

# Thermal behavior of cross-linked carboxymethylchitosan membranes

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## Abstract

Carboxymethylchitosan (CMCh) was submitted to reaction with genipin to result in cross-linked membranes (M-CMCh) exhibiting different average degrees of crosslinking ( $\overline{CrD}$ ) according to the concentration of genipin used during the reaction. The effects of the viscosity average molecular weight ( $0.4 \text{ kDa} < M_v < 1.9 \text{ kDa}$ ) of the parent CMCh, average degree of crosslinking ( $3 \% < \overline{CrD} < 18 \%$ ) and degree of crystallinity ( $14 \% < CI < 18.9 \%$ ) of the resulting M-CMCh on its thermal behavior and crystallinity were evaluated by carrying out TGA and XRD analysis, respectively. Results show that the cross-linked membranes (M-CMCh) are significantly less stable and exhibit lower crystallinity as compared to the parent CMCh. The viscosity average molecular weight of the parent CMCh has little effect on the thermal stability of M-CMCh while increasing the  $\overline{CrD}$  slightly decreased the thermal stability as well as the crystallinity of the cross-linked membranes.

**Keywords:** USAD chitosan, carboxymethylchitosan, genipin

## 1. Introduction

Chitin and Chitosan are a linear copolymer of  $\beta(1\rightarrow4)$ -linked glucosamine (2-amino-2-deoxy-D-glucopyranose; GlcN) and *N*-acetylglucosamine (2-acetamido-2-deoxy-D-glucopyranose; GlcNAc). Generally, chitosan is produced from chitin, a widely spread polysaccharide which is abundantly found in crustacean shells and mollusks as well as in the exoskeletons of insects [1; 2; 3]. The conversion of chitin into chitosan, named as the *N*-deacetylation of chitin, is carried out by the hydrolysis of the acetamido groups of GlcNAc units of chitin producing GlcN units.

Chitosan displays limited solubility in aqueous media but its carboxymethylation results in a water soluble chitosan derivative, namely carboxymethylchitosan (CMCh), presenting interesting physicochemical properties and biological activities, such as biocompatibility [4], biodegradability, low immunogenicity [5], antimicrobial activity [6; 7; 8; 9], antioxidant activity [10; 11], capacity to form gels [12; 13] and solubility in a wide range of acidity [14; 15]. Thus, carboxymethylchitosan has been proposed for a number applications in Pharmacy, Biomedicine and Tissue Engineering [15].

The development of self-sustained films and membranes exhibiting suitable physicochemical and mechanical properties is an important breakthrough aiming to enlarge the range of applications of carboxymethylchitosan, particularly those targeted by the Tissue Engineering. To produce stable membranes from carboxymethylchitosan, it can be cross-linked by using different crosslinker agents such as glutaraldehyde [16],

epichlorohydrin [17], 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride [18; 19; 20] and genipin [20; 21]. However, some of the commonly used crosslinker agents, mainly those of synthetic origin, exhibit high cytotoxicity and low biocompatibility [22]. Thus, when biomedical applications are aimed, it is important to avoid the use of such crosslinker agents to prevent the occurrence of undesirable harmful effects.

Recently, genipin, an iridoid isolated from the fruits of *Gardenia jasminoides* Ellis [23], has been used to produce biomaterials for tissue regeneration [24; 25; 26]. Genipin exhibits much lower cytotoxicity as compared to glutaraldehyde [27; 28] and it promotes the crosslinking of polymers by reacting with primary amine groups, functional groups present in proteins and in chitosan as well. Indeed, Sung H. W. et al. [29] reported that the genipin-crosslinked gelatin-based bioadhesive is 5,000 times less cytotoxic as compared to glutaraldehyde-crosslinked bioadhesive.

The polymers processing frequently involve melting, extrusion or sterilization. Thus, the thermal stability is important for application of this polymers and derivatives to produce stable gels, solutions, films, membranes or nanoparticles. Studies indicate that the chitin is thermally more stable as compared to chitosan, the stability and thermal behavior being affected by the average degree of acetylation [30], average molecular weight and crystallinity index [31]. De Abreu F. R. e Campana S. P. [14] showed that the carboxymethylation of the chitosan resulted in lower crystallinity and lower thermal stability when compared to chitosan.

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In the present study, emphasis is given on the thermal behavior of genipin-cross-linked carboxymethylchitosan membranes.

## 2. Experimental

### 2.1. Chitosan and carboxymethylchitosan

High molecular weight extensively deacetylated, from now on named as sample Ch0, was produced by submitting beta-chitin extracted from squid pens (*Doryteuthis spp.*) to the ultrasound-assisted deacetylation [32]. Thus, beta-chitin was suspended (1/10 w/v) in aqueous 40% NaOH, the suspension was poured in a double walled glass reactor ( $\theta_{int} = 3.5$  cm) coupled to a thermostat ( $60 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$ ) and then it was submitted to the ultrasound-assisted deacetylation process (USAD) for 50 min. A Hielscher Sonifier UP400S ultrasonic device ( $\nu = 24$  kHz) coupled to the sonotrode ( $\theta = 22$  mm) was employed and pulsed irradiation at constant ultrasound power (200 W) was adjusted. The above described process was repeated three times consecutively to yield USAD chitosan named as sample Ch0.

Sodium carboxymethylchitosan (CMCh) was prepared by reacting chitosan with monochloroacetic acid in water/isopropanol. Thus, sample Ch0 (10.0 g) was suspended in 100 mL of water/isopropanol (1/4 v/v) containing 13.5 g of NaOH, the suspension was stirred for 1 h at  $30 \text{ }^\circ\text{C}$ , then monochloroacetic acid (15 g) dissolved in isopropanol (20 mL) was slowly added to the suspension and the reaction was allowed to proceed for 4h at  $30 \text{ }^\circ\text{C}$  [7; 33]. The resulting carboxymethylchitosan, named as sample CMCh0, was isolated upon filtration, extensive washing and drying at room temperature. Aliquots of an aqueous solution of sample CMCh0 ( $10.0 \text{ g L}^{-1}$ ) were submitted to ultrasound irradiation for 1 h and 3 h to result in lower molecular weight samples, named as CMCh1 and CMCh3, respectively. The samples CMCh0, CMCh1 and CMCh3 were purified by dissolving them in aqueous  $0.1 \text{ mol L}^{-1}$  NaCl, filtering the resulting solutions to remove insoluble particles and gels, followed by the slow addition of ethanol to provoke the precipitation of the polymers, which were extensively washed with ethanol and dried at room temperature.

The average degrees of acetylation ( $\overline{DA}$ ) and of substitution ( $\overline{DS}$ ) of the polymers were determined from the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra according to the literature [33; 34; 35; 36] using a Bruker AVANCE III spectrometer ( $\nu = 400$  MHz) at  $80 \text{ }^\circ\text{C}$ . The viscosity average molecular weight ( $\overline{M}_v$ ) of the polymers were calculated from the values of intrinsic viscosity ( $[\eta]$ ), which were determined by using the AVS-360 (SCHOTT) viscometer coupled to the automatic burette (TITRONIC universal - SCHOTT). For doing so, chitosan was dissolved in aqueous solution of acetic acid  $0.3 \text{ mol L}^{-1}$  / sodium acetate  $0.2 \text{ mol L}^{-1}$  (pH = 4.5) while carboxymethylchitosan was dissolved in aqueous solution of NaCl ( $0.1 \text{ mol L}^{-1}$ ). The values of intrinsic viscosity were determined from the curves of the reduced viscosity ( $\eta_{sp}/C$ ) vs. polymer concentration (C) at infinite dilution while the values of  $\overline{M}_v$  were determined according to the Mark–Houwink–Sakurada relationship (equation 1):

$$[\eta] = KM_v^a \quad (1)$$

where  $[\eta]$  is the intrinsic viscosity,  $M_v$  the viscosity average molecular weight, and K and a, are the constants for a given polymer/solvent system and temperature [37; 38].

### 2.2. Cross-linked Carboxymethylchitosan Membranes

Aqueous solutions of CMCh (0.5 % (w/v)) and genipin ( $1.0 \times 10^{-4} \text{ mol L}^{-1}$ ,  $3.0 \times 10^{-4} \text{ mol L}^{-1}$  or  $5.0 \times 10^{-4} \text{ mol L}^{-1}$ ) were mixed and submitted to ultrasound treatment in an ultrasonic bath (UltraSonic Cleaner, model SC-1800) to eliminate air bubbles. The resulting solutions were poured into Petri dishes (solution volume/disk area =  $0.5 \text{ mL cm}^{-2}$ ) and maintained at room temperature for 24 h to allow the crosslinking reaction to occur. Following, they were submitted to freeze-drying, neutralized with acetic acid buffer  $0.3 \text{ mol L}^{-1}$  / sodium acetate  $0.2 \text{ mol L}^{-1}$  (pH = 4.5) and washed with distilled water to remove the excess of salts and genipin. After washing, the cross-linked membranes were freeze-dried again. The resulting membranes were named as M-CMChxy, where "x" (0, 1 or 3) identifies the parent CMCh samples (CMCh0, CMCh1 and CMCh3) while "y" (1, 3 or 5) stands for the concentration of genipin ( $1.0 \times 10^{-4} \text{ mol L}^{-1}$ ,  $3.0 \times 10^{-4} \text{ mol L}^{-1}$  or  $5.0 \times 10^{-4} \text{ mol L}^{-1}$ ) employed in the crosslinking reaction.

### 2.3. Average degree of crosslinking

The average degree of crosslinking ( $\overline{CrD}$ ) of the M-CMChxy samples was determined by using the ninhydrin assay [39]. Thus, the sample was freeze-dried for 24 h, weighed and then suspended in 10 mL of aqueous ninhydrin solution (2% w/v). The suspension was heated at  $100 \text{ }^\circ\text{C}$  for 20 min. and the optical absorbance of the solution was measured in a spectrophotometer UV-VIS JASCO model V-630 at  $\lambda = 400$  nm.

### 2.4. Thermogravimetry

The thermal stability of the samples was investigated by thermogravimetric analysis carried out in a TGA-50 (Shimadzu) under a dynamic atmosphere of synthetic air at a flow rate of  $20 \text{ mL min}^{-1}$ . Initially, the sample (8 mg) was heated at a rate of  $10 \text{ }^\circ\text{C min}^{-1}$  from room temperature to  $100 \text{ }^\circ\text{C}$  (staying at  $100 \text{ }^\circ\text{C}$  for 10 min to eliminate water) and then it was heated up to  $800 \text{ }^\circ\text{C}$ . Before carrying out such analysis, the sample was conditioned for 15 days in a desiccator containing silica gel.

### 2.5. X-ray diffraction

The X-ray diffraction analysis were carried out in a Bruker diffractometer model D8 Advance equipped with Lynxeye detector, employing Cu radiation ( $K\alpha = 1.5418 \text{ \AA}$ ), in the range of  $5\text{-}50^\circ$ . The crystallinity index (CI) was estimate according to [Osorio-Madrado A., David L., Trombotto S., Lucas J.-M., Peniche-Covas C. e Domard A. \[40\]](#) by using equation 2:

$$CI = \frac{A_t - A_c}{A_t} \times 100 \quad (2)$$

where  $A_c$  and  $A_t$  correspond to the area of the signals concerning the crystalline domains and the amorphous regions, respectively.

### 3. Results and Discussion

The results show that the USAD process was effective to convert beta-chitin into chitosan as sample Ch0 exhibits high average molecular weight ( $\overline{M}_v \approx 8.0 \times 10^5 \text{ g mol}^{-1}$ ) and low degree of acetylation ( $\overline{DA} \approx 4.3\%$ ). The carboxymethylation of sample Ch0 resulted in sample CMCh0, characterized by its average degree of substitution ( $\overline{DS} = 0.98$ ) and viscosity average molecular weight ( $\overline{M}_v \approx 1.9 \times 10^5 \text{ g mol}^{-1}$ ). Samples CMCh1 and CMCh3, which were produced by depolymerization of sample CMCh0 via ultrasound treatment, presented  $\overline{M}_v \approx 0.9 \times 10^5 \text{ g mol}^{-1}$  and  $\overline{M}_v \approx 0.4 \times 10^5 \text{ g mol}^{-1}$ , respectively. Thus, such data show that the carboxymethylation of USAD chitosan (sample Ch0) successfully produced carboxymethylchitosan (sample CMCh0) with defined degree of substitution ( $\overline{DS} = 0.98$ ) and viscosity average molecular weight ( $\overline{M}_v \approx 1.9 \times 10^5 \text{ g mol}^{-1}$ ). Also, the ultrasound-induced depolymerization of sample CMCh0 resulted in lower molecular weight samples, namely CMCh1 and CMCh3, as evaluated by capillary viscosity. Moreover,  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy shown that the depolymerization procedure did not provoke any changes in the polymers structure, the average degree of substitution of the three CMCh samples being identical.

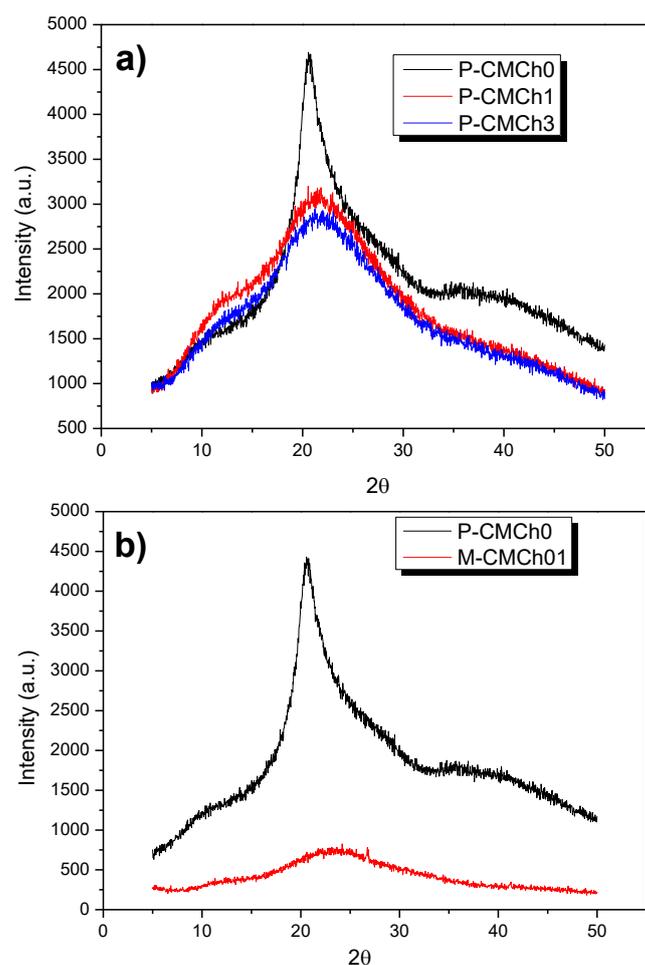
The genipin cross-linked CMCh membranes, named as M-CMChxy samples, were fully insoluble in water and its average degree of crosslinking ( $\overline{CrD}$ ) was dependent on the concentration of genipin used in the cross-linking reaction as well as on the viscosity average molecular weight ( $\overline{M}_v$ ) of the parent carboxymethylchitosan (CMCh) (Table 1). Indeed, such data show that the higher the concentration of genipin used during the crosslinking reaction, the higher the  $\overline{CrD}$  of the M-CMChxy sample while the higher the  $\overline{M}_v$  of the parent CMCh, the lower the average degree of crosslinking, regardless of the concentration of genipin. Therefore, the  $\overline{CrD}$  of M-CMChxy sample varies linearly with the concentration of genipin only when low molecular weight CMCh (sample CMCh3) was submitted to the crosslinking reaction.

**Table 1.** Average degree of crosslinking ( $\overline{CrD}$ ) of the M-CMChxy samples as a function of the viscosity average molecular weight ( $\overline{M}_v$ ) of the parent CMCh and the concentration of genipin used in the crosslinking reaction.

CMChx	$\overline{M}_v \times 10^5$ $\text{g mol}^{-1}$	[genipin] x $10^{-4} \text{ mol L}^{-1}$	M-CMChxy*	$\overline{CrD}$ (%)
CMCh0	1.9	1.0	M-CMCh01	$3.3 \pm 1.9$
CMCh0	1.9	3.0	M-CMCh03	$9.5 \pm 1.4$
CMCh0	1.9	5.0	M-CMCh05	$12.5 \pm 0.8$
CMCh1	0.9	1.0	M-CMCh11	$4.2 \pm 0.6$
CMCh1	0.9	3.0	M-CMCh13	$10.3 \pm 1.1$
CMCh1	0.9	5.0	M-CMCh15	$14.0 \pm 1.1$
CMCh3	0.4	1.0	M-CMCh31	$5.1 \pm 0.6$
CMCh3	0.4	3.0	M-CMCh33	$11.6 \pm 0.5$
CMCh3	0.4	5.0	M-CMCh35	$17.8 \pm 1.0$

\* "x" (0, 1 or 3) identifies the parent CMCh samples (CMCh0, CMCh1 and CMCh3) while "y" (1, 3 or 5) stands for the concentration of genipin ( $1.0 \times 10^{-4} \text{ mol L}^{-1}$ ,  $3.0 \times 10^{-4} \text{ mol L}^{-1}$  or  $5.0 \times 10^{-4} \text{ mol L}^{-1}$ ) employed in the crosslinking reaction.

To evaluate the effect of cross-linking on the solid state arrangements of the resulting M-CMChxy samples, its XRD patterns were compared to those of the parent CMCh samples. The DRX patterns of the parent CMCh samples in powder form show an intense signal at  $2\theta \approx 20^\circ$  which intensity clearly depends on the polymer molecular weight, *i.e.* the higher the molecular weight the more intense the signal (Figure 1 a). Also, it is observed that such a signal is sharp in the case of sample CMCh0 while it is much broader in the XDR patterns of samples CMCh1 and CMCh3, suggesting that these latter samples are less crystalline as compared to the former one. The XRD patterns of the genipin-crosslinked membranes also presented much less intense signals as compared to the corresponding CMCh in powder form, that one of sample M-CMCh01 (Figure 1 b) being representative of the whole set of M-CMChxy samples.



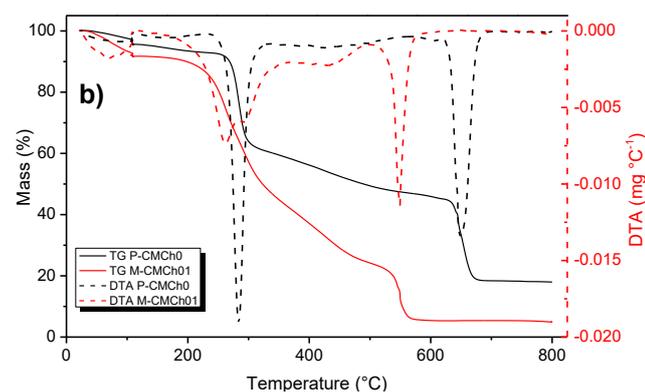
**Figure 1.** X-ray diffraction patterns of: a) samples CMCh0, CMCh1 and CMCh3 in powder form (P-CMCh); b) sample CMCh0 in the powder form (P-CMCh0) and as crosslinked membrane (M-CMCh01).

Indeed, the quantitative treatment of the XRD patterns allowed the determination of the crystallinity index ( $CI$ ) of the samples, clearly showing that the sample CMCh0, that one displaying the higher average molecular weight, is much more crystalline as compared to samples CMCh1 and CMCh3 (Table 2). Moreover, comparing the crystallinity index of M-CMChxy samples reveals that carrying out the crosslinking reaction resulted in an

important decrease of crystallinity, regardless of the molecular weight of the parent CMCh.

To evaluate the thermal stability of the samples produced in this work, firstly the parent carboxymethylchitosans in powder form are compared. Accordingly, comparing the TG curves of samples CMCh0, CMCh1 and CMCh3 in powder form (**Figure 2 a**) reveals the occurrence of the following three main thermal events: *i*) elimination of water loosely bound to the polymer network, at 25 – 110 °C; *ii*) first stage of thermal decomposition of the polymer, in which the pyrolysis rate achieves a maximum value, at 200 – 400 °C; *iii*) complementary decomposition stage, in which the products of the previous decomposition step are further degraded, at 550 – 655 °C. As in the latter thermal degradation stage the polymer structure has been significantly changed due to degradation reactions, the focus of the following discussions will be centered in the loss of water and in the first decomposition event.

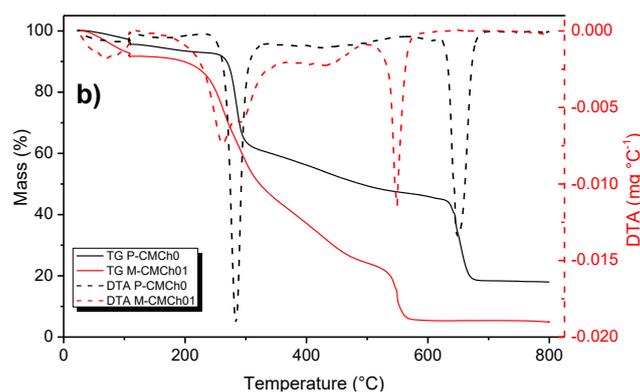
The thermal behavior of CMCh samples in powder form can be better evaluated by comparing the weight loss due to elimination of water and the onset temperature related to the first stage of thermal decomposition (**Table 2**). Such data show that the higher the crystallinity of the CMCh



**Figure 2.** TG-DTA curves: a) powder carboxymethylchitosan (P-CMCh) and; b) high molecular weight carboxymethylchitosan (CMCh0) in the powder form (P-CMCh0) and in the uncrosslinked membrane (M-CMCh01).

The TG curves of the M-CMCh<sub>xy</sub> samples show the same main three thermal events as observed in the case of CMCh in powder form, that TG curve of sample M-CMCh01 (**Figure 2 b**) being representative of the whole set of samples M-CMCh<sub>xy</sub>. The quantitative treatment of the TG curves of M-CMCh<sub>xy</sub> samples reveals that carrying out the crosslinking reaction of CMCh resulted in improved adsorption of water and lower thermal stability as compared to the parent polymers in powder form (**Table 2**). As already discussed in the cases of samples CMCh in powder form, the crystallinity index seems to be the key parameter to affect the adsorption of water and the thermal behavior of the samples M-CMCh<sub>xy</sub> too. In fact, the crosslinking degree of the M-CMCh<sub>xy</sub> samples slightly affects the adsorption of water and  $T_{onset}$  of the cross-linked membranes, a trend of higher amount of adsorbed water and lower thermal stability being observed the higher the cross-linking degree (**Table 2**). Generally, the thermal stability of a given polymer is strongly affected by its degree of order in the solid state, as the polymer chains acquires enough energy to break the interchain interactions, starting sliding on each other. Thus,

sample, the lower the water content and the higher the onset temperature of the polymer. Thus, taking into account such thermal behavior and the crystallinity index of the CMCh samples in powder form, it is concluded that the water content and the thermal stability of the polymers are directly related to solid-state arrangement of the polymer chains. Accordingly, it is observed that the more crystalline is the CMCh sample in the powder form, the lower the amount of water it can adsorb and the more thermally stable it is. It is also observed from the Tg curves that the CMCh samples in powder form presented higher ash content (e. g. P-CMCh0 presented ash content  $\approx 18\%$ ) as compared to the cross-linked membranes (e. g. M-CMCh01 presented ash content  $\approx 5.5\%$ ). This is attributed to the high content of  $\text{Na}^+$  present in the CMCh samples in powder form as they were isolated as sodium carboxylate salts and taking into account the average degree of substitution ( $\overline{DS} = 0.98$ ), the maximum ash content would be  $\approx 20\%$ . In contrast, the CMCh cross-linked membranes were submitted to neutralization with acetic acid buffer 0.3 mol L<sup>-1</sup> / sodium acetate 0.2 mol L<sup>-1</sup> (pH = 4.5) followed by extensive washing with deionized water after the crosslinking reaction and before the second freeze-drying step. Thus, electrostatic interactions involving carboxylate and ammonium groups of M-CMCh were established while the excess of sodium was washed out.



as polymers displaying low crystallinity generally present a high degree of chain entanglement, it results in an important restriction to macromolecular movements and a less efficient dissipation of thermal energy, increasing the likelihood of breakage of chemical bonds at lower temperatures.

**Table 2** – Onset temperature ( $T_{onset}$ ), water content and crystallinity index ( $CrI$ ) of CMCh samples in the powder form (P-CMCh0; P-CMCh1; P-CMCh3), and genipin-crosslinked membranes (M-CMCh01/03/05, M-CMCh11/13/15, M-CMCh31/33/35).

Sample	$T_{onset}$ (°C)	water %	CI %
P-CMCh0	271	6,4	25.8
M-CMCh01	231	8,3	17.1
M-CMCh03	229	9,8	17.9
M-CMCh05	228	10,2	18.9
P-CMCh1	264	7,7	18.5
M-CMCh11	230	8,4	14.0
M-CMCh13	229	9,2	16.3
M-CMCh15	225	12,0	14.8
P-CMCh3	261	9,4	17.4
M-CMCh31	229	12,5	14.6
M-CMCh33	228	12,6	15.6
M-CMCh35	225	14,8	15.8

#### 4. Conclusion

The results show that genipin-crosslinked carboxymethylchitosan membranes are less thermally stable the higher its crosslinking degree and the lower the viscosity molecular weight of the parent carboxymethylchitosan.

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