J Mater Sci (2010) 45:1046–1051
DOI 10.1007/s10853-009-4039-x

Influence of cross-linking density on swelling and estradiol permeation of chitosan membranes

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Received: 22 July 2009 / Accepted: 12 November 2009 / Published online: 24 November 2009
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Abstract Chitosan membranes were obtained by a casting/solvent evaporation method and cross-linking with sodium tripolyphosphate. The effect of cross-linking time was studied and the cross-linked membranes were characterized with respect to equilibrium water content, sodium content, IR spectroscopy and permeability to a model drug (Estradiol). Sodium content increased with increasing cross-linking time. Equilibrium water content was higher in the membrane with the lowest cross-linking time. This membrane also showed a higher flux of Estradiol compared with the other which exhibited an increment in equilibrium water content and flux of Estradiol with the increase in cross-linking duration. These dissimilar results could be explained by a significant correlation between the hydrophilicity of the membrane and the hydrophobicity of Estradiol.

Introduction

Chitosan (CHT) is an abundant, low-cost, biodegradable, biocompatible and non-toxic polysaccharide with good membrane and gel-forming properties. Membranes have found applications in drug delivery systems, skin permeation simulation, scaffolding in cell culture, etc. [1]; wastewater treatment, depolluting agents, ultrafiltration, reverse osmosis and coagulation [2, 3]; packaging in food industry [4] and textile dyeing [5].

Depending on the nature of the cross-linker, membranes can be covalently or ionically cross-linked. Ionically cross-linked CHT membranes can be prepared by dipping membranes into a solution containing a cross-linking agent. Cross-linking occurs by electrostatic interactions between the negative charges of the cross-linker and the positively charged amino groups of chitosan. Cross-linked membranes can swell, retain water, and selectively permeate low molar mass molecules. Those properties are mainly influenced by the ionic interactions, which depend on the cross-linking density set during the formation of the network, size of the cross-linker, charges of CHT and cross-linker, the degree of deacetylation and the molecular weight of the CHT and the pH of the medium [1]. The cross-linking agent tripolyphosphate (TPP) can diffuse into CHT membranes and can have a high charge density [6]. Several research groups [6, 7] have reported that with more cross-linked sites formed (i.e. prolongation of cross-linking time or the increase of TPP concentration) swelling and drug release/diffusion decreased.

Nghiem et al. [8] investigated recycling of domestic wastewater effluent using thin-film composite nanofiltration membranes for the removal of Estradiol (E2) and estrone, its metabolite. These authors concluded that the transport mechanism of E2 through the membrane is diffusion rather than convection; however, the role of water in facilitating this transport remains unclear. Braeken et al. [9] evaluated the relation between the hydrophobicity of organic compounds and their retention (steady state) in nanofiltration. They tested E2 which is characterized by a high hydrophobicity and saw that retention was lower than expected, based on the molecular weight cut-off of the membranes.

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It was mentioned that two of the uses of CHT membranes are wastewater treatment and the development of drug delivery systems. Pharmaceutical formulations containing E2 are used by women for the treatment of menopausal symptoms. This female hormone is also a contaminant present in wastewater effluent.

The aim of this study is to increase knowledge on the E2 flux through CHT/TPP cross-linked membranes focusing on the water equilibrium content and sodium content at different cross-linking times.

Materials and methods

Materials

CHT was purchased from Polymar (Brazil). Sodium tripolyphosphate (TPP), E2 (purity 99%), deuterium oxide and deuterium chloride were purchased from Sigma (USA). Sodium chloride (NaCl), potassium chloride (KCl), potassium phosphate monobasic (KH_2PO_4), sodium phosphate dibasic dehydrate (Na_2HPO_4 $2H_2O$), and sodium acetate ($NaCH_3COO$) (Anedra, Argentina) were analytical grade. Acetic acid and ethanol were both PA > 99.5% (Cicarelli, Argentina). Acetonitrile was HPCL grade (Merck, Germany). The water was of Milli-Q quality.

Isotonic phosphate buffer saline (PBS, pH 7.4) was prepared by dissolving 8 g of NaCl, 0.2 g of KCl, 0.2 g of KH_2PO_4 and 1.44 g of $Na_2HPO_4{\cdot}2H_2O$ in 1 L of water.

Characterization of CHT powder

The degree of deacetylation (DD) was 77% (^{1}H NMR evaluated method referring to Lavertu et al. [10]). Briefly, a solution of CHT was prepared by dissolving 10 mg in a mixture composed of 1.96 mL of deuterium oxide and 0.04 mL of deuterium chloride, ^{1}H NMR spectra were acquired on a Bruker AVANCE 300 MHz spectrometer and the experiments were run at 70 °C. At this temperature, the solvent peaks do not interfere with any of chitosan's peaks. Viscosimetric molecular weight (MW) was 600 kDa, obtained from viscosity measurements and the Mark–Houwink relationship [11]. Briefly, the intrinsic viscosity was measured with an Ostwald capillary viscometer in 0.2 M acetic acid/0.1 M sodium acetate aqueous solution at 30 °C; then, using the Mark–Houwink equation MW was obtained.

Preparation and cross-linking of CHT membranes

Membranes were prepared by modifying the method reported by Remuñán-López and Bodmeier [7]. A 3.5 w/v% CHT solution was prepared by dissolving CHT in 2 M acetic acid solution. The solution was centrifuged during 30 min at 4000 rpm to remove air bubbles and debris. A portion was poured on a polycarbonate Petri dish and subjected to drying at room temperature until constant weight. The dried membranes were stored in polyethylene bags till use.

The dried membranes were cut into 5 cm^2 circular sections and were cross-linked by dipping in a TPP solution (10 mL, 5 w/v%) during 5–55 min. Subsequently, the membranes were washed three times with water to remove excess of TPP. All membranes were weighed and the thickness was determined (with a micrometer) before and after the cross-linking reaction. Freshly cross-linked membranes were used for water content evaluation, atomic absorption spectroscopy studies and in vitro permeation experiments. In order to verify the influence of cross-linking time with TPP on membranes properties, uncross-linked membranes were prepared by dipping CHT membranes in water without TPP during 5–55 min.

Membrane characterization

Water content

The water content was gravimetrically determined. Uncross-linked and cross-linked membranes were separated into two sets. Group 1 was conditioned in a desiccator containing silica gel until constant weight, and then introduced into bottles having 10 mL of swelling medium (40:60 v/v% ethanol:PBS solution). At predetermined times, the membranes were taken out; the excess water was removed carefully with filter paper from the membrane surface and then weighed immediately. Directly after cross-linking, group 2 was immersed into bottles with the swelling medium and weighed at predetermined times in the same way as the first group. This procedure was repeated until the membranes reached constant weight (equilibrium water content).

The water content of the cross-linked membranes was calculated according to the following equation:

$$\text{Water content per membrane (g g}^{-1}) = (W_s - W_0)/W_0 \quad (1)$$

where W_0 and W_s are the weights of dry and swollen membranes measured at different time periods.

Atomic absorption spectroscopy for sodium in CHT membranes

Sodium in CHT membranes was determined according to Method 200.3 for Sample Preparation Procedure for

Spectrochemical Determination of Total Recoverable Elements in Biological tissues [12]. Uncross-linked, freshly cross-linked membranes and membranes that reached equilibrium water content were analyzed. The determined sodium concentration is reported in microgram per membrane (μg mg^{-1}).

Infrared absorption spectroscopy

Uncross-linked and cross-linked CHT membranes were scratched to obtain the powder, then it was dried at 40 °C for 24 h. Subsequently, the powder was blended with potassium bromide and compressed to obtain discs. The IR spectrums were recorded on a FTIR-8001 PC Shimadzu spectrophotometer in the frequency range of 4000–400 cm^{-1}.

In vitro diffusion experiments

Experiments were conducted using a vertical Franz diffusion cell with a diffusion area of 1.77 cm^2 (Permegear Inc., USA). The receptor and donor compartments contained a 40:60 (v/v%) ethanol:PBS solution. The receptor fluid was thermostatically regulated to 37 °C under moderate stirring. Firstly, a freshly cross-linked membrane was clamped between donor and receptor compartments. The membrane was allowed to equilibrate overnight with the diffusion medium. Next morning the donor fluid was exchanged with a fresh donor solution containing E2 (500 μg mL^{-1}) in 40:60 (v/v%) ethanol:PBS mixture. Aliquots (200 μL) were withdrawn from the receptor compartment and assayed by HPLC. An equal volume of fresh medium was added to maintain a constant volume. The HPLC instrumentation (Shimadzu model LC-10) consisted of a ternary team of high-performance liquid chromatography with UV detection by diode array. The chromatographic system and conditions of analysis were performed, assembling USP Pharmacopoeia recommendations for E2 [13], as follows: C18 column (Spherisorb ODS2, 250 × 4.6 mm, 5 mm internal diameter), mobile phase acetonitrile:water (50:50), flow rate 1 mL min^{-1}, oven temperature 30 °C, wavelength 280 nm, stop time 8 min and injection manual volume 20 μL.

E2 flux was calculated from the slope of the linear portion of the cumulative amount permeated per unit area versus time plots. Figure 1 shows the amount of E2 diffused through CHT membrane as a function of time in a selected permeation experiment repeated 3 times demonstrating excellent reproducibility. Comparison of the values of cumulative E2 when the steady state was reached proved no statistically significant difference between the three experiments ($p > 0.05$).

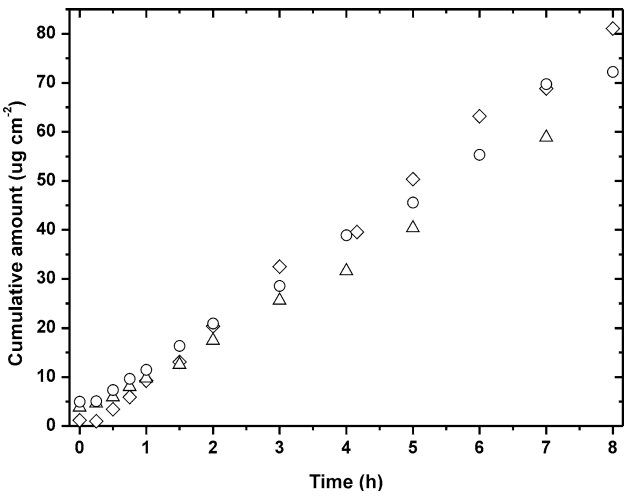

Fig. 1 Cumulative amount permeated per unit area versus time plots in a selected permeation experiment repeated 3 times (cross-linking time 30 min)

Statistical analysis

ANOVA and data analysis were done employing Statgraphics Plus 5.1. Results were considered statistically significant if $p < 0.05$.

Results and discussion

The weight and thickness of dried membranes used for all the characterization experiments resulted 0.111 g (SD: 0.010) and 170 μm (SD: 15), respectively.

All uncross-linked membranes broke during water content experiments and for this reason the water content value tended to decrease, not to the equilibrium. Also, this weight loss could be related to an erosion process, small amount of CHT could be solubilized at longer times [14]. Atomic absorption spectroscopy data show that the sodium content was lower in uncross-linked membranes than in the cross-linked membranes. However, water and sodium content did not correlate with immersion time in water. The disruption of the uncross-linked membrane is proposed as explanation.

These studies with uncross-linked membranes are strong evidence that the reaction with TPP is responsible for the integrity and the characteristics of the cross-linked membranes that are discussed hereafter.

All the cross-linked membranes retained their flexibility and integrity in the ethanol:PBS mixture and were translucent. Membranes with the lowest cross-linking time presented the highest weight after cross-linking reaction (Table 1). Cross-linking time had no effect on the thickness after cross-linking ($p > 0.05$).

Table 1 Results

	CLt (min)				
	5	15	30	45	55
Weight after CL (g)	0.367 (0.044)	0.263 (0.018)	0.297 (0.019)	0.289 (0.010)	0.285 (0.021)
Thickness after CL (μm)	330 (52)	271 (28)	297 (17)	322 (8)	311 (22)
Equilibrium WC (g g^{-1})[a]	0.976 (0.020)	0.427 (0.022)	0.422 (0.014)	0.462 (0.019)	0.468 (0.016)
Equilibrium WC (g g^{-1})[b]	1.731 (0.026)	0.440 (0.006)	0.503 (0.010)	0.581 (0.010)	0.647 (0.009)
Na^+ (μg mg^{-1}) after CL	6.73 (0.51)	19.36 (3.10)	25.43 (1.59)	34.15 (2.96)	35.55 (7.22)
Na^+ (μg mg^{-1})[a]	4.14 (0.34)	5.93 (1.94)	2.23 (0.10)	14.80 (0.21)	22.10 (0.20)
Na^+ (μg mg^{-1})[b]	2.93 (0.13)	14.99 (0.46)	18.80 (0.79)	24.58 (0.58)	28.20 (0.37)
E2 flux (μg cm^{-2} h^{-1})	9.300 (0.180)	2.458 (0.075)	8.582 (0.551)	ND	12.743 (0.365)

SD in parentheses, $n \geq 3$

CLt cross-linking time, *CL* cross-linking, *WC* water content, *ND* not determined

[a] Group 1

[b] Group 2

From equilibrium water content experiments, the membranes with the lowest cross-linking time presented the highest hydrophilicity in both groups. This could be attributed to improper cross-linking. Group 1 shows no differences ($p > 0.05$) with an increased cross-linking time (15–55 min). However, for Group 2, hydrophilicity was found to increase with an increase in cross-linking time (15–55 min) and the differences in water content were significant ($p < 0.05$). In Fig. 2, it is observed that membranes from Group 1 reached the equilibrium water content after 60 min of exposure to the ethanol:PBS solution, while membranes from Group 2 reached the equilibrium at approximately 30 min. The decrease in water content in Group 2 could be attributed to dehydration effect by ethanol which was also reported for silastic membranes [15]. This dehydration can be seen *prima facie* because the membranes became slightly brittle and compact; however, the membranes retain water inside the polymer matrix, and this water content is increasingly dependent on cross-linking time.

Atomic absorption spectroscopy data show that an increase in cross-linking time increased the Na^+ content in cross-linked CHT membranes (Table 1, Na^+ after CL) up to 45 min; after this time, it seems to reach a plateau. Moreover, the Na^+ content in both groups of membranes that were subjected to equilibrium water content was found to decrease as compared to the freshly prepared membranes, indicating that during in vitro permeation and equilibrium water content experiments the cross-linking ionic network would have broken down. This could be attributed to the pH 9 of the TPP cross-linking solution and the low DD of CHT (77%). At this pH, the ionization degree of amine groups in CHT is <10% [6], so the electrostatic interactions between the negative charge of TPP

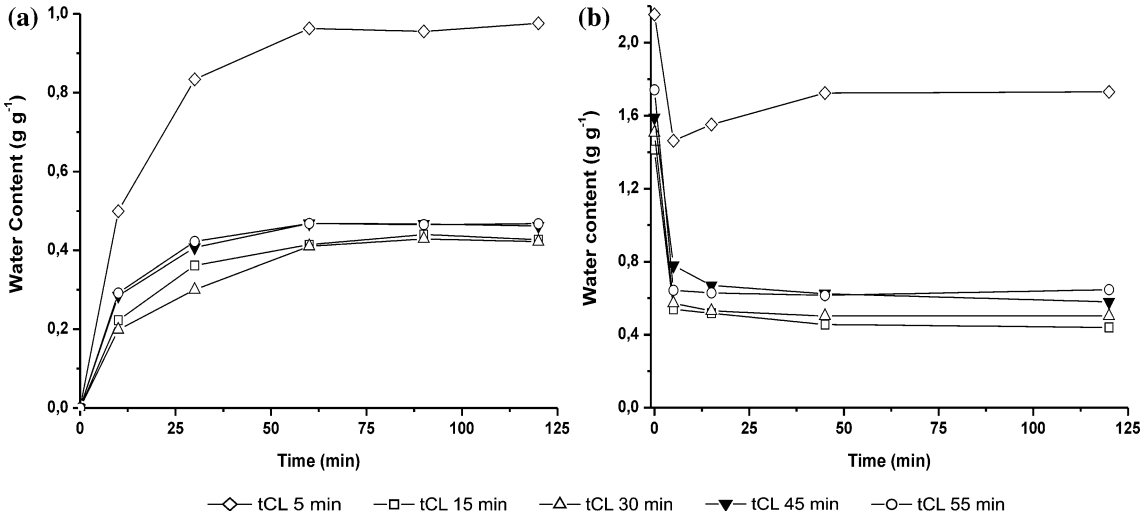

Fig. 2 Equilibrium water content: **a** Group 1, **b** group 2. *tCL* cross-linking time (min)

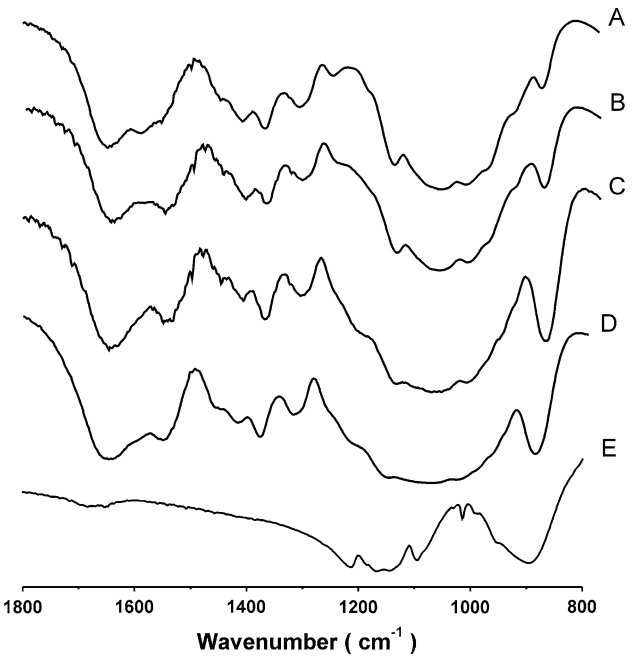

Fig. 3 IR spectra of *A* uncross-linked membrane; cross-linked membranes with cross-linking time of *B* 5 min, *C* 15 min, *D* 55 min, and *E* sodium tripolyphosphate

and the reduced positive charge of the CHT were not too extensive, but enough to allow the membranes to preserve their integrity. Nevertheless, in Group 1, there is no correlation between the decrease in Na^+ content and cross-linking time, and in Group 2 the remaining Na^+ content increases with an increase in cross-linking time.

The IR spectra of uncross-linked CHT membranes, cross-linked CHT membranes and TPP are shown in Fig. 3. Comparing all the spectra, differences were observed in the intensities of peaks at 800–1800 cm^{-1} between the uncross-linked and cross-linked membranes. Bhumkar and Pokharkar [16] reported that the peak at 1655 cm^{-1}, characteristic of amide I, can disappear in cross-linked membranes and two new peaks at 1645 and 1554 cm^{-1} appear. The disappearance of the band could be attributed to the linkage between the TPP and CHT. This can be observed in Fig. 3. Also, Bhumkar and Pokharkar [16] reported that CHT cross-linked at higher pH (pH 9) are more porous, increasing free space for diffusion and allowing the membranes to retain more water molecules inside the network. The intensities of peaks at 900–1200 cm^{-1} increase with an increase in cross-linking time, which is also the region where the peaks of TPP appear. This suggests the presence of phosphonate linkage between $–NH_3^+$ of CHT and $–O_2PO^-$ moieties of TPP during cross-linking process. This could be the reason for the increase in Na^+ content with an increase in cross-linking time.

It has been reported that with more cross-linked sites formed, swelling and drug release/diffusion decreased [6, 7]. However, in Table 1 it can be seen that the flux of E2 increases with an increase in cross-linking time from 15 to 55 min. The high flux at 5 min may be due to an improper cross-linking.

In this study, membranes with the highest cross-linking time had the highest equilibrium water content and the highest E2 flux. The explanation could be the existence of a significant correlation between the hydrophilicity of the membrane and the low water solubility of E2. Braeken et al. [9] evaluated the relation between the hydrophobicity of organic compounds, expressed by the logarithm of the octanol–water partition coefficient (log P), and their retention (steady state) in nanofiltration. These authors used three different membranes which are of hydrophilic nature and among the organic compounds they tested E2 which is characterized by a high hydrophobicity (log P: 2.72). They found that a compound with a high value of log P (hydrophobic compound) permeates relatively easily through the membranes, while a molecule with a high affinity for the water phase (negative value of log P) will be rejected. This occurs because molecules with a low (negative) value of log P generally have more polar groups, which can form hydrogen bonds with the water molecules. Hydrophobic compounds have less polar groups and are thus less solvated. Because of their smaller size, they can enter more easily into the membrane pores and permeate due to the pressure difference over the membrane. This was the explanation for the low retention observed for E2, based on the molecular weight cut-off of the membranes. So, the increase in E2 flux with an increase in equilibrium water content may be explained by the relation between hydrophobicity and hydrophilicity proposed by Braeken et al. [9].

The equation $M_t/M_\infty = Kt^{0.5}$, proposed by Higuchi [17], describes drug release as a diffusion process based on Fick's law and can be applied to analyze the kinetics of drug diffusion/release. In this equation, M_t is the amount of drug diffused at time t, M_∞ is the total amount of the drug diffused at infinite time, and K is the rate constant of drug diffusion. This constant can be determined from the slope of the plot of M_t/M_∞ versus $t^{0.5}$. An initial portion of the curve indicates that the diffusion follows a Higuchi mechanism. Since many processes can be represented by a coupling of Fickian and non-Fickian mechanisms, Ritger and Peppas [18] introduced the power law equation $M_t/M_\infty = Kt^n$ to characterize the controlled release behavior of a drug from polymeric matrixes. In this equation, the exponent n is dependent on the mechanism of drug diffusion. Both equations are applicable only to the first 60% of the release. The values of n can be calculated from the slope of ln M_t/M_∞ versus ln t. Generally for a thin film or slab, $n = 0.5$ is indicative of Fickian release and $0.5 > n > 1$ indicates anomalous transport due to swelling.

In our case, none of the membranes tested followed a Higuchi model (r^2 for the fit was less than 0.96). The correlation was better for the power law ($r^2 > 0.99$) and the n exponent was between 0.8 and 1.0. These results are in agreement with the increase in E2 flux with an increase in equilibrium water content due to an increase in cross-linking time. Thein-Han and Stevens [19] and Neto et al. [14] also reported an anomalous transport (combination of diffusion and swelling) through CHT membranes. Remuñán-López and Bodmeier [7] said that the main mechanism of chlorpheniramine maleate (a water soluble drug) diffusion through the polymeric films was that of diffusion through the water-filled pores. For this reason they saw a decrease in permeability with a reduction in water uptake and swelling, which was accompanied by the increased degree of cross-linking and thus the decreased free volume of the film.

Conclusions

The data presented in this work concern the interaction between E2 (a model drug) and CHT, a widely used biopolymer. The findings of in vitro permeation studies were explained on the basis of the contribution of the water content to the membrane. The results suggest that the existence of pore filled water inside the polymeric matrix make E2 diffusion possible because of its hydrophobicity and less solvation. This explains the increased flux with increasing water content. However, in many swollen porous polymer systems, drug diffusion could occur simultaneously through water-filled pores and through the swollen polymer per se. TPP cross-linked CHT membranes with different hydrophilicity and permeation properties can be prepared. This knowledge could have a great potential application in the recycling of wastewater effluents using CHT membranes for the removal of E2 (a currently contaminant) or in the pharmaceutical industry for the development of transdermal delivery systems.

Acknowledgements The authors thank CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas) and LIF SE (Laboratorio Industrial Farmacéutico SE-Provincia de Santa Fe) for the financial support. The authors also thank Susana Gervasio and Ignacio Rintoul for their substantial contributions and technical assistance.

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