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Hydrophilic and hydrophobic interactions in cross-linked chitosan membranes

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HIGHLIGHTS

- NMR was used to characterization of chitosan–water interactions.
- Sodium tripolyphosphate (TPP) was used as crosslinker for membrane preparation.
- First evidence of hydrophilic and hydrophobic interactions in chitosan/TPP systems.
- Results explain increase in estradiol flux with increase in crosslinking density.
- This knowledge can have potential application in medicine and pharmaceuticals.

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ABSTRACT

Chitosan membranes with different cross-linking density were prepared by modifying cross-linking time. Sodium tripolyphosphate was the cross-linking agent. A pulsed nuclear magnetic resonance study was performed on uncross-linked and cross-linked membranes. Different fraction of water molecules were identified in different zones within the membranes. The ratio of water molecules per chitosan repeating unit were calculated. A maximum of twelve water molecules were tightly coordinated to the chitosan repeating unit. Also, a very small water molecule fraction was identified but it was mobile enough as not to contribute to the dipolar interactions. The cross-linking reaction could lead to the formation of hydrophilic and hydrophobic interactions. These two types of interactions could result in the coexistence of a network formed by hydrophilic and hydrophobic micropores. This knowledge could be useful for the interpretation of results of hydrophobic drugs permeation across hydrophilic membranes. For example, the increment of estradiol fluxes across chitosan membranes with an increase in cross-linking density.

1. Introduction

Chitosan (CHT) is a polycationic polymer derived from chitin. CHT has a number of applications in agriculture, water treatment and biomedical fields. Those applications exploit the water solubility, adhesion to negatively charged surfaces, storage and transport of drugs, biocompatibility and biodegradability, among other properties of CHT based materials [1]. Commonly, the applications of CHT are intimately related to its strong interaction with charged and polar molecules. Fig. 1 presents the repeating unit of a CHT molecule. Clearly, 8-9 sites per CHT repeating unit may be able to form hydrogen bonds with polar molecules.

CHT can react with negatively charged molecules leading the formation of network through ionic bridges between polymeric chains [2]. This network can behave as a hydrogel. Hydrogels are three-dimensional hydrophilic polymer networks capable of swelling in water or biological fluids, and retaining a large amount of fluids in the swollen state. The water content in the hydrogel affects permeability, mechanical and surface properties and biocompatibility [3,4].

With the increasing use of hydrogels in applications where the diffusive transport of the water molecules is important, such as drug delivery platforms, tissue scaffolding, and membranes filtration devices, it is of great importance to understand how the polymer network changes the behavior of the imbued water [5]. Water state studies in ionic hydrogel membranes are necessary to

Abbreviations: CHT, chitosan; TPP, sodium tripolyphosphate; A, signal amplitude; T_2, spin–spin relaxation time; M_2, second moment.

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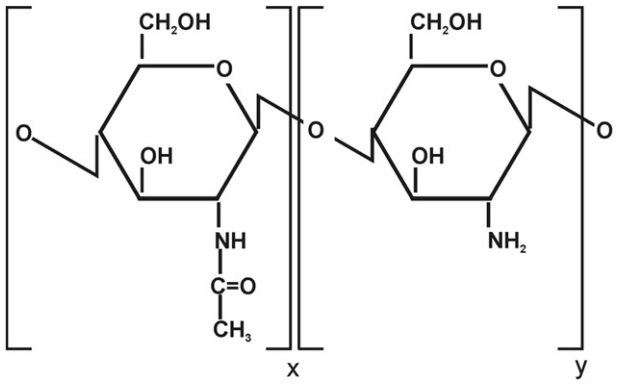

Fig. 1. Chemical structure of chitosan.

understand their dynamic and equilibrium swelling behavior as well as to analyze solute transport and other diffusive properties of such systems. In particular, the understanding of the water function in hydrogels intended for controlled drug delivery is crucial. Swelling, water penetration into the device via pores or through continuous polymeric network, creation of water-filled pores or pore closing due to polymer swelling are some of the reported mechanisms affecting the drug delivery. In addition, the water function may interfere with the type, concentration and combination of drugs and excipients, the environmental releasing conditions and the geometry and dimensions of the drug delivery system [6]. Finally, water may also act as a plasticizer or antiplasticizer depending on the concentration, temperature, structural organization of the absorbed water and the intrinsic polymer mobility, which also depends on the cross-linking density [7].

Specifically, the understanding of the water function and state in ionically cross-linked CHT membranes may provide reasonable explanations to some controversial results. On the one hand, an increase in the cross-linking density of CHT based hydrogels has been reported to decrease the final swelling and the rate of drug release [2,8]. On the other hand, a simultaneous increment of both, the equilibrium water content and the flux of estradiol, a highly hydrophobic drug, with the increase of the cross-linking density in CHT membranes was reported. The relation between the hydrophobic nature of estradiol and the hydrophilic nature of the polymer was proposed as hypothesis to explain these results [9].

Characterization of polymer–water interactions can be performed by nuclear magnetic resonance (NMR). NMR provides insight into the dynamics of water molecules and their environment [10–14]. This technique is fast, low-cost and non destructive. Samples can be recovered for other studies. Several examples of functions of water molecules in different systems using NMR spectroscopy were reported [15,16]. Richardson et al. [17,18] have determined different zones of water mobility in corn starch, depending on water content. Vackier et al. [19] have carried out a study of the state of water in gelatine gels. These authors proved that adding water to the system the mobility of the solid matrix increased. They also suggested that proton exchange became more significant as multilayer water is introduced, allowing the fraction of exchangeable gelatine protons to be added to the mobile pool. McConville and Pope [20] and McConville et al. [5] have demonstrated that valuable information about the mobility of water in contact lenses can be extracted from NMR experiments. The average mobilities of both water and the polymer are largely governed by the equilibrium water content. It explains why hydrogels with greater equilibrium water content dehydrate more on the eye.

The state of water in a hydrogel network can be studied by measuring the mobility of the protons participating in the chemical structures composing such hydrogel.

The aim of this study is to increase knowledge about water state in ionically cross-linked CHT membranes. The study is based on pulsed NMR experimental information and focused on the water equilibrium content at different cross-linking densities. This information could help to clarify the influence of water–polymer interactions in controlled release of drugs.

2. Experimental

2.1. Materials

Technical grade CHT with degree of deacetylation 77% and viscosimetric molecular weight 600 kDa (Polymar, Brazil) was selected as case study polymer. Analytical grade sodium tripolyphosphate (TPP) (Sigma, USA) was used as ionic crosslinker. Analytical grade sodium chloride (NaCl), potassium chloride (KCl), potassium phosphate monobasic (KH_2PO_4) and sodium phosphate dibasic dehydrate ($Na_2HPO_4{\bullet}2H_2O$) (Anedra, Argentina) were used for the preparation of isotonic phosphate buffer saline (PBS). PBS at pH 7.4 was prepared by dissolving 8.0 g of NaCl, 0.2 g of KCl, 0.2 g of KH_2PO_4 and 1.44 g of $Na_2HPO_4{\bullet}2H_2O$ in 1 L of water. Acetic acid and ethanol were PA >99.5% (Cicarelli, Argentina). The water was of Milli-Q quality.

2.2. Preparation of cross-linked CHT membranes and water content evaluation

CHT membranes were prepared by a casting solvent evaporation technique. A 3.5 w/v% CHT solution was prepared by dissolving the polymer in 2 M acetic acid solution. The solution was centrifuged; 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 at room temperature for a maximum of 7 days. Bags were kept in a closed container under darkness conditions until use.

The dried membranes were cut into circular sections and were cross-linked by dipping in a TPP solution (5 w/v%) during 5, 15, 30, 45 and 55 min. Immediately after the specified immersion periods, the membranes were withdrawn from the TPP solution and washed three times with water to remove excess of TPP and then introduced into glasses having 10 ml of swelling medium (40:60 v/v% ethanol:PBS solution). At a predetermined time membranes were taken out; the excess water was soaked carefully with filter paper from the membrane surface, and then weighed immediately. A constant weight indicated that membranes reached equilibrium water content.

2.3. ^{1}H NMR spectroscopy

Subsequently, after reaching equilibrium water content cross-linked CHT membranes were placed in standard NMR tubes and subjected to ^{1}H NMR measurements. Experiments were performed at 23 °C on a Bruker Minispec PC 120 spectrometer operating at a resonance frequency of 20 MHz. The magnitudes evaluated were the spin–spin relaxation time, T_2 (ms); the second moment, M_2 (ms^{-2}) and the signal amplitude, A (volts). The Carr-Purcell-Meiboom-Gill (CPMG) pulsed sequence was the method used to T_2 measurements: 90° pulse, τ, 180° pulse, 90° shift phase, 2τ, 180° pulse, 90° shift phase, 2τ. Delay time (τ) is the time between two pulses and was kept at 19 μs ($2\tau = 38$ μs). The selected 2τ value was not trivial and it depends on the T_2 value. Hence, before performing the CPMG experiments, preliminary measurements were carried out and the 2τ values were properly selected for a complete spins diphase, allowing the effective T_2 measurement. To obtain the NMR signal, 40 scans and 1560 data points were collected for each

membrane. Each membrane corresponding to each cross-linking time was analyzed three times. Experimental data were fitted according to Equation (1).

$$A(t) = A_{11}e^{\left(\frac{-a_1^2t^2}{2}\right)}\frac{\sin b_1 t}{b_1 t} + A_{12}e^{\left(\frac{-a_2^2t^2}{2}\right)}\frac{\sin b_2 t}{b_2 t} + A_2 e^{\left(\frac{-t^2}{T_{22}}\right)^{n_2}} + A_3 e^{\left(\frac{-t^2}{T_{23}}\right)^{n_3}} \quad (1)$$

van den Dries et al. [12] and Kumagai et al. [21] have proposed Equation (1) to fit the total amplitude ($A(t)$) of the resonance signal of a pulsed NMR experiment specifically designed to monitor the differential mobility of the protons composing the hydrogel network. In such conditions, protons can be classified as mobile and immobile protons. Furthermore, mobile protons can be subclassified in highly mobile and poorly mobile protons.

In Equation (1), (A_{1i}) represents the contributions of the immobile protons. A_2 and A_3 represent the contributions of the poorly and highly mobile protons, respectively. T_{22} and T_{23} are the spin–spin relaxation times of the mobile proton fractions. Protons characterized by T_{22} are less mobile than those characterized by T_{23}. b is the half-width of a rigid proton spectrum (assumed to be rectangular) and a is the standard deviation of the Gaussian line shape which is convoluted with the rigid proton spectrum. In order to monitor the mobility of the immobile protons the second moment (M_2) was calculated using Equation (2), proposed by van den Dries et al. [12] and Kumagai et al. [21].

$$M_2 = a^2 + \frac{b^2}{3} \quad (2)$$

3. Results and discussion

As stated, CHT can react with negatively charged molecules leading to the formation of network through ionic bridges between polymeric chains. TPP was selected as cross-linker due to that it is known that it can possess a high charge density and it is able to diffuse into CHT membranes during cross-linking reaction. Its use as ionic cross-linking agent has been extensively reported [9,22,23].

Water molecules are constantly in motion, even in heterogeneous environments such as polymer cross-linked networks. The attracting or binding forces rendered by the polar groups of the macromolecule on the surrounding water molecules diminish with the distance from the macromolecule. Therefore, it may be reasonable to consider that the mobility of water molecules in such a system declines, as the water molecules are close to the macromolecule (bound water). Those layers far from the macromolecule have the highest mobility (free water) [24]. In fact, the mobility of water in a hydrogel is modified relative to the bulk form due to the presence of boundaries and interfaces arising from the network structure of the polymer, which physically constrain the mobility of water molecules through electrostatic interaction and hydrogen bonding. In principle, water molecules in a hydrogel network can be found as free water or as bound water. Free water may behave as water in its bulk form. Bound water refers to water molecules attached some how to the polymer network. In general, the bound water can be divided into rotationally bound water if the water molecule forms a single hydrogen bond to the macromolecule and irrotationally bound water if the water molecule forms two or more hydrogen bonds with the macromolecule, which restrict the rotational motion of the water molecule [25].

Uncross-linked and cross-linked membranes with different crosslinking degrees, and thus, different water contents were subjected to NMR measurements. Fig. 2 shows typical normalized NMR signal versus time plots for uncross-linked membranes, and membranes with 30 min of cross-linking time. The presence of fast and slow relaxing components is observed. The first component (fast decay signal) can be regarded as immobile proton contributions, and the second component (slow decay signal), as the mobile proton contributions. The experimental data fit was satisfactorily described using values of $n_2 = n_3 = 1$ (Equation (1)).

3.1. Immobile protons

The contribution of immobile protons is described in Equation (1) by the damped sinusoidal part. Table 1 presents the fitting parameters corresponding to the immobile proton fractions. Membranes with 45 and 55 min of cross-linking time presented two rigid fractions, while membranes with other cross-linking times only one. This component in CHT structure (Fig. 1) is due to: the protons directly bound to the rings, the protons of the alcohol groups (CH_2), the protons of the acetylated and deacetylated amino groups and that of the hydroxyl groups. In addition, the water molecules in the first layer closest to the macromolecule have lowest mobility (bound water) and may contribute to the immobile protons fractions.

3.2. Mobile protons

A biexponential function resulted in the best adjustment during the fitting of the NMR signal for uncross-linked membranes and membranes with 30 min of cross-linking time. Membranes with other cross-linking times resulted better fitted with monoexponential functions. Table 2 shows the fitting parameters corresponding to the mobile protons fractions. Protons characterized by T_{22} presented relaxation times of hundreds of microseconds therefore they correspond to rotationally bound water [25]. Relaxation times of the most mobile protons characterized by T_{23} did not exceed the unity of millisecond therefore correspond also to rotationally bound water [25]. The shorter the T_2 is the stiffer the system is. Typical values are 1–2 s for liquids, 10^{-2} to 10^{-3} s for macromolecular systems in solution and 10^{-5} to 10^{-6} s for solids [13]. In addition, the lower limiting value of T_2 is 10^{-5} s and corresponds to an upper limit of the rotation correlation time (τ_R) of 3 x 10^{-6} s. Protons with τ_R values of 3 $\times$ 10^{-6} s will be called

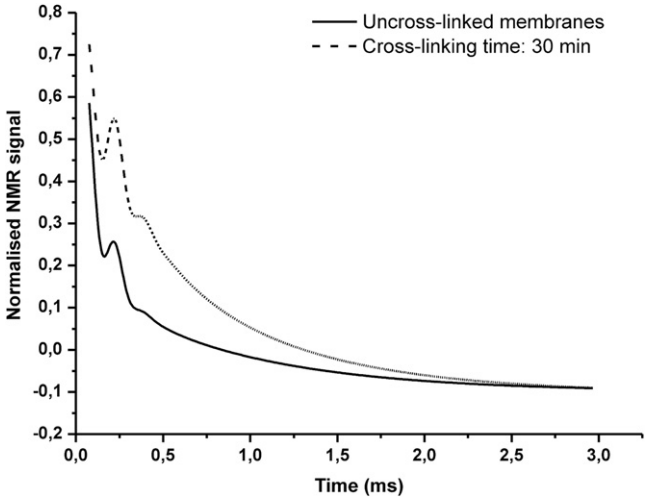

Fig. 2. NMR signal decay for water swelled uncrosslinked chitosan membranes and chitosan membranes crosslinked with TPP during 30 min.

Table 1
Immobile protons. Fitting parameters satisfying the first two terms of Equation (1) for chitosan membranes at different crosslinking times.

	Cross-linking time (minutes)				
	0.0	15.0	30.0	45.0	55.0
a_1 (ms^{-1})	7.7	4.4	6.7	5.3	2.2
b_1 (ms^{-1})	32.7	33.0	33.5	32.3	32.6
M_{21} (ms^{-2})	416.3	381.4	419.6	376.2	358.1
A_{11} (volts)	39.2	30.1	41.8	70.7	4.8
a_2 (ms^{-1})	–	–	–	4.3	9.0
b_2 (ms^{-1})	–	–	–	22.0	29.9
M_{22} (ms^{-2})	–	–	–	180.5	379.7
A_{12} (volts)	–	–	–	15.9	209.8

immobile. If the T_2 value obtained by fitting to Equation (1) becomes smaller than 10^{-5} s, it would indicate that there is no longer a mobile proton fraction and the data are only fitted to the first part of Equation (1) [26].

3.3. Cross-linking reaction and water arrangement

Membranes with 5 min of cross-linking time presented one rigid fraction and one mobile fraction with a relaxation time of 1.98 ms, corresponding to free water because relaxation time was greater than 1 ms.

When the cross-linking reaction begins a conformational variation occurs and the polymer may act in such a way to expose the hydroxyl groups to the solvent [13]. This was observed in membranes with 5 min of cross-linking time because relaxation time was greater than 1 ms. After 15 min of cross-linking time water molecules were not free but were rotationally linked due to the interactions between CHT and TPP and the stabilization of the three-dimensional network.

A reduction of M_2 takes place if the proton density in the sample decreases, because dipolar interactions decrease with the sixth power of the distance between the protons [12,27]. Table 1 shows that M_2 of membranes with 30 min of cross-linking time was greater than M_2 of membranes with 15 min of cross-linking time. Proton density decreased when distance between the axes of two linked polymer molecules increased. Table 2 shows an increase in the mobility of the most mobile protons; T_{23} of membranes with 30 min was greater than T_{23} of membranes with 15 min of cross-linking. This result seems to disagree with the remark previously stated from the analysis of the second moment: if the distance between two polymer molecules decreases in membranes with 30 min of cross-linking time, the mobility of protons from rotationally bound water should decrease. Despite acetyl group is a segmental chain and could have some mobility, the protons fraction (A_3) characterized by T_{23} did not correspond to this group. The mobility of loosely bound water is characterized by relaxation time values of several hundreds of microseconds. In addition, the percentages of the most mobile protons were different from the percentages of acetyl group. The explanation for the discordant results previously mentioned could be that the location of the most mobile water molecule fraction was not the reticulum formed by the CHT and TPP. Therefore, the cross-linking reaction with TPP could lead to the formation of hydrophilic and hydrophobic interactions. These two types of interactions could result in the total entanglement of the membrane in the coexistence of hydrophilic and hydrophobic micropores. The structure formed by the ionic interaction between CHT and TPP contributes to the formation of hydrophilic micropores, which was corroborated by the intra and intermolecular dipolar interactions registered by an increase in the second moment. The acetyl groups lead to the formation of the hydrophobic micropores because these groups do not contribute to the dipolar interactions. A very small water molecule fraction, but mobile enough as not to contribute to the dipolar interactions, would be located in the hydrophobic micropore. At this point it must be taken into consideration that the CHT used has a degree of deacetylation of 77%. Therefore, the presence of acetyl groups is high.

Table 2
Mobile protons. Fitting parameters satisfying the last two terms of Equation (1) of chitosan membranes at different crosslinking times.

	Cross-linking time (minutes)				
	0.0	15.0	30.0	45.0	55.0
T_{22} (ms)	0.106	–	0.263	–	–
A_2 (volts)	0.814	–	0.437	–	–
T_{23} (ms)	0.854	0.591	0.816	0.591	0.527
A_3 (volts)	0.362	0.823	0.602	0.806	0.868

The results presented in this work are the first evidence of hydrophilic and hydrophobic interactions in CHT/TPP systems (Fig. 3), though they are similar to those suggested for CHT/β-glycerophosphate [28]. Ruel-Gariépy et al. [28] reported that CHT has a strong potential for chemical interactions with anionic and hydrophobic materials, since it acts simultaneously as a polycationic molecule and a promoter of hydrophobic interactions due to its remaining acetyl groups. In addition, during the formation of CHT/β-glycerophosphate hydrogels, monomers of the same type (either acetylated or deacetylated) interact together. These interactions were suggested to lead the formation of two types of domains: hydrophilic domains containing the deacetylated charged monomers and most of the negatively charged β-glycerophosphate and hydrophobic domains containing the acetylated uncharged monomers [10,28].

The results of water arrangement were different for membranes with 45 and 55 min of cross-linking time and can be explained considering water content.

3.4. Cross-linking reaction and water content

According to the second moment values (Table 1), the distance between the axes of two linked polymer molecules in the hydrophilic micropores increased and the mobility of the water molecule fraction in the hydrophobic micropore decreased (Table 2).

Table 3 shows percentages of NMR signal corresponding to the different proton fractions. Membranes with 5 min of cross-linking time showed one rigid fraction whose percentage of NMR signal was 94.3% (A_{11}) and one mobile fraction with 5.7% of NMR signal. Fig. 4 shows the signal amplitude corresponding to the different

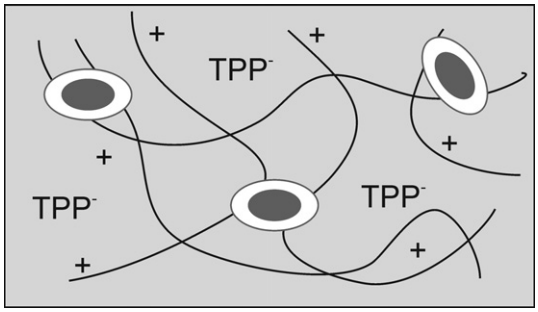

Fig. 3. (⬭) Hydrophobic zone due to hydrophobic interactions (●) between uncharged acetylated monomers; (▭) hydrophilic zone due to ionic interactions between positively charged deacetylated monomers and negatively charged tripolyphosphate.

Table 3
Percentages of NMR signal.

	Cross-linking time (minutes)				
	0.0	15.0	30.0	45.0	55.0
A_{11}	97.09	97.34	97.58	80.91	2.23
A_{12}	–	–	–	18.17	97.37
A_2	2.01	–	1.02	–	–
A_3	0.90	2.66	1.41	0.92	0.40

proton fractions versus cross-linking time. A sharp increase of spin density (proportional to signal amplitude) is observed in rigid proton fractions ($A_{11} + A_{12}$) for membranes with 45 and 55 min of cross-linking time. The percentages of NMR signal corresponding to these fractions were almost 100%. For uncross-linked membranes and the others cross-linking times were about, 97% (Table 3).

The water content in cross-linked membranes was gravimetrically determined. Membranes reached the equilibrium water content at approximately 30 min of immersion in swelling medium. As reported in a previous article [9] estradiol flux increased with the cross-linking time. Fig. 5 shows the increase in water content and estradiol flux with the increase in cross-linking time. Membranes with 5 min of cross-linking time presented a high hydrophilicity and a high estradiol flux. This may be attributed to improper cross-linking and disruption in the membrane conformation.

The amount of water protons corresponding to the different proton fractions was calculated considering the water content values and the percentages of NMR signal shown in Table 3. Subsequently, the ratios of water molecules per CHT repeating unit corresponding to the different proton fractions were calculated (Table 4).

It can be observed that membranes with 15 min of cross-linking time were able to bind 7.83 water molecules per CHT repeating unit while for 30 min, 9 water molecules (A_{11} fraction). Fig. 1 shows that there are 8–9 sites per CHT repeating unit to form hydrogen bonds. In CHT membranes with 15 and 30 min of cross-linking time water molecules were located in the first hydration layer, the closest to the macromolecule, and one rigid proton fraction was observed (A_{11} fraction). Membranes with 45 min of cross-linking time bound 10.8 water molecules in two fractions with different dipolar moments. The most rigid fraction bound 6.74 water molecules and the least rigid fraction bound 4.09. For membranes with 55 min of cross-linking time all of the water molecules (12.2) were located in the second rigid fraction (A_{12}). These membranes presented two rigid fractions with similar rigidity (Table 1). In addition, Table 4 shows that water molecules per CHT repeating unit in fraction A_3 decreased as the cross-linking time increased. This fraction represents the hydrophobic micropore whose hydrophobicity increased with an increase in cross-linking time. The enhancement of hydrophobicity would explain the increment of estradiol flux with the increase in cross-linking density (Fig. 5). Water molecules would be preferentially located or partitioned at hydrophilic sites. Additionally, water molecules in fraction A_3 for membranes with 30 min of cross-linking time presented the highest mobility (highest T_{23} value). The explanation could be that the first hydration layer was completed and the water molecules in the second layer (A_2) were not rigidly bound. T_{23} value decreased for membranes with 45 and 55 min of cross-linking time.

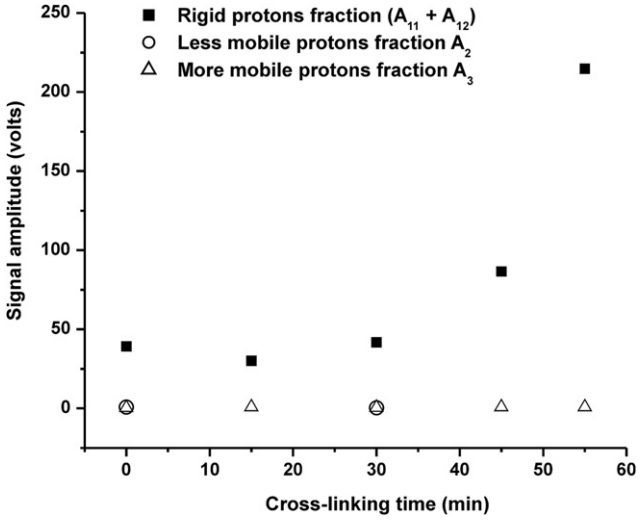

Fig. 4. Signal amplitude corresponding to the different protons fractions versus cross-linking time plots.

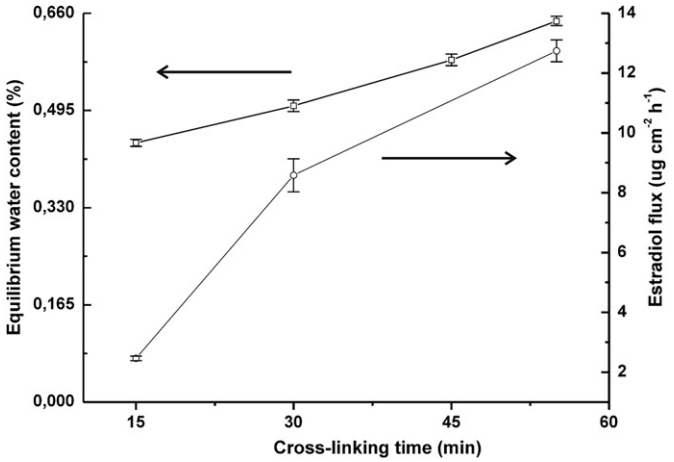

Fig. 5. Water content and estradiol flux versus cross-linking time plots.

Table 4
Water molecules per CHT repeating unit.

	Cross-linking time (minutes)			
	15.0	30.0	45.0	55.0
Total	8.36	9.56	11.03	12.29
A_{11} fraction	7.83	9.04	6.74	–
A_{12} fraction	–	–	4.09	12.20
A_2 fraction	–	0.22	–	–
A_3 fraction	0.53	0.30	0.20	0.09

4. Conclusion

NMR results showed that uncross-linked and cross-linked membranes presented one or two slowly relaxing fractions, in addition to the fast relaxing fraction attributed to the polymer network and tightly bound water. During the cross-linking reaction, a conformational rearrangement occurs in the three-dimensional structure of the hydrogel. Polymer and water molecules interact and the result of the interaction is the formation of different water molecule fractions within the hydrogel. The cross-linking reaction with TPP could lead to the formation of hydrophilic and hydrophobic interactions. As the cross-linking density with TPP increases, the hydrophilic and hydrophobic nature of the micropores increases. These two types of interactions could result in the total entanglement of the membrane in the coexistence of hydrophilic and hydrophobic micropores. The structure formed by the ionic interaction between CHT and TPP would contribute to the

formation of hydrophilic micropores. The acetyl groups would contribute to the formation of the hydrophobic micropores. The knowledge of coexistence of hydrophilic and hydrophobic interactions could have a great potential application in CHT membranes technology. In addition, could be useful for the interpretation of results of hydrophobic drugs permeation across hydrophilic membranes. For example, the increment of estradiol fluxes across chitosan membranes with an increase in cross-linking density.

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