Multilayer Biopolymer Membranes Containing Copper for Antibacterial Applications

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ABSTRACT: Chitosan/alginate multilayer dense membranes, with micrometric thicknesses, were produced by casting the solution of each polymer alternately on a Petri dish. Copper ions were also incorporated into these membranes, which were then characterized with regard to morphology, chemical and crystallographic structures, ions diffusivity, and bacteriostatic properties. Results indicate that the casting approach for multilayers production was successful, since the membranes produced were formed by organized and alternated polymer layers. The pH dependence of the metal adsorption resulted in a higher concentration of the copper in the alginate layers, as shown by SEM-EDS (scanning electron microscopy - energy dispersive X-ray spectroscopy) analysis. Fourier transformed infrared spectroscopy confirmed the Coulombic interaction between the chitosan amino groups and sodium alginate carboxyl groups. The copper ions were distributed homogeneously, without the formation of clusters, and were bound mainly to oxygen and nitrogen atoms located on the alginate and chitosan functional groups, respectively, according to extended X-ray absorption fine structure (EXAFS) results. The antibacterial properties of this membrane indicate that this material may hold promise for numerous applications, such as for water treatment or antibacterial functional coatings with controlled release. © 2012 Wiley Periodicals, Inc. J Appl Polym Sci 000: 000–000, 2012

Key words: adsorption; membranes; biopolymers; chitosan; alginate

INTRODUCTION

Over the last decades, studies regarding preparation methods and functionalization of membranes have attracted much interest in Materials Science. Along with the increase in the number of applications involving membranes, there exists a need to create structures that meet specific demands, without loss in functionality. In this context, some processes have been proposed to achieve multilayered membranes.[1] Multilayered membranes or films of natural polymers have been studied in recent years for a variety of applications in the biomedical area, such as antibacterial coatings for medical devices,[2,3] biocompatible coatings of biomaterials,[4,5] and controlled drug delivery,[6–8] and also in separation process, such as pervaporation separation.[9–11] Chitosan (CHI) and sodium alginate (SA) are natural polymers with characteristics that allow the production of multilayer membranes.[9,12]

CHI is a linear copolymer of glucosamine and *N*-acetyl glucosamine, linked by β-1,4 glucosidic

bonds. CHI is derived by alkaline *N*-deacetylation from chitin, which is the major component of crustaceans shells and is the second most abundant biopolymer in nature.[13] SA is produced from brown seaweeds by the use of dilute alkali. It consists mainly of the sodium salt of alginic acid, a polysaccharide composed of β-D-mannuronic acid (M) and α-L-guluronic acid (G). The relative amount and sequential distribution of homogeneous M-M segments (M-blocks), homogeneous G-G segments (G-blocks), and alternating M-G segments (MG-blocks), which represent the primary structure of SA, depend on the producing species, and for marine sources, on seasonal and geographical variations.[14]

Interactions between CHI and SA give an electrostatic complex[15–17]; polyelectrolyte complexes (PECs) are formed as association complexes due to the electrostatic interaction between oppositely charged polyions.[18] The negatively charged carboxylic acid groups of mannuronic and guluronic acid units in SA interact electrostatically with the positively charged CHI amino groups to form a PEC. SA is one of the most studied anionic polyelectrolytes in complexation with CHI, because the PEC formed between these two polymers is biodegradable and biocompatible, as well as mechanically stronger at lower pH values where CHI dissolves.[16]

For multilayer membranes, by using CHI and SA, it is possible to control the degradation rate of each
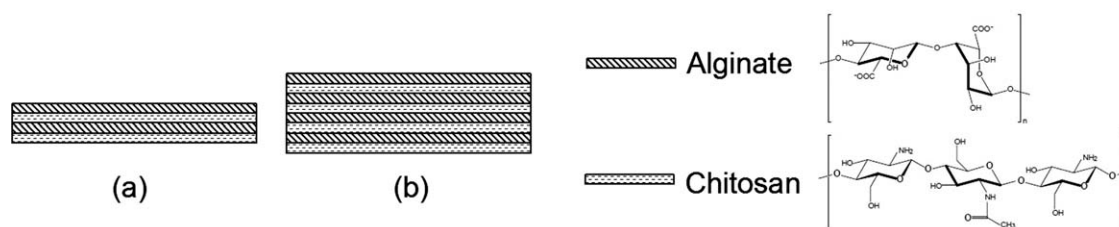
Figure 1 Schematic illustration of the synthesized membranes: (a) two bilayer and (b) four bilayer.

layer of polymer, thus controlling the release of any desirable component incorporated in the polymer layer. The polymers may be combined to fine-tune the material's properties to meet a variety of requirements; this is accomplished by simply changing the set of polymers and their layer thicknesses during the production of the membrane.[19]

Among the main characteristics of CHI and SA, their ability to interact with metal ions is well known.[20,21] Metallic ions, such as copper and silver, can be used as a sanitizing agent, ensuring hygiene, due to its antimicrobial properties.[22] Ruparelia et al.[23] studied the growth of three kinds of microbial cultures in the presence of copper and silver. This study showed that silver and copper nanoparticles hold great promise as antimicrobial agents against *E. coli*, *B. subtilis*, and *S. aureus*. The best results for *E. coli* and *S. aureus* were obtained with the silver nanoparticles, while the copper nanoparticles demonstrated better antibacterial activity toward *B. subtilis*. The authors proved that the combination of silver and copper nanoparticles may give rise to a more complete bactericidal effect against a mixed bacterial population.[23]

Although some studies have shown that copper has a lower antibacterial activity than silver, in this study, copper was chosen due to its stronger tendency to form complexes with CHI and SA. In addition, with a history of using copper since ancient times, this bacteriostatic agent may be less harmful to human health, when compared with other metals used for this purpose.

We describe, herein, a method to produce CHI/SA multilayer membranes with uniform and homogeneous layers, by casting the solutions of CHI and SA alternately on a petri dish. Additionally, the ability of copper adsorption by this multilayer membrane was evaluated, as well as its bacteriostatic potential, to evaluate its potential for antibacterial applications.

MATERIALS AND METHODS

Materials

CHI was purchased from Sigma (high molecular weight), USA, with 85% deacetylation degree. SA was purchased from Vetec, Brazil. All other reagents

were of analytical grade (Synth, Brazil). Milli-Q® ultrapure water was used to produce all solutions.

Multilayer membrane preparation

The CHI/SA multilayer membranes were prepared by casting and solvent evaporation. Briefly, CHI was dissolved in aqueous acetic acid solution (3% v/v) and stirred for 3 days, until obtaining a homogenous solution of 1% (w/v). SA solution was prepared by dissolving 1% (w/v) SA in $1M$ NaOH aqueous solution. Both the CHI and SA solutions were filtered to remove any impurities. The CHI solution was then cast on a polystyrene petri dish, followed by partial solvent evaporation in an oven at 50°C for $\sim$ 2 h. Subsequently, the SA solution was cast on the partially dried CHI layer and kept at 50°C. This process allowed the formation of a bilayer membrane (with one CHI layer and one SA layer). In this study, CHI/SA two- and four-bilayered membranes were produced, as illustrated in Figure 1(a,b), respectively. During casting, the CHI solution had a pH = 3.6, while the SA solution had a pH = 7.2. The functional groups were neutralized by immersing the dried membranes in $0.1M$ sulfuric acid solution in 50 vol % ethanol, for 24 h. The neutralized CHI/SA membrane was washed with ultrapure water to remove the sulfuric acid residues and stored in ultrapure water at 4°C.

Pristine CHI and SA membranes were also prepared to compare their characteristics with the multilayer membranes.

Copper incorporation

The process of copper incorporation into the CHI/SA membranes was achieved by soaking the membranes in a copper sulfate solution with [Cu (II)] = 15.7 mM, for 48 h at 25°C. A proportion of 0.30 g of absorbent/25 mL of copper solution was used.[24] The pH during the copper incorporation was chosen based on the metallic speciation curve for salt copper sulfate (data not shown). The Hydrochemical Equilibrium-Constant Database (HYDRA) software[25] was used to plot the diagram for [Cu (II)] = 15.7 mM and showed that copper is present predominately in its dissociated form Cu (II) at 2 < pH < 4. Thus, the pH for copper incorporation was adjusted
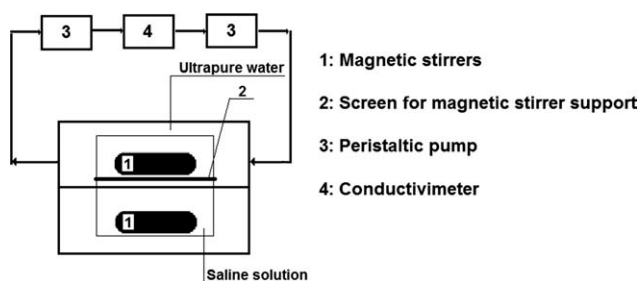
Figure 2 Schematic representation of the ion diffusion experiments.

initially to four and no corrections were made throughout the test.

Characterization

The surface and cross-sectional microstructure of CHI/SA and CHI/SA-copper membranes were observed by scanning electron microscopy (SEM—LEO 440 Electron Microscopy Ltd, Cambridge, England). Samples were prepared by cryofracture in liquid nitrogen and coated with gold. The membranes were also exposed to energy dispersive X-ray to analyze the copper distribution.

The structures of the CHI, SA, and CHI/SA multilayer membranes, with and without copper, were investigated in the range of 600–2000 cm^{-1}, using Fourier transformed infrared spectroscopy (FTIR—Protégé 460, Nicolet), and using a horizontal attenuated total reflectance (ATR) ZnSe cell. The ATR device allows us to obtain information about the chemical structure of the material's surface.

Extended X-ray absorption fine structure (EXAFS) was used to characterize the copper bindings with the polymeric chains of CHI and SA. The experiments were carried out with the D04B - XAS beamline at the National Synchrotron Light Laboratory (LNLS, Campinas, Brazil). X-ray absorption spectra were collected around the Cu (8970 eV) K edges. The X-ray energy incident on the sample was defined using a double-crystal Si (111) monochromator. Each spectrum corresponded to an average over three independent scans. The spectra were energy calibrated by simultaneous measurement of the transmission spectrum of Cu foils, where the energy of the first inflection point for the reference sample absorption edge, E_0, was defined as 8970 eV. The software used in the data analysis was IFEFFIT and their graphical interfaces, ATHENA and ARTEMIS, were used for background subtraction and fitting.[26]

Ion diffusion experiments

Ion diffusion experiments were performed according to Beppu et al.,[27] to observe interaction differences

among sodium ions and the membranes with or without copper. The diffusion cell (Fig. 2) was connected to a peristaltic pump and a conductivimeter. The cell consisted of two separate compartments with 1M NaCl solution in the lower portion and ultrapure water in the upper compartment. The concentration in the upper compartment was determined by a conductivimeter, over time. To carry out these measurements, a calibration curve of conductivity versus concentration was obtained for the NaCl solution. In the inferior compartment, a mass balance was used to calculate ion concentration.

Through the mass balance and Fick equation, it was possible to obtain the coefficient of diffusion (D), by eq. (1).

$$\ln\left(\frac{C_f^1 - C_f^2}{C_0^1 - C_0^2}\right) = -\frac{\beta}{L} \cdot D \cdot t, \qquad (1)$$

where C is the final or initial concentration in the compartments, L is the membrane thickness, t is the diffusion time, D is the coefficient of diffusion, and β is the cell constant, defined by eq. (2).

$$\beta = A \cdot \left(\frac{1}{V_1} + \frac{1}{V_2}\right) \qquad (2)$$

where A is the area of transference, L is the membrane thickness, and V is the volume of the compartments.

Antibacterial tests

For the antibacterial tests, CHI, SA, and CHI/SA multilayer membranes, with and without copper, were used. The antimicrobial potentials of the membranes were determined by the disk-diffusion method.[28] The incubation of the plates was performed at 32°C for 48 h after placing the membranes over a culture dish with bacteria seeds. The tests were conducted with a representative of Gram-negative bacteria (*Escherichia coli*) and another representative of Gram-positive bacteria (*Staphylococcus aureus*). The technique of the inhibitory concentration halo was used to characterize the antibacterial activity of the membranes.

RESULTS AND DISCUSSION

Scanning electron microscopy

Morphological characterization of the membranes (transversal sections obtained by cryofracture in liquid nitrogen) was carried out using scanning electron microscopy (SEM). The SEM images presented in Figure 3(a,b) show the well-defined CHI/SA-multilayered structure formed with two and four
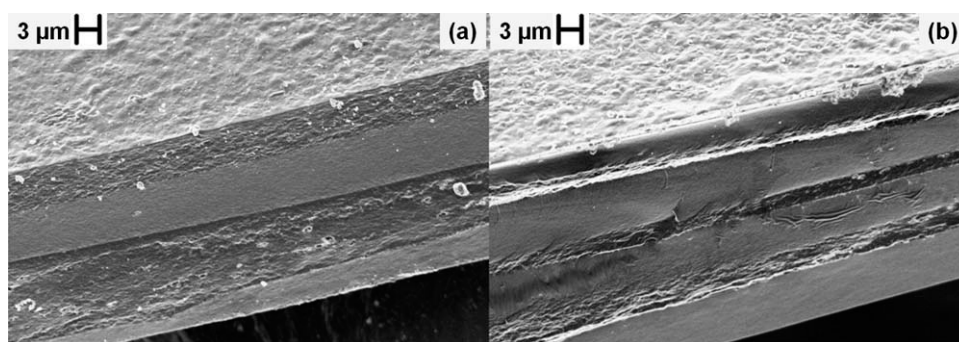
Figure 3   SEM micrographs of cryofractured cross-sections of two bilayer (a) and four bilayer (b) membranes.

bilayers, respectively. A good cohesion between the layers could be observed, caused by the interaction between the opposite charges of CHI and SA.[15] Figure 3 also shows that the thicknesses of the SA and CHI layers are visually different, even though the same quantities of solution for each biopolymer were used. This fact can be explained by the difference between the densities of the solutions, in this case $\rho_{CHI} > \rho_{SA}$.[29]

Figure 4 depicts the images and mapping of the copper of the two bilayer membrane. According to the EDS images, copper was found in a higher quantity in the SA layers. This result can be explained by the pH dependence of the metal adsorption. At low pH values, the polymer surface is positively charged, while at high pH values the polymer surface is negatively charged. From the electrostatic interaction point of view, the positive charge of samples under acidic solution conditions could favor the adsorption of negatively charged species and, in basic solution, conditions may enhance the adsorption of positively charged species. During casting, the CHI solution presented pH = 3.6, while the SA solution presented pH = 7.2, and the pH of copper incorporation was adjusted to four. At these experimental conditions CHI was found protonated; thus copper was preferentially linked to the SA negatively charged sites. The copper mapping of the four bilayer membrane (data not shown) presented the same aspect as that of the two bilayer membrane (Fig. 4).

FTIR

Figure 5 shows the FTIR-ATR spectra of CHI, SA and CHI/SA multilayer membranes, without copper incorporation. Sulfuric acid acted as an ionic cross-linker for CHI, but as an insolubilizing agent for SA. SA is converted to the free alginic acid form, which is insoluble in water, by sulfuric acid treatment.[9] The band at about 1726 cm^{-1} [Fig. 5(c)], indicates the formation of alginic acid. The 1631 and 1411 cm^{-1} bands were attributed to the asymmetric and symmetric COO$^-$ bond, respectively. Also the characteristic peaks related to C—O (1200–1250 cm^{-1}) and OH (1035 cm^{-1}) bonds were observed.[30] For the CHI membrane [Fig. 5(d)], it is possible to identify the CHI characteristic peaks at 1637 and 1498 cm^{-1}, attributed to amide I vibration and C—N deformation, respectively. The peak located at 1459 cm^{-1} is due to CH$_2$ vibration, while the peak at 1369 cm^{-1} is attributed to the symmetric CH$_3$ deformation.

The prepared CHI/SA multilayer membranes showed different FTIR spectra, when compared with CHI and SA membranes. The peak indicating an interaction between the NH$_3^+$ of CHI and COO$^-$ group of SA was observed at 1419 cm^{-1} [Fig. 5(a,b)], which was assigned to the symmetric stretching peaks of the carboxylate group.[31] This evidence confirms the formation of a PEC, where CHI acted as a polycation and SA as a polyanion. The Coulombic interaction between the CHI amino groups and the
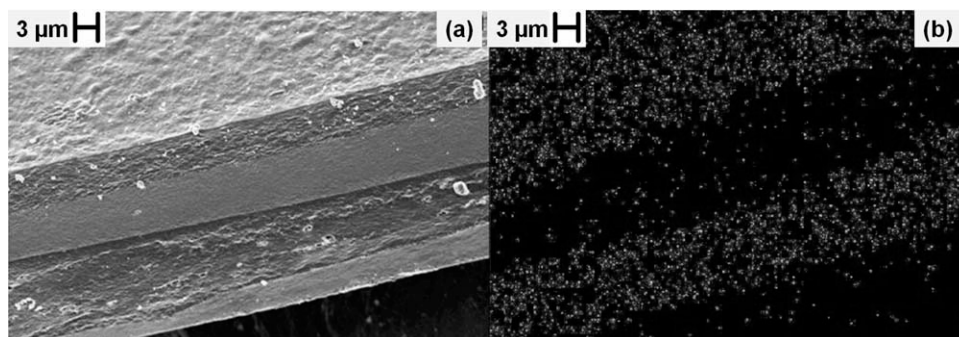

Figure 4   SEM micrograph of two bilayer membrane (a) and copper ion mapping (b).
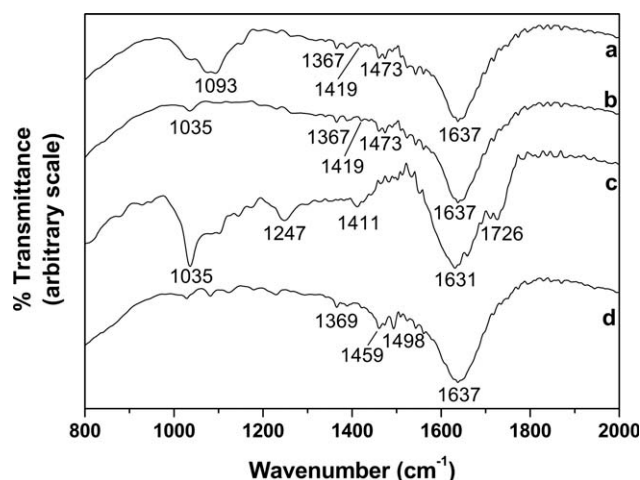
Figure 5 FTIR-ATR spectra of (a) four bilayer, (b) two bilayer, (c) alginate, and (d) chitosan membranes without copper.

SA carboxyl groups promotes the formation of a very dimensionally stable multilayer membrane (without delamination).

The FTIR-ATR spectra of the membranes after copper incorporation can be seen in Figure 6. The differences after copper incorporation were most pronounced between the spectra of the SA and CHI membranes [Figs. 5 and 6(c,d)]. This is indicative that the interaction between CHI and SA on two and four bilayers membranes changed the behavior of copper adsorption, when compared with the pristine versions.

The CHI and SA copper membranes peaks [Fig. 6(c,d)] located in the range of 1050–1150 cm^{-1} implies that the alcoholic hydroxyl groups are involved during the metal adsorption.[32] The amide I vibration (1637 cm^{-1}) of the copper-loaded CHI
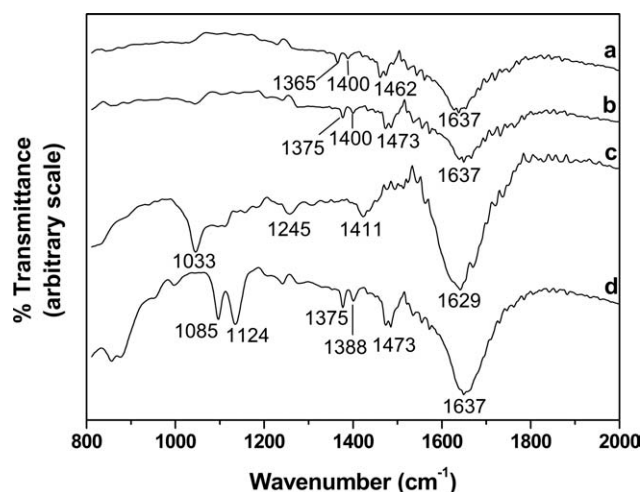

Figure 6 FTIR-ATR spectra of (a) four bilayer, (b) two bilayer, (c) alginate, and (d) chitosan membranes after incorporation of copper.
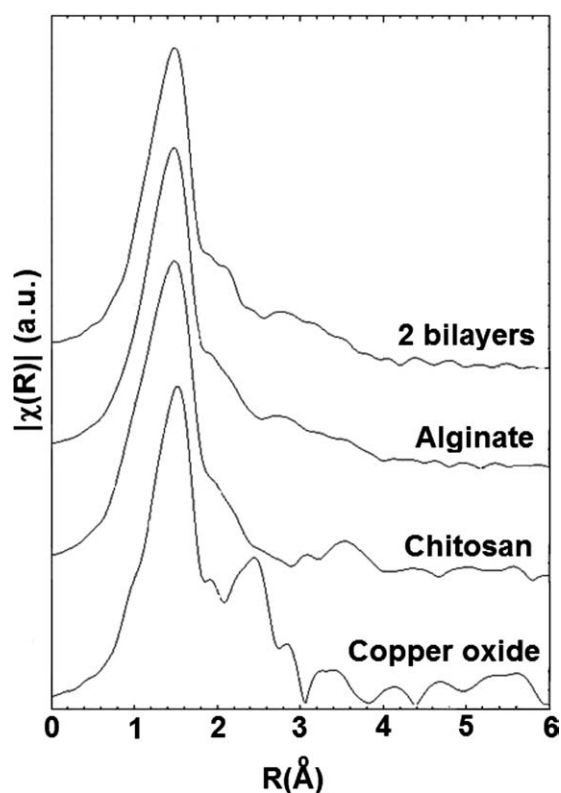

Figure 7 Function of radial distribution obtained by EXAFS for copper element in copper-incorporated membranes and in standard copper oxide

membrane [Fig. 6(d)] has a narrower peak than the pristine membrane, which confirms that amine groups interacted with copper during the metal adsorption. Copper addition resulted in a decrease in the vibration intensity in the peaks of Figure 5(a,b), as observed in Figure 6(a,b), due to the Coulombic interaction between the CHI amino groups and the SA carboxyl groups. Probably, Cu (II) ions competed with the COO^- group of SA to interact with the NH_3^+ group of CHI, thereby reducing the peak regarding the CHI-SA interaction.

EXAFS

EXAFS analysis was performed by evaluating the absorption spectra, μ (E), without any adjustment in measurements [space (k)] and the radial distribution functions (FDR) for each type of membrane. The experimental values for the edges of absorption, represented by peaks in the absorption spectra were 8986.96, 8989.99, 8982.26 eV, for the membranes of SA, CHI/SA (two bilayers), and CHI, respectively.

According to Figure 7, the CHI, SA, and CHI/SA copper membranes presented peaks at distances of 1.9, 2.1, and 2.2 Å. The shortest distance of R refers to the first level of coordination (binding distance of 1.9 Å), while the other two distances are the second
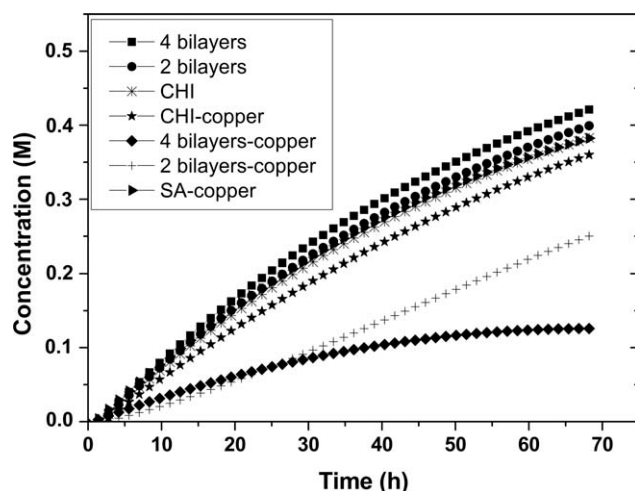
Figure 8 Ions diffusion curves of chitosan, alginate, and multilayers membranes, with and without copper.

TABLE I
Diffusion Coefficients of NaCl Through the Membranes, With and Without Copper

Membrane	Diffusivity (cm^2/s)
Four bilayers with copper	3.58×10^{-8}
Four bilayers without copper	3.26×10^{-7}
Two bilayers with copper	7.62×10^{-8}
Two bilayers without copper	1.99×10^{-7}
Alginate with copper	4.03×10^{-8}
Alginate without copper	NA
Chitosan with copper	6.69×10^{-8}
Chitosan without copper	8.68×10^{-8}

level of coordination (distances of binding of 2.1–2.2 Å). In a radial distribution function of the copper oxide, more defined peaks are noticed in positions R, within the 2 to 2.5 Å range. This difference may occur due to the fact that the standard is an inorganic compound, with substantially higher crystallinity, compared with the membranes studied. For the CHI and CHI/SA membranes, the peaks depicted in Figure 7 were related to the Cu-O and Cu-N interactions. However, it was not possible to evaluate, using the EXAFS technique, whether the Cu (II) was preferentially linked to the amino, hydroxyl or carboxyl groups. Similar results were obtained when CHI membranes were used to adsorb mercury.[33] In EXAFS analysis, atoms of nitrogen and oxygen are hardly differentiated, due to the bond distances, which are very similar.[34] Furthermore, no interference in the interaction of copper with its polymer chains due to the number of bilayers of the membrane (single layer or multilayer) was observed.

When EXAFS studies were applied to evaluate the chromium binding in CHI membranes, it was possible observe that Cr was preferably linked to the amino groups with well-defined peaks at 1.3 and 1.7 Å. This study also showed that the heavy metal ions

adsorption to CHI depended on the chemical modification of polymeric matrix, or primarily on the chemical groups available in polymeric matrices.[33]

Ion diffusion tests

The results for the ion diffusion through the different membranes, with and without copper, are shown in Figure 8. Table I describes the calculated values for diffusivity. As expected from a diffusion test, all samples presented a tendency to strike a balance between the two chambers of the diffusion cell (asymptotic curve).

In the CHI/SA multilayer membrane, the new arrangement of the chains propitiated the transport of chloride and sodium ions in larger quantities, when compared with the CHI membrane. Unfortunately, it was not possible to perform this experiment with the SA membrane, due to its low mechanical resistance, which did not support the osmotic pressure in the diffusion cell.

The diffusion coefficient values of the membranes containing copper are smaller than the values found for membranes without metal incorporation. This result shows that the addition of copper reduced mobility of polymer chains, leading to a higher difficulty for ions to diffuse through the membrane. In the two bilayers-copper membrane, the time of diffusion was higher than that observed for the CHI and CHI/SA (two and four bilayers) membranes. The copper incorporation caused a rearrangement of the chains,

TABLE II
Results of Antibacterial Test of Chitosan, Alginate, and Multilayer Membranes, With and Without Copper—
Escherichia coli

Copper incorporation	CHI	SA	CHI/SA
No	No inhibition zone present (Level 3)	Formation of a weak inhibition zone (Level 0)	Formation of a weak inhibition zone (Level 0)
Yes	No inhibition zone present (Level 0)	Formation of a weak inhibition zone (Level 0)	Formation of a 3 mm inhibition zone (Level 0)

Level 0, no growth; Level 1, weak growth; Level 2, moderate growth; Level 3, intense growth.

Results of Antibacterial Test of Chitosan, Alginate, and Multilayer Membranes, With and Without Copper—
Staphylococcus aureus

Copper incorporation	CHI	SA	CHI/SA
No	No inhibition zone present (Level 3)	No inhibition zone present (Level 1)	No inhibition zone present (Level 2)
Yes	Strong inhibition zone (Level 0)	Strong inhibition zone (Level 0)	Strong inhibition zone (Level 0)

Level 0, no growth; Level 1, weak growth; Level 2, moderate growth; Level 3, intense growth.

limiting the flow of the ions through the membrane. Furthermore, as diffusion progressed, saturation of the membrane occurred, causing the docking of the ions into the more concentrated chamber with saline solution.

Antibacterial tests

According to Table II, SA membranes exhibited antibacterial characteristics when exposed to *E. coli* culture. The same behavior was not observed in Table III for *S. Aureus*. Similar results were obtained by Pielesz et al.[35] when they exposed SA gels to *E. coli* and *S. Aureus* cultures. Although CHI is known for its antibacterial properties, in this study, CHI permitted bacterial growth, as can be seen in Tables II and III. This is probably due to the form in which CHI was molded (in the form of membranes) and, mainly, due to the necessary chemical modifications for the production of the membrane (neutralization with sulfuric acid). Heterogeneous CHI is not an appropriate material for the inhibition of bacterial growth, since its ability for bacteria inactivation is attributed to its capacity in interacting with the cellular walls of the microorganisms and, in the form of a membrane, which is chemically stable, this interaction is not possible.

Tables II and III show that all membranes prepared after copper incorporation presented the same behavior for inhibition growth: proliferation of bacteria was not observed (images of the antibacterial tests are provided as Supporting Information). The main advantage of using multilayer membranes for the incorporation of copper is the possibility of modulating the copper concentration in each layer, through an appropriate selection of polymers. In this study, CHI and SA had different degrees of affinity for this metal (caused by the pH adsorption dependence), so the concentration of copper in each of the layers was different. Future studies could include the synthesis of multilayer membranes with two or more metals adsorbed. Thus, a membrane with layers of copper and silver, for example, may have a great antibacterial potential against *E. coli*, *B. subtilis*, and *S. aureus*, as tested by Ruparelia et al.[23]

CONCLUSIONS

In this study, a simple technique of applying polycations (CHI) and polyanions (SA) was used to prepare CHI/SA multilayered membranes by casting and solvent evaporation. Additionally, the adsorption of copper ions was performed in these membranes, to access their potential for antibacterial applications. The pH dependence of the metal adsorption resulted in a higher concentration of the copper in the SA layers, as shown by scanning electron microscopy - energy dispersive X-ray spectroscopy (SEM-EDS) analysis. FTIR confirmed the Coulombic interaction between the CHI amino groups and SA carboxyl groups. EXAFS spectra identified peaks related to Cu-N and Cu-O bonds. However, EXAFS analysis did not reveal whether the Cu (II) was preferentially linked to amino, hydroxyl or carboxyl groups. Also, it was found that the ion diffusion behavior was influenced by the presence of copper. In addition, the CHI/SA-copper multilayer membrane demonstrated a proven use as a system of protection against bacterial growth, since proliferation of bacteria on these membranes was not observed.

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