



Polyelectrolyte complexes based on pectin–NH₂ and chondroitin sulfate

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ABSTRACT

This work reports on the formation and characterization of a polyelectrolyte complex based on pectin (PT), functionalized with primary amine groups (PT–NH₂), and chondroitin sulfate (CS). From the simple mixture of PT–NH₂ and CS, in acid conditions, it was formed a polyelectrolyte complex, labeled as PT–NH₂/CS complex, which was confirmed through FTIR spectroscopy. The electrostatic interactions among the protonated amine groups from PT–NH₂ and the sulfate groups from CS are responsible by complex formation. XRD patterns and thermal analysis showed that the complex formation disrupts some interactions present on the PT–NH₂ and CS, but on the other hand, others are created. SEM images showed that the PT–NH₂/CS complex presents a porous and rough morphology. PT–NH₂/CS complex is new material that maintains the properties of CS with synergic association of properties from both polymers, which could maximize its applicability as biomaterial, for example.

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1. Introduction

Chondroitin sulfate (CS) is a highly water-soluble biopolymer, classified as glycosaminoglycan (Kirker, Luo, Nielson, Shelby, & Prestwich, 2002; Sinha & Kumria, 2001). It was first extracted and purified in the 1960s and is currently manufactured from natural sources (shark/beef cartilage or bovine trachea, for instance) (Volpi, 2004) or by synthetic means (Victor et al., 2009). CS is an important structural component of connective tissues and a key component of extracellular cartilage matrix (ECM). In connection with tissues, it forms 3D networks with the collagen that allow passage of macromolecules e.g., globular proteins (Block, Oegema, Sandy, & Plaas, 2010; Lammers, Tjabringa, Schalkwijk, Daamen, & van Kuppevelt, 2009; Staub, Hinzmann, & Rosenthal, 2002). This biopolymer is used for a long time in the combat and pain reduction of some diseases like atherosclerosis and osteoarthritis (Saylers, 1979; Theocharis, Tsolakis, Tzanakakis, & Karamanos, 2006). The consensus supports the use of CS for improving symptoms and stopping (or possibly reversing) the degenerative process of osteoarthritis. One of the interesting features exhibited by CS is its degradation by anaerobic bacteria, *Bacteroides thetaiotaomicron* and *Bacteroides ovatus*, which reside in the large intestine (Saylers & O'Brien, 1980; Sintov, Di-Capua, & Rubinstein, 1996). This fact suggests that the CS could be potentially a good candidate to act as a device for drug delivery in the colon region (Rubinstein, Nakar, & Sintov, 1992). However, the administration of CS in solid state

is limited due to its high solubility in water. A way to overcome this limitation is to associate the CS with other natural polymer to obtain a stable formulation allowing controlling the solubility (Wang, Chen, Wang, & Chen, 2007). Several papers report and discuss about the association of CS with other polysaccharides (Huang, Sui, Wang, & Jiao, 2010; Huang & Yang, 2008; Piai, Lopes, Fajardo, Rubira, & Muniz, 2010).

Pectin (PT), a heterogeneous polysaccharide from vegetal sources, is extracted from several kinds of fruits and vegetables and its application is concentrated in the food industry mainly (Sutar, Mishra, Pal, & Banthia, 2008). PT also shows interesting features as non-toxicity, biocompatibility, and biodegradability, which allow its association with other polysaccharides to develop drug delivery systems (Naggar, El-Khawas, Ismail, & Boraie, 1992; Sungthongjeen, Pitaksuteepong, Somsiri, & Sriamornsak, 1999). Another very interesting and attractive aspect of PT is its abundant availability in the Brazilian biodiversity. It can be obtained by extraction from natural sources (Mata, Blázquez, Ballester, González, & Muñoz, 2009) or from industrial wastes (Yoshimura, Sengoku, & Fujioka, 2005) (sugar cane or sugar beet bagasses and orange and lemon peels, for instance), which are usually discarded. This last possibility is a very positive aspect in the economic point of view.

This work discusses about the formation and characterization of a polyelectrolyte complex based on PT and CS, where PT was previously functionalized with primary amines groups. Polyelectrolyte complexes based on PT–NH₂/CS could form effective systems and maintain the properties of the CS in addition to associate the interesting properties of both polymers. The formation and discussion about the properties and behaviors exhibited by this

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polyelectrolyte complex is not yet reported in scientific literature. Furthermore, the development of this material can also be interesting with potential to act as a device for wide range of applications (tissue engineering, wound dressing, etc.).

2. Materials and methods

2.1. Materials

Pectin (PT) from apple (Galacturonic acid $\geq 74.0\%$) purchased from Sigma (USA); chondroitin sulfate, lot OP1141/08, kindly supplied by Solabia (Maringá, Brazil) with viscometric molar mass (M_v) equal to $22,000 \text{ g mol}^{-1}$ (Fajardo, Piai, Rubira, & Muniz, 2010). Ethylenediamine (99.8%) purchased from Fluka (USA); 1-ethyl-3-[3-dimethyl aminopropyl] carbodiimide (EDC) (pellets with 200–400 Mesh, 2% crosslinked with divinylbenzene) purchased from Sigma (USA); and sodium hydroxide purchased from Nuclear (Brazil). All the reactants were used as received, without previous purification.

2.2. Functionalization of PT with primary amines

Firstly, to improve the capacity of functionalization of PT with primary amine, the degree of esterification (DE) of PT was reduced through an alkaline hydrolysis reaction. A properly amount of PT was solubilized in NaOH aqueous solution (molar ratio PT/NaOH equal 1:1). The hydrolysis reaction was kept under magnetic stirring, at 50°C for 24 h. After this, the PT was collected through precipitation in ethanol and then dried by lyophilization process (Christ Gefriertrocknungsanlagen equipment, at -55°C for 24 h). The powder obtained was characterized by Fourier Transformed Infra Red spectroscopy technique (FTIR).

From the PT deesterified it was prepared an aqueous solution (2 wt%) in which were added 3 mL of ED (molar ratio between carboxyl groups on PT/ED 1:50). The resultant solution was stirred vigorously until the complete homogenization. Thus, the pH of the solution was adjusted to 5.0 with addition of HCl solution (0.2 M). Separately, an amount of EDC was solubilized in distilled water to form a solution with molar ratio between carboxyl groups on PT/EDC equal to 1:18. This last solution was added to the first one by dropping and the final solution was kept under magnetic stirring, at room temperature for 24 h to complete the functionalization reaction (Katav et al., 2008). Afterward, the PT functionalized was collected through precipitation in ethanol and put in a Soxhlet extractor for 12 h (~60 cycles) with ethanol to eliminate the ED/EDC not reacted and others possible dross. The functionalization of PT with primary amine (PT-NH₂) was accompanied by FTIR and proton nuclear magnetic resonance (^1H NMR) spectroscopy techniques.

2.3. Formation of PT-NH₂/CS complex

At first, an amount of PT-NH₂ (2 wt%) was solubilized in a 2 (v/v)-% acid acetic solution under magnetic stirring at 50°C for 4 h. A second solution was prepared by solubilization of CS (20 wt%) in distilled water at room temperature. Afterward, both the solutions were slowly mixed at room temperature to allow the polyelectrolyte complexation reaction happen. The resultant solution was stored overnight to complete complex formation. Therefore, the formed material was collected and dried by lyophilization process (-55°C for 12 h). FTIR, Thermogravimetry (TGA), and X-ray diffraction technique (XRD) characterized the material. The morphology was evaluated through SEM images.

2.4. Characterization techniques

2.4.1. FTIR

The FTIR spectra of PT, PT deesterified, PT-NH₂, CS, and PT-NH₂/CS complex powders were obtained in a Bomem (Model MB 100-C26) spectrometer, operating in the region from 4000 to 500 cm^{-1} , resolution of 4 cm^{-1} . The dried samples were blended with KBr powder and pressed into tablets before spectrum acquisition.

2.4.2. ^1H NMR

^1H NMR spectra of PT deesterified, and PT-NH₂ was recorded in Water-d₂ in a Varian spectrometer (Model Oxford 300) at a frequency of 300 MHz, using 3-(trimethylsilyl) propionic acid-d₄ sodium salt as intern reference. The relaxation delay and the angle pulse used were 30 s and 90° , respectively. The line broadening of 3 Hz was used.

2.4.3. TGA

TGA curves from PT deesterified, PT-NH₂, CS, and PT-NH₂/CS complex were performed in a Simultaneous Thermal Analysis System, Netzsch (Model STA 409 PG/4/G Luxx) with a scanning rate of $10^\circ\text{C min}^{-1}$ under N_{2(g)} atmosphere with flow of 20 mL min^{-1} in a range of temperature of 22 – 400°C .

2.4.4. XRD

The diffraction patterns of PT-NH₂, CS, and PT-NH₂/CS complex powders were obtained in a X-ray Diffractometer Shimadzu (Model D6000) with Cu-K α 40 KV and 30 mA source ($\lambda = 0.15418 \text{ nm}$).

2.4.5. SEM

The morphology of PT-NH₂/CS complex was evaluated through SEM images. For this, a PT-NH₂/CS complex sample were freeze-drying in N_{2(l)} and then fractured into small pieces. Thereafter, the pieces surfaces were coated with gold by sputtering, to make them electrically conductive and allow viewing by SEM. The images were obtained at room temperature in a Shimadzu (Model SS-550 Superscan) SEM microscope, by applying an acceleration voltage of 12 keV.

3. Results and discussion

3.1. Synthesis of PT-NH₂

The first step performed to functionalize the PT with primary amine was to reduce the degree of esterification, or DE, of the raw PT through an alkaline hydrolysis reaction. Both the raw PT and the deesterified PT were characterized through FTIR spectroscopy (Fig. 1(i)).

The FTIR spectra of raw PT (Fig. 1(i)a) exhibited a broad band at 3438 cm^{-1} assigned to stretching of -OH groups; a band at 2916 cm^{-1} assigned to C-H vibrational stretching; bands at 1751 and 1627 cm^{-1} which are assigned to C=O stretching of esterified carboxylic groups (-COOCH₃) and free carboxylic groups (-COOH), respectively. In addition, bands at 1411 , 1101 and 1019 cm^{-1} assigned to -CH₂ scissoring, >CH-OH stretching of aliphatic cyclic secondary alcohol and >CH-O-CH< stretching (Sutar et al., 2008). The FTIR spectrum of deesterified PT (Fig. 1(i)b) exhibited similarity with the FTIR spectrum of raw PT although it can be seen some clear differences between them. The most important differences are the reduction in the band intensity assigned to C-H vibrational stretching (at 2916 cm^{-1}) and the complete disappearance of the band assigned to C=O stretching of esterified carboxylic groups (at 1751 cm^{-1}). Furthermore, the band at 1627 cm^{-1} , which are assigned to C=O stretching of free carboxylic groups, has its

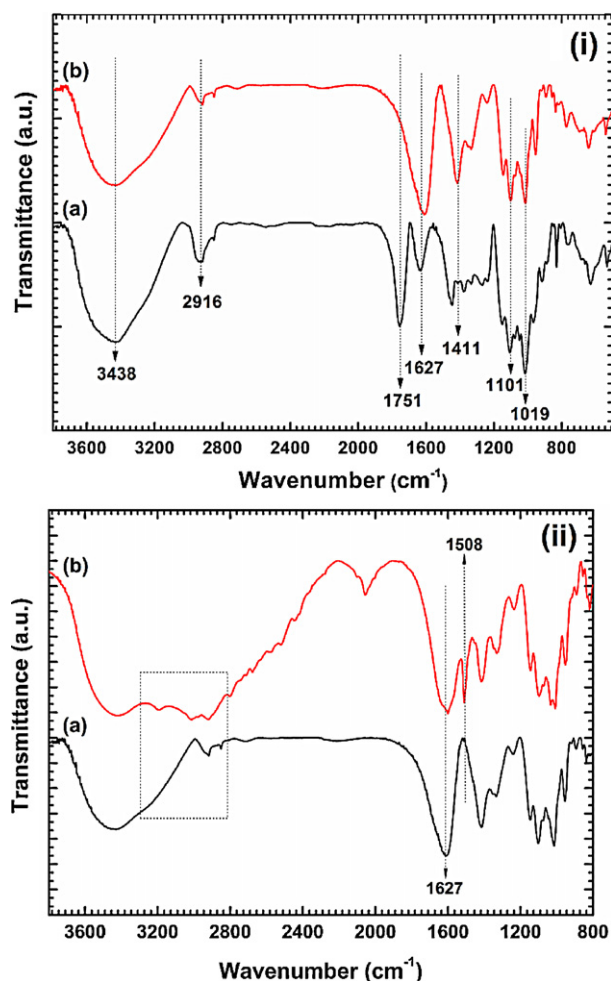


Fig. 1. (i) FTIR spectra of (a) raw PT and (b) deesterified PT and (ii) FTIR spectra of (a) deesterified PT and (b) PT-NH₂.

intensity increased. These facts show that the alkaline hydrolysis was efficient to deesterify completely the raw PT.

The deesterified PT was functionalized with primary amine from the ED in presence of EDC. EDC is a water-soluble carbodiimide applied as crosslinker agent used to couple carboxyl groups to primary amines in physiologic solutions without adding organic solvents. The insertion of primary amines on the PT chains was characterized through FTIR and ¹H NMR techniques. Fig. 1(ii) shows the FTIR spectra of deesterified PT and PT-NH₂, respectively. It is possible to verify the enlargement of the band close to 3100–3300 cm⁻¹ in the PT-NH₂ spectra, which can be assigned to N–H stretching of primary amines. At 1508 cm⁻¹ is observed a band assigned to N–H bend of those primary amines inserted on

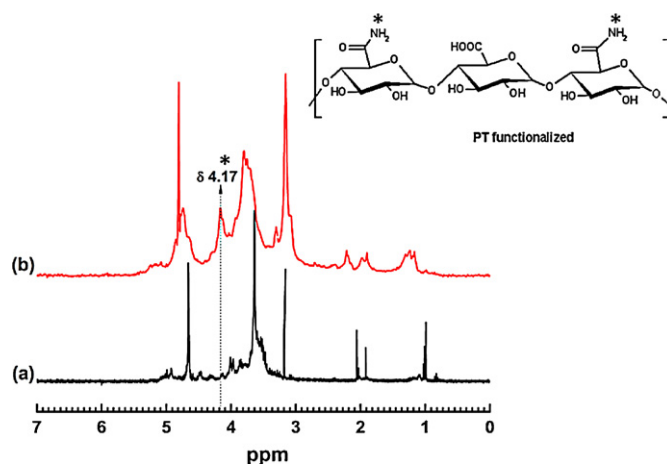


Fig. 2. ¹H NMR spectra of (a) deesterified PT and (b) PT-NH₂.

the PT chains. ¹H NMR spectra was obtained to confirm the functionalization of PT and the spectra of deesterified PT and PT-NH₂ are shown in Fig. 2.

The ¹H NMR spectrum of PT-NH₂ shows a resonance peak at δ 4.17 ppm, which was not observed in the spectrum of deesterified PT. This resonance peak can be related to hydrogen bonded to nitrogen of primary amine inserted on the PT chains. The results found by the spectroscopy techniques (FTIR and ¹H NMR) allow to infer that the PT, previously deesterified, was functionalized with primary amine groups. An illustrative scheme is presented in Fig. 3 depicting both reactions performed with the pectin.

3.2. PT-NH₂/CS complexes

The functionalization of PT with primary amines from ED using EDC, as crosslinking agent, allows obtaining amide bonds with neutral linkage. However to form a polyelectrolyte complex with CS, a polysaccharide with polyanionic feature, the PT-NH₂ must show a polycationic feature. Therefore, to create a positive charge density in the amine groups from PT-NH₂, it was solubilized in an acid medium (pH ≤ 1.5) (–NH₂ + H⁺ → –NH₃⁺). After the mixing of PT-NH₃⁺ and CS solutions (resultant pH ≈ 3), the final solution was cloudy and there was the formation of some colloidal particles. After a while, the particles precipitated and compacted to form a rigid and compact structure. The material was collected from the supernatant and dried by lyophilization. The FTIR spectroscopy technique was used to characterize PT-NH₂, CS, and PT-NH₂/CS complex (Fig. 4).

The FTIR spectrum of CS (Fig. 4a) shows a broad band between 3100 and 3600 cm⁻¹ assigned to –OH and to N–H vibrational stretching in which the –OH stretching overlaps the N–H one. The bands close to 1650 and 1050 cm⁻¹ are assigned to amide bands

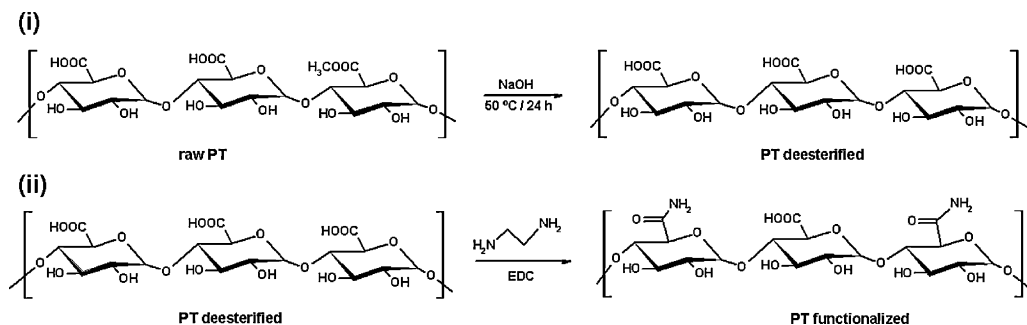


Fig. 3. Illustrative scheme of (i) deesterification reaction and (ii) functionalization of PT with primary amines.

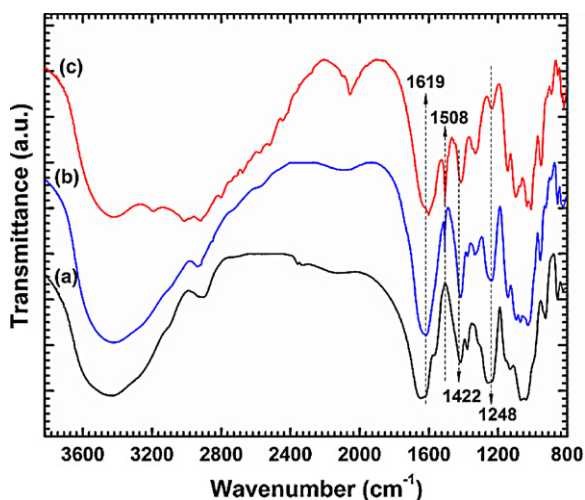


Fig. 4. FTIR spectra of (a) pure CS, (b) PT-NH₂/CS complex, and (c) PT-NH₂.

and to C–O vibration stretching. A quite broad band appears at 1422 cm⁻¹, which is assigned to C–O stretching and to O–H angular coupling vibration. This band indicates the existence of free carboxyl groups. The band assigned to the vibrational stretching of S=O bonds from sulfate groups, characteristic of CS, appears at 1238 cm⁻¹. The FTIR spectrum of PT-NH₂/CS complex (Fig. 4b) exhibited the characteristic bands from CS and PT-NH₂. On the other hand, it is possible to notice a decrease in intensity of the band in the region of 3100–3200 cm⁻¹ in relation to the band presented on the FTIR spectrum of PT-NH₂. This band is assigned to N–H stretching and its decreasing in intensity can be associated with the interaction between the protonated amine groups from PT-NH₂ (–NH₃⁺ groups) and the sulfate groups from CS (–OSO₃H groups). Other memorable differences are related to the band at 1508 cm⁻¹, which is assigned to the N–H bend of that primary amines inserted from PT-NH₂, and to the band at 1248 cm⁻¹, which is assigned to stretching of S=O from sulfate groups of CS. In the FTIR spectrum of PT-NH₂/CS complex the band at 1508 cm⁻¹ suffered a decrease in intensity whereas the band the 1248 cm⁻¹, which proves the presence of CS on the complex network, also appears with less intensity than the band observed to this wavenumber in the spectrum of pure CS. It can be inferred that these changes are due to electrostatic interaction between the amine groups from PT-NH₂ and the sulfate groups from CS.

The electrostatic interactions between these two functional groups happen because in the pH of the medium in which the complex was formed (ca. 3) the sulfate groups from CS, which present pKa close to 2.6 (Larsson, Nilsson, & Tjalve, 1981), are in the neutral form (–OSO₃H ⇌ –OSO₃⁻ + H⁺). Therefore, the –OSO₃⁻ groups from CS interact with the protonated amine groups from PT-NH₂. Other fact that contribute to form the complex is the fact that the carboxylic groups from PT-NH₂ have pKa close to 3.6 (Chronakis, Kasapis, & Abeysekera, 1997) and at pH 3 they are in their protonated form. This avoids that possible anion–anion repulsion between the –COO⁻ and –OSO₃⁻ groups happens and harm/hinder the complex formation. The pKa of amine groups from PT-NH₂ was calculated through the computational software ACD/pKa DataBase® and the average value obtained was 15.5.

Fig. 5 shows the powder XRD patterns of pure CS, PT-NH₂, and PT-NH₂/CS complex, respectively. The X-ray patterns provide additional information about the inter- and intra-molecular interactions about the complex network. The XRD pattern of pure CS (Fig. 5a) shows a broad and low-intensity diffraction peak at 2θ = 23.69°, which shows that CS has a backbone with low crystalline profile. According to Lutz, Aserin, Wicker, and Garti (2009)

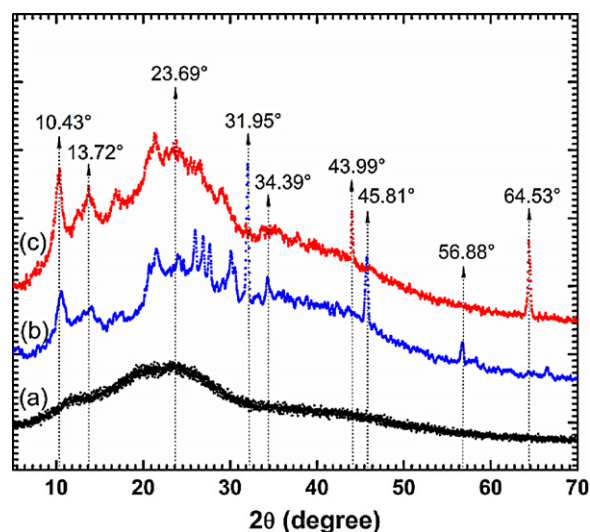


Fig. 5. Powder XRD patterns of (a) pure CS, (b) PT-NH₂, and (c) PT-NH₂/CS complex.

despite the pectins present semi-crystalline patterns the modified ones are more crystalline than the raw PT and present more diffractions peaks as observed for the XRD pattern of PT-NH₂. The XRD pattern of PT-NH₂ shows several sharp and quite intense diffraction peaks distributed throughout the 2θ-scanned region. This suggests that the ordered regions inside the PT-NH₂ backbone possess different periodic distance between itself. In addition, the appearance of these diffraction peaks reveals that the functionalization of raw PT contributed to increase the ordering of its backbone and consequently increase of its crystallinity. The insertion of primary amines, with neutral charge, into PT chains allows that hydrogen bonds among the –NH₂ groups to be created. The hydrogen bonds contribute to backbone ordering and help the formation of crystalline patterns. The X-ray pattern of PT-NH₂/CS complex (Fig. 5c) presents as well as the PT-NH₂ pattern, several diffractions peaks where some of them are in the same 2θ degree. On the other hand, it is possible to realize that the broad diffraction peak (ca. 2θ = 23.69°) proceeding from CS is present on the X-ray pattern of PT-NH₂/CS complex. In addition, as result of the electrostatic interactions between the charged functional groups from PT-NH₂ and CS, some diffraction peaks appeared or disappeared. For example, the sharp peaks at 2θ = 43.99° and 64.53° appeared while the peaks at 2θ = 31.95° and 45.81°, observed in the X-ray pattern of PT-NH₂, disappeared. The protonation of –NH₂ groups on PT-NH₂ disrupt the hydrogen bonds, which causes disappearance of some ordered regions, and the electrostatic interaction between the –NH₃⁺ and –OSO₃⁻ causes the formation of novel ordered regions within the complex network. Therefore, it can be inferred that the data from XRD technique corroborate with the FTIR data. In other words, the mixing of acid solution of PT-NH₂ with CS solution allows forming a polyelectrolyte complex where the main interaction is due to the electrostatic interaction between the –NH₃⁺ (from PT-NH₂) and the –OSO₃⁻ (from CS).

Fig. 6 shows the TGA (Fig. 6a) and DTG (Fig. 6b) curves of PT, PT-NH₂, CS, and PT-NH₂/CS complex. It can be observed two stages of weight loss. The first one (ca. 50–150 °C) is attributed to the loss of water and other volatile compounds. The TGA curve of CS shows that this first stage happens in a higher temperature and the weight loss verified is quite higher when compared with the other curves. This indicates that the amount of moisture in the CS structure is greater than in PT, PT-NH₂ or PT-NH₂/CS complex.

The second stage in the range of 200–300 °C was attributed to the thermal degradation of the samples. Fig. 7b shows the DTG data obtained from the TGA curves first derivate. Comparing the DTG

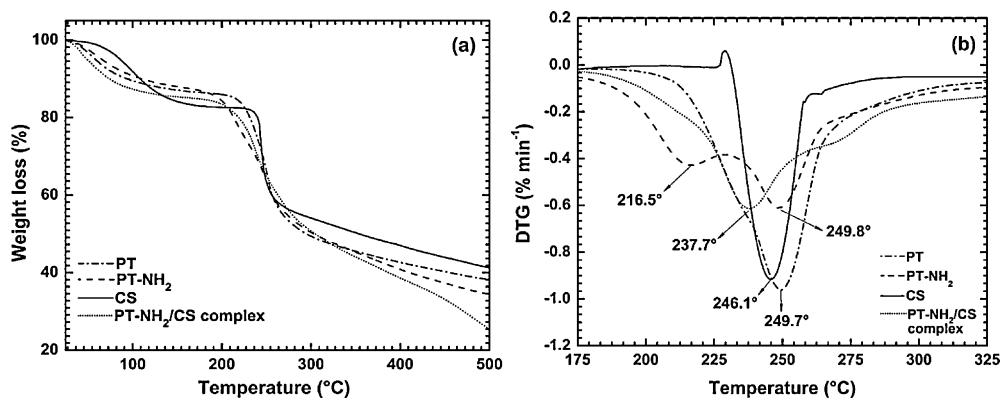


Fig. 6. (a) TGA and (b) DTG curves of PT, PT-NH₂, CS, and PT-NH₂/CS complex.

curves of PT and PT-NH₂ it is possible to verify that differently of PT, PT-NH₂ shows two temperatures for thermal degradation. The first one is verified at 216.5°C while the second is at 249.8°C. The functionalization of PT with primary amines contributes to form regions into PT-NH₂ backbone with low thermal stability, possibly due to the hydrogen bonds between the amine groups, which are weaker than the hydrogen bonds between the carboxylic groups present on the PT not functionalized. For this reason, it was observed that the second temperature of thermal degradation of PT-NH₂ happens in the same one of PT (ca. 249.7°C). This thermal profile presented by PT-NH₂ corroborates with the FTIR and ¹H NMR data demonstrating that the PT was partially functionalized with the primary amines.

For the PT-NH₂/CS complex, it was observed that its thermal degradation happens at 237.7°C. The complex is formed by electrostatic interactions between the protonated amine groups from PT-NH₂ and the sulfate groups from CS, as discussed above. Therefore, the hydrogen bonds between the amine groups from PT-NH₂ cease to exist, due to the cation–cation repulsion, and then the thermal degradation referent to these interactions is not observed in the complex DTG curve. On the other hand, the complex shows a lower thermal stability than pure CS, which degrades at 246.1°C. The complex formation although disrupts some interactions present on the pure CS and PT-NH₂ and to form another ones, as observed by the XRD patterns (see Fig. 5), does not exhibit synergistic effect in relation to the thermal stability.

The morphology of PT-NH₂/CS complex was investigated through SEM images (Fig. 7). As can be seen, PT-NH₂/CS complex shows a porous and irregular morphology with a rough

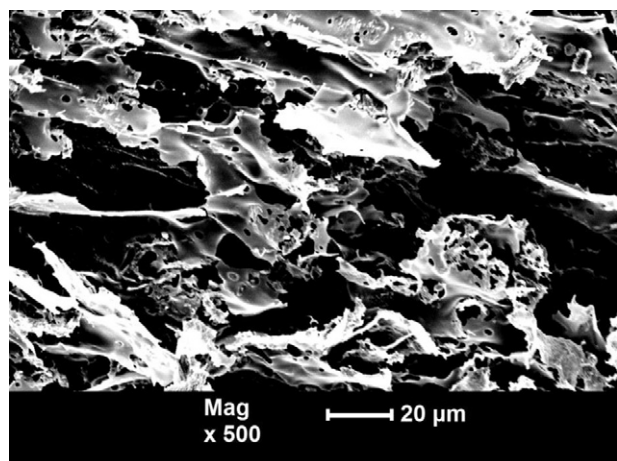


Fig. 7. SEM image of PT-NH₂/CS complex (mag. 500×).

aspect. The pores are distributed in an irregular way with different sizes too. The average pore size was calculated by means of the computational software *Size Meter*[®], version 1.1 with differentiation threshold set according to the image scale of 20 μm. Since the pore shape was undefined, the measurements were taken between the extreme points of the pores. The average was calculated from the measurement of 100 randomly selected pores. The average pore size is 2.401 ± 1.60 μm for the PT-NH₂/CS complex.

It is well-defined consensus that porous materials have desirable features to be applied as scaffold for cellular growing (Jayakumar, Prabakaran, Nair, & Tamura, 2010; Langer, 2009). This kind of material can be improved by the presence of CS, which is present in the complex developed in this work, because it could induce the cells to produce extracellular matrix components and accelerate cell growth (MacDonald, Rodriguez, Shah, & Hammond, 2010; Uygun, Stojish, & Matthew, 2009).

4. Conclusions

Pectin (PT) was successfully functionalized with primary amine groups through the reaction with ethylenediamine/EDC. The PT functionalized (PT-NH₂) was characterized by FTIR and ¹H NMR spectroscopy techniques and from the data it was possible to infer that PT was partially functionalized. From the simple mixture of PT-NH₂ and chondroitin sulfate (CS), in acid conditions, it was formed a polyelectrolyte complex labeled as PT-NH₂/CS complex. The complex is formed by the electrostatic interactions of the protonated amine groups from PT-NH₂ ($-NH_3^+$) and the unprotonated sulfate groups from CS ($-OSO_3^-$). The electrostatic interaction among the charged functional groups is strong enough to form a compact material. FTIR spectra, XRD patterns, and thermal analysis (TGA and DTG) were applied to characterize the complex. It was confirmed through FTIR spectra the interaction among the functional groups from PT-NH₂ and CS. XRD patterns and thermal analysis showed that the complex formation disrupts some interactions present on the PT-NH₂ and CS but on the other hand, others are created. It was inferred that the insertion of amine groups on PT backbone allows the formation of new ordered regions, which are formed by hydrogen bonds among the amine groups. In acid media, the amine groups acquire positive charge and the hydrogen bonds are disrupting due to cation–cation repulsion. Moreover, it is possible to realize through the TGA/DTG data that PT-NH₂ shows regions into its backbone with different thermal stability and after the complex formation, this behavior does longer occur. SEM images showed that the PT-NH₂/CS complex presents a porous and rough morphology, with the pores distributed in an irregular way.

The development and characterization of this new material, formed by polysaccharides, amplify the number of materials with potential to be applied in several fields. From the results and discussions raised in this work, it is possible in the future to test and apply its features as a biomaterial in cell growth, for example.

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