when considering the high enzyme concentration and the long evaluation period. The low degradation values obtained can be explained by the characteristics of the chitosan used in this work (Tomihata and Ikada, 1997; Senel and Mcclure, 2004) and the physical characteristics of the membranes (Ratner *et al.*, 1996). Among other factors, the rate of chitosan membranes degradation by lysozyme depends on chitosan deacetylation degree, since this enzyme seems to target acetylated residues (Suh and Matthew, 2000). According to Ratner *et al.* (1996), porous membranes are more easily degraded than dense ones. Since the chitosan used in this work had high deacetylation degree (e.g.>83%) and the produced membranes are dense, the low enzymatic degradation rates observed were, in fact, expected.

3.7. *In vitro* cell adhesion

The average results obtained for *in vitro* cell adhesion test are given in Fig. 3.

After two hours of Vero cells exposure to chitosan membranes, the statistical analysis showed, in all conditions, absorbance values lower than that obtained for the positive control (polystyrene plate) and for the negative control (Teflon® disks). Chitosan solution concentration and chitin and glycerol incorporation did not show influence on the adhesion of Vero cells on the membranes surface since the absorbance values obtained for the different membranes composition did not show statistical difference. Once absorbance values have direct connection to the number of metabolically active cells, the results obtained showed that the membranes were not favorable to the cells adhesion on their surface. Since chitosan membranes do not show cytotoxic effect to Vero cells (Marreco *et al.*, 2004), a probable explanation for the low adhesion values observed could be the short exposure time of the cells to the membranes.

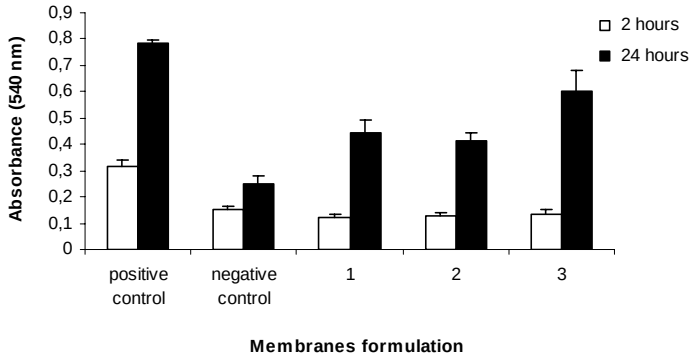

Figure 3. Results obtained from *in vitro* cell adhesion test using MTT reagent.

After 24 hours, it was observed an increase in the absorbance values, although all the absorbance values obtained for the different chitosan membranes composition were lower than the observed for the positive control. Differently from observed after two hours of materials exposure to Vero cells, while chitosan solution concentration did not show influence on the adhesion of Vero cells on the materials surface, incorporation of chitin and glycerol in the membranes composition increased the absorbance values when compared to the membranes composed exclusively by chitosan. However, the values obtained were lower than the observed for the positive control. These results show that cell adhesion on membranes surface was not improved, even though the exposure time was increased from two to 24 hours. These results were confirmed by microscopy analysis (results not shown).

3.8. Stability in aqueous solutions

The non-neutralized membranes composed exclusively by chitosan (formulations 1 and 2) dissolved after five minutes of immersion in the aqueous media. Membranes composed by chitosan, chitin and glycerol (formulation 3) conserved their shapes, losing 12.65 ± 1.20% mass when exposed to distilled water and 8.64 ± 0.14% mass when exposed to PBS. A possible explanation can be associated to the presence of glycerol in the polymeric structure, since this plasticizer improves acid acetic withdrawal during solvent evaporation in the oven (Brown *et al*., 2001).

The neutralized membranes showed high stability when immersed in the aqueous solutions. Mass losses were lower when the membranes were exposed to distilled water than when contacting PBS.

4. Conclusion

While initial chitosan solution concentration showed influence on the membranes cost, cristallinity, and swelling ratio in distilled water, incorporation of chitin and glycerol in the chitosan solutions affected all the analyzed membranes properties. Strong cell adhesion was not observed in any of the tested membranes formulation.

The overall results indicate, therefore, that the prepared membranes meet the performance requirements of temporary non-biodegradable burn dressings, since they presented low values of *in vitro* cell adhesion on their surfaces, low degradation when exposed to lysozyme solution, besides, high stability in aqueous solutions when properly neutralized.

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7. Responsibility notice

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