Study of Cellulose Nanocrystals and Zinc Nitrate Hexahydrate Addition in Chitosan Hydrogels

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In this study, chitosan hydrogels were produced with 0, 2, 4 and 6 wt% of cellulose nanocrystals (CNC) using hexahydrate zinc nitrate ($Zn(NO_3)_2.6H_2O$) as a catalyst for chitosan crosslinking reaction. CNC´s size was estimated by dynamic light scattering (DLS) and surface charge by zeta potential. Hydrogels were characterized by rotational rheometer, swelling test, uniaxial compression test, in vitro degradation, scanning electron microscopy (SEM) and microtomography. Results showed that zinc nitrate and CNC addition did not influence mechanical properties, degradation, and morphology of the hydrogels. However, zinc nitrate decreased 36.54% of the gel time and 41.37% of the swelling degree, and increased the crosslinking degree of the chitosan hydrogels, proving not only its catalytic effect but also its participation in the crosslinking reaction. Porosity was slightly reduced after addition of zinc nitrate and incorporation of CNC. In the mechanism of crosslinking reaction, a competition between CNC and zinc nitrate was observed.

Keywords: *chitosan hydrogel, crosslinking, glutaraldehyde, zinc nitrate, cellulose nanocrystals.*

1. Introduction

Hydrogels designed for medical applications are inherently biocompatible due to the hydrophilicity and anatomical similarity to humans, so, they can be used as a biomaterial to simulate a bone, cartilage, and others. They are three-dimensional polymer networks capable of swelling in an aqueous environment without dissolving, and they are formed by crosslinking between polymer chains, using a crosslinking agent[1,2].

Chitosan is a natural polymer of great interest in the production of hydrogels for medicine due to its biocompatibility, biodegradability and bacteriostatic activity. It can be used in polymeric scaffolds for tissue engineering, wound healing, microcapsules for drug controlled release and many others medical applications[3-5]. This polymer is also sustainable, cheap and easy to find, once it is obtained from the chitin, the second most abundant natural polymer in the biosphere[3].

Glutaraldehyde is the primary agent used in chitosan hydrogels crosslinking[6,7]. This reaction is more efficient when using catalysts such as zinc nitrate ($Zn(NO_3)_2$)[8,9]. Zinc nitrate is a material of low toxicity and often used in the synthesis of catalysts, for example, in carbon nanotubes and biodiesel[10,11].

Chitosan has low mechanical strength, then, many studies have added nanoparticles to their matrices to improve

properties. An example of nanoparticle used is the cellulose nanocrystal (CNC)[12,13].

Cellulose is the renewable organic material most abundant in the biosphere. It is a fibrous substance that has crystalline and amorphous regions. When the crystalline regions are isolated by enzymatic or acid hydrolysis, CNCs are obtained[14-16].

CNC possess many desirable properties, such as large surface area, excellent colloidal stability, high tensile strength and stiffness, and hydroxyl groups that have potential for reactions[17]. They are needle-shaped nanostructures used in composites as reinforcement to improve essential properties in materials such as thermal stability, water and mechanical resistance[18-22]. They can be used for drug delivery, protein and enzyme immobilization, support cell cultures, biosensing and bioethanol production[17,23-25].

When used as a crosslinking agent, glutaraldehyde may react with amine groups ($-NH_2$) present in chitosan and with the hydroxyls groups ($-OH$) present in the CNC, making possible different reactions like CNC-CNC molecules, CNC-chitosan molecules and between the chitosan molecules[26].

CNC addition into chitosan matrices can improve its properties, and the use of $Zn(NO_3)_2$ as a catalyst can increase the crosslinking efficiency[9,11,19]. To our knowledge, there is no report in the literature about chitosan/cellulose nanocrystals hydrogels nanocomposites using glutaraldehyde as the crosslinking agent and zinc nitrate as the catalyst,

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what makes, therefore, this work innovative and important regarding the hydrogels research.

2. EXPERIMENTAL

2.1 Materials

Chitosan with minimum deacetylation degree of 95% was supplied by Primex(r), glutaraldehyde from Sigma-Aldrich and zinc nitrate hexahydrate ($Zn(NO_3)_2.6H_2O$) from Synth(r). CNC, produced by acid hydrolysis and dried by spray dryer, were supplied by CelluForce.

2.2 Characterization of CNC

2.2.1 Dynamic Light Scattering (DLS) and Zeta Potential

The average particle diameter and zeta potential values were obtained on a Beckman Coulter Delsa Nano equipment. CNC were suspended in deionized water, and the measurements were performed at 25 ºC at an angle of 15º. For the zeta potential calculation from mobility values, the Smoluchowski equation was used, with refractive index, dielectric constant and water viscosity values at 25 ºC.

2.3 Production of Chitosan/CNC Hydrogels

A solution of chitosan 1% (w/v) with acetic acid 2% (v/v), hexahydrate zinc nitrate ($Zn(NO_3)_2.6H_2O$) and different volumes of CNC suspension was prepared to obtain nanocomposites with the desired CNC concentration (2, 4 and 6 wt%). This solution was kept in magnetic stirring during 1h, and then it was sonicated for 5 min in an ultrasonic bath (Logen Scientific LS UC2-120-3.0). After, the glutaraldehyde was added with vigorous stirring for 1 min. The resulting solution was poured into tubes internally covered with aluminum foil and kept for 24h. Then, the hydrogels were manually removed and washed three times with deionized water.

The molar ratio of glutaraldehyde:amine groups in chitosan was 0.7: 1 (mol/mol) and between $Zn(NO_3)_2.6H_2O$:glutaraldehyde was 0.05:1 (mol/mol). The hydrogels were produced according to Table 1.

2.4 Characterization of Chitosan/CNC Hydrogels

2.4.1 Rheological Measurements

The crosslinking hydrogels were studied by dynamic viscoelastic behavior as a function of time. The viscoelastic behavior was analyzed by dynamic stress sweep test at constant angular frequency (1 hz) and temperature in the range from 0 to 70 Pa in the sample of quitosan without glutaraldehyde, zinc nitrate and cellulose nanocrystals.

The gel time was determined by dynamic time sweep tests at 0.1 Pa and 25 ± 0.5ºC using a rotational rheometer (Haake - Reostress 6000) with parallel plates geometry.

2.4.2 X-Ray Microtomography

3D images and porosity values were obtained by X-ray microtomography (SkyScan 1272 Bruker microCT, Kontich, Belgium) with pixel resolution of 7.4 µm. The X-ray source was defined 20 kV of energy and 175 µA of current. Images of the cross sections of the hydrogels were obtained with a XIMEA xiRAY16 (2419 dpi) camera. Porosity values were obtained using a CTAn software (Bruker). Round cuttings of 500 µm (diameter) and with fixed height were made in different points of the hydrogels. Five measurements were performed, and the results are given as the average value. The samples were previously lyophilized to perform the analysis.

2.4.3 Scanning Electron Microscopy (SEM)

The hydrogels surface fracture morphology was studied in a FEI - Quanta Scanning Electron microscope, operating at 15 KeV, with secondary electron detectors. The samples were frozen at -80ºC, dried for 24 hours in a Labconco FreeZone 2.5 Plus lyophilizer and, then, fractured in liquid nitrogen.

2.4.4 Water Absorption Capacity Test (Swelling Test)

The hydrogels were immersed in distilled water and placed in an incubator at 37ºC and 120 rpm of agitation. The samples were removed and weighed every 15 minutes until the sample had a constant mass. The assay was performed in triplicate, and the swelling degree (SD) was calculated using the Equation 1.

$$SD(\%) = \frac{M_t - M_o}{M_o} \times 100 \qquad (1)$$

Where M_t is the swollen hydrogel mass at "t" time and M_0 is the dry hydrogel mass.

2.4.5 Uniaxial Compression Test

The uniaxial compression properties of the produced hydrogels were analyzed in a CT3 Texture Analyzer. The cylindrical samples had 10 mm of diameter and height. They were submitted to uniaxial compression with speed of 0,2 mm s^{-1} until fracture. The assay was performed at room temperature.

2.4.6 In Vitro Degradation

To evaluate in vitro degradation behavior, hydrogels were placed in saline solution (pH 6) for 70 days. In the first 28 days, the hydrogels were removed from the solution every 7 days, washed with distilled water, dried with absorbent

Table 1. Hydrogels description

Hydrogel	Description
C	Without zinc nitrate and without CNC
C/N	With zinc nitrate and without CNC
C/N/CNC 2	With zinc nitrate and 2% CNC
C/N/CNC 4	With zinc nitrate and 4% CNC
C/N/CNC 6	With zinc nitrate and 6% CNC

paper and weighed; but from 29th to 70th day, this procedure occurred every 14 days. The saline solution was changed at each weighing. The test was performed on 5 samples of each hydrogel. Weight loss evaluated the degradationas a function of the time. In Equation 2, M is the percentage of remaining mass in relation to the initial mass, M_0 is the initial hydrogel mass and M_f is the hydrogel mass after degradation.

$$M(\%) = \frac{M_f}{M_o} \times 100 \qquad (2)$$

3. Results and Discussions

3.1 Cellulose Nanocrystals Characterization

3.1.1 Dynamic Light Scattering (DLS) and Zeta Potential

The average CNC particle size in water suspension estimated by dynamic light scattering was 118 nm with a polydispersity of 0.317. The zeta potential in deionized water was - 45 mV. Then, this might be considered a stable CNC suspension, because the modulus of the obtained value is greater than 25 mV[27]. This value of zeta potential is suitable for the production of aqueous chitosan solution, once it will result in a stable colloidal suspension, resulting in a good dispersion of CNC into de hydrogels.

Although DLS technique assumes that each particle has spherical shape to calculate the hydrodynamic diameter, results can be used to prove the nanometric size and to compare particle size from others studies. Montanheiro *et al*. reported average diameter for CNC between 42 and 130 nm[28], which is in accordance with the results obtained.

3.2 Chitosan/Cellulose Nanocrystals Hydrogels Characterization

3.2.1 Rheological Measurements

The rheological properties of the hydrogels were studied by dynamic viscoelastic behavior as a function of time.

Figure 1 shows the viscoelastic test of the chitosan without zinc nitrate, CNC and glutaraldehyde (uncrosslinked hydrogel), that is the solution of chitosan used in hydrogels production. Viscoelastic behavior was found at 0.1 Pa. Thus, this stress was used to analyze gel point of the hydrogels.

Gel point is defined as the beginning of cross-linking during a gelation reaction, in which elastic and viscous modulus (G 'and G") intersect, and, from that instant on, G' becomes larger than G"[29]. Figure 2 (a-e) shows dynamic time sweep tests with G 'and G" of the hydrogels C, CN, C/N/CNC2, C/N/CNC4 and C/N/CNC6. Gel time (t gel) and critical shear modulus (Gc) values of this samples are in Table 2.
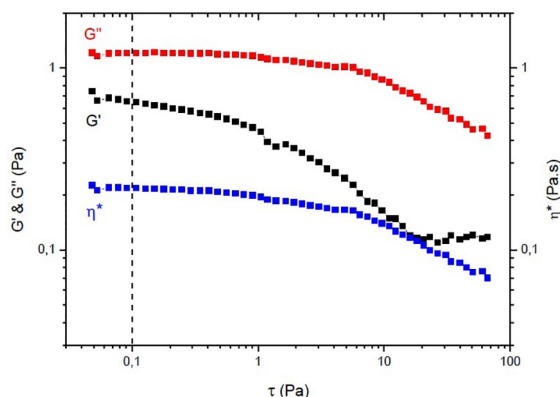

Figure 1. Dynamic stress sweep test of the uncrosslinked chitosan hydrogel.

According to Figure 2 and Table 2, it can be observed that the gel time, comparing sample C to CN, decreased 36.54%, confirming the catalytic effect of the zinc nitrate[8,9]. However, when CNC was added, for all concentrations, the gel time increased compared to the C/N sample. Glutaraldehyde may react with amine groups (-NH$_2$) present in chitosan and with the hydroxyls groups (-OH) present in the CNC, making possible different reactions like CNC-CNC molecules, CNC-chitosan molecules and between the chitosan molecules. This behavior can generate competition in the mechanism of the crosslinking reaction, changing its kinetics and its efficiency[26].

The graphs of the complex viscosity (η^*) as a function of time (t) of the crosslinked chitosan hydrogels are shown in Figure 3. It is observed that the zinc nitrate addition also increased the viscosity of hydrogel (samples C and NC). The increasing of viscosity can be related to an increase in the crosslinking degree of the chitosan hydrogels. It was proved that, zinc nitrate not only act decreasing the crosslink time but also contributed increasing the crosslinking degree of the chitosan hydrogel. However, CNC addition decreased the hydrogels viscosity and also the crosslinking degree. This behavior can also be explained by competition in the mechanism of the crosslinking reaction, previously mentioned[26]. Therefore, increasing CNC concentration in C/N hydrogel causes, at first, a decrease in viscosity of hydrogel, as verified for C/N/CNC 2 and C/N/CNC 4 hydrogels (Figure 3).

Opposite behavior was found by Zhou *et al*.[30-31] with polyacrylamide-CNC hydrogels. The CNC accelerated the formation of hydrogels and increased the effective crosslink density of hydrogels. Thus, the authors found that CNC were not only a reinforcing agent for hydrogel, but also acted as a multifunctional cross-linker for gelation.

Addition of fillers in polymer solutions hampers the polymer chairs flow and increase viscosity of the mixture. This way, although CNC filler causes a decrease of viscosity due the decrease of crosslinking degree, higher CNC concentrations
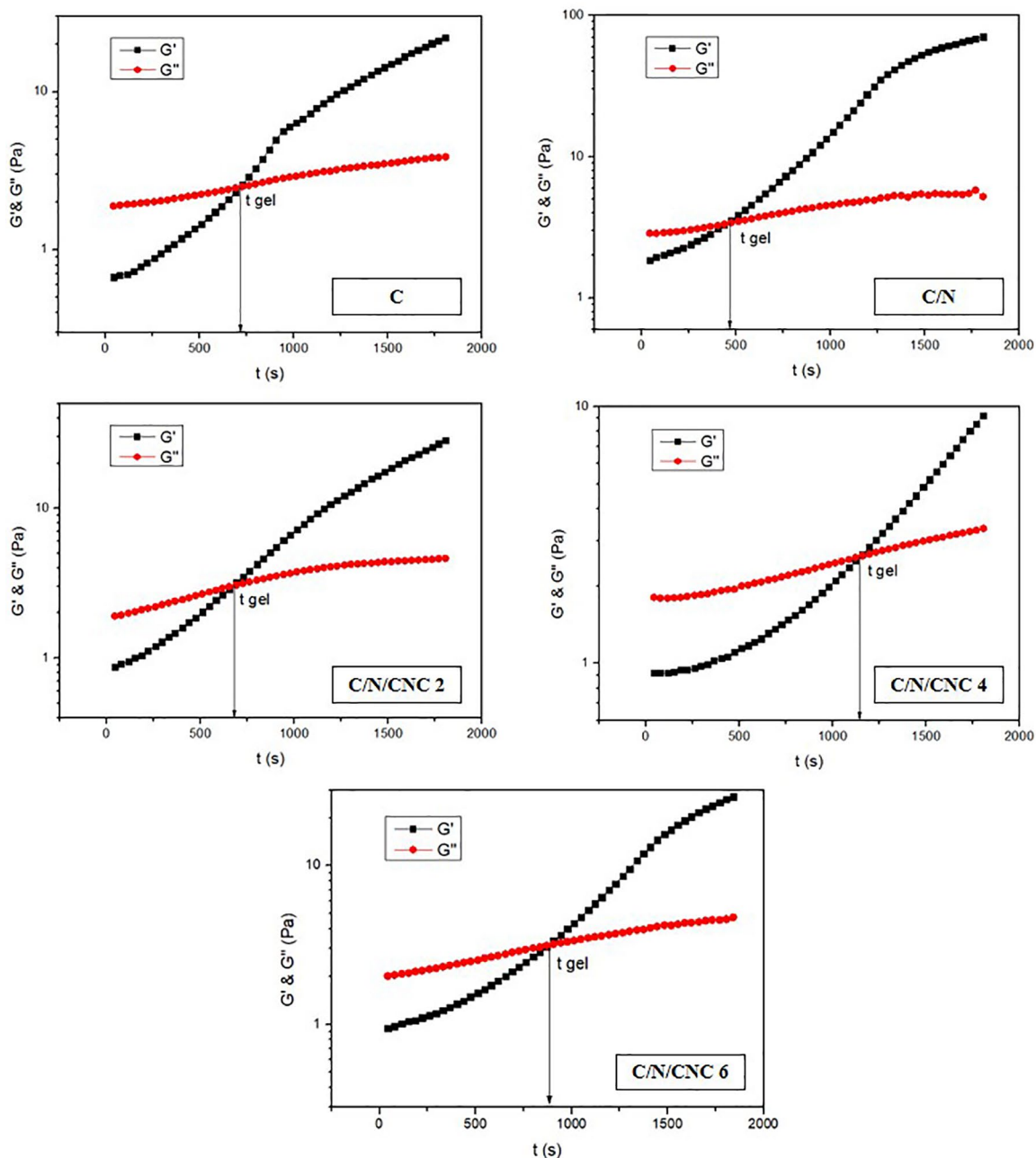
Figure 2. Time dependence of G' and G" for the C, C/N, C/N/CNC2, C/N/CNC4 and C/N/CNC6 hydrogels.

Table 2. Gel time (t gel) and critical shear modulus (Gc) values of the hydrogels.

Hydrogel	t gel (s)	Gc (Pa)
C	717	2.51
C/N	455	3.40
C/N/CNC 2	676	3.04
C/N/CNC 4	1152	2.63
C/N/CNC 6	905	3.36

promote, simultaneously, an increase in the viscosity of the mixture. Therefore, C/N/CNC 6 hydrogel showed higher viscosity than C/N/CNC 4 hydrogels.

3.2.2 X-Ray Microtomography

Figure 4 shows 3D images of the hydrogels obtained by X-ray microtomography. It can be observed, for all samples, highly porous morphology, without density variations, since

X-rays have distinct behavior when passing through solids with different densities. So, in the case of density variations, it would be possible to observe color changes in the scaffolds. It may be concluded then, that CNCs were satisfactorily dispersed among the samples[32].
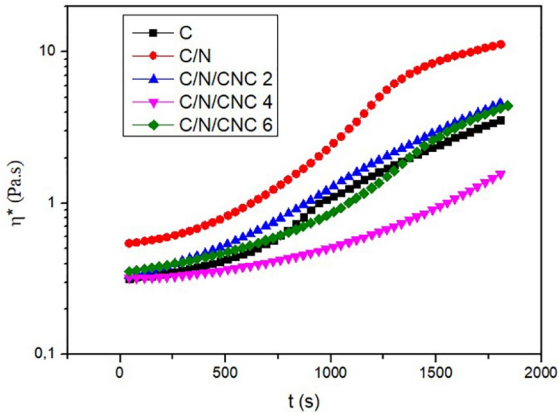
Porosity could also be determined, and it is shown in the graph from Figure 5. It can be noted that the values are all higher than 80%, and the standard deviations are small, meaning that the pores were homogeneously distributed in the samples. Despite it seems that the values are all very
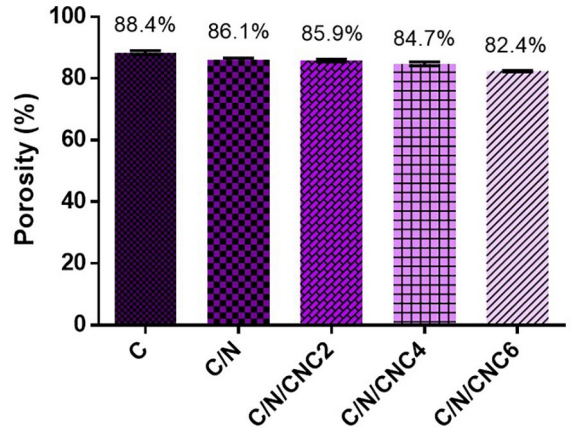

Figure 3. Graph of the complex viscosity (η^*) as a function of time (t) of the hydrogels.

Figure 5. Porosity values of the hydrogels obtained by X-Ray microtomography. Results are given as mean ± SD (n = 5).

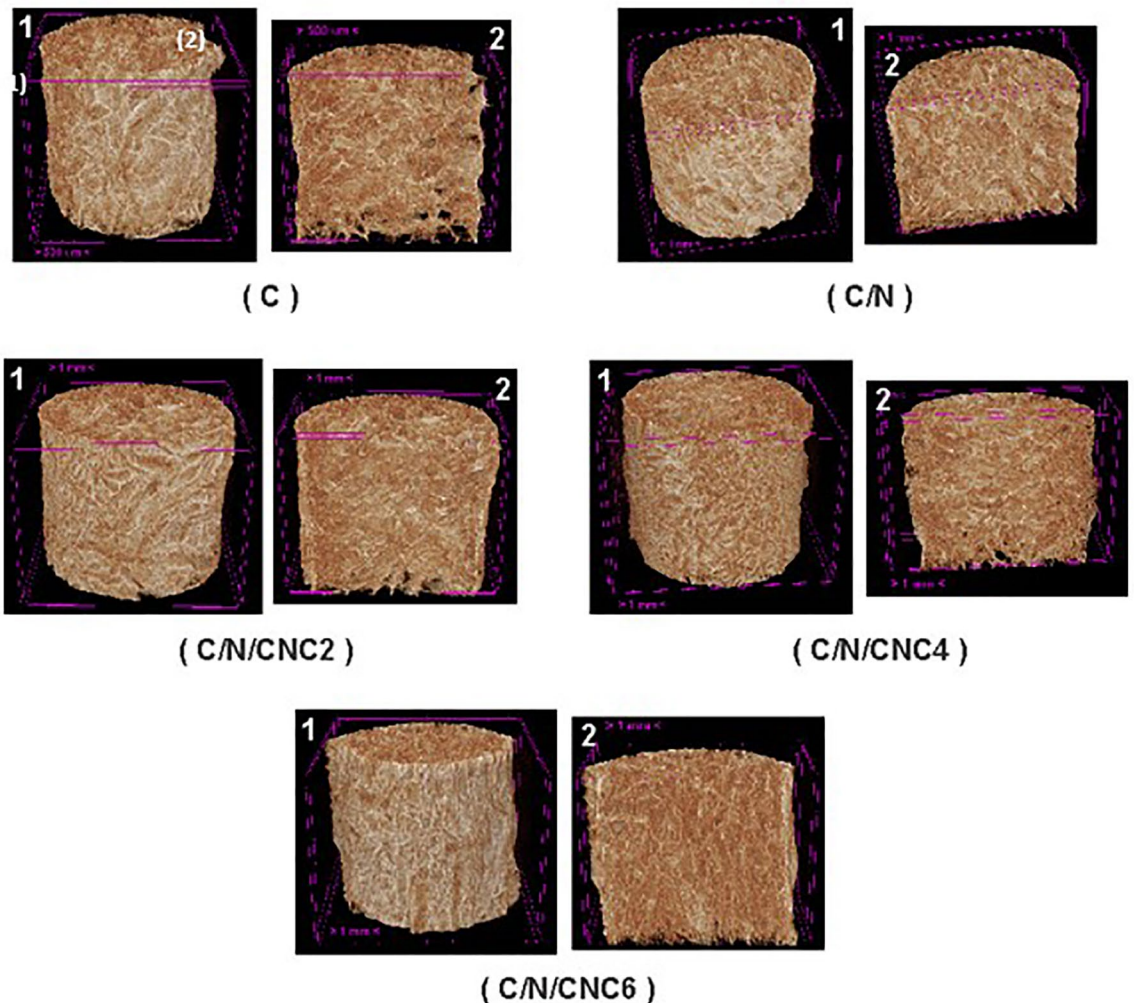(C)

(C/N)

(C/N/CNC2)

(C/N/CNC4)

(C/N/CNC6)

Figure 4. 3D view of C, C/N, C/N/CNC2, C/N/CNC4 and C/N/CNC6 hydrogels. Front view (1) and cross section (2).

close from each other (mean standard deviation 0.5%), statistical differences were found between samples (P<0.05 was considered significant). The addition of 2% CNC did not change significantly the porosity of the hydrogel, compared to the sample without CNC, but, except this case, there are statistical differences between all samples. The porosity was reduced with the addition of zinc nitrate and CNC.

As discussed on rheological results, the addition of zinc nitrate increased the viscosity of hydrogel and the crosslinking degree, what resulted in lower porosity. However, the CNC addition decreased the crosslinking degree as verified by the decreasing of hydrogels viscosity, but also reduced porosity, what was not expected.

Zeng et al.[33] produced crosslinked chitosan membranes, using glutaraldehyde as the crosslinking agent. Authors changed glutaraldehyde concentration and evaluated porosity of the obtained membranes. They reported that increasing the crosslinking agent concentration, and, consequently the crosslinking degree, led to a reduction in the porosity. This behavior was similar to the observed with hydrogels in the present work and was attributed to the formation of a continuous and more compact network, resulting in a less porous structure.

3.2.3 Scanning Electron Microscopy (SEM)

The scanning electron micrographs of the hydrogels' fracture surfaces are shown in Figure 6. As already observed on microtomography images, the images of different hydrogels did not show any change in terms of size, quantity or distribution of the pores, so, it can be concluded that zinc nitrate and CNC addition did not change the chitosan hydrogels morphology. Similar morphology was observed in the study of Shen et al.[34], which produced chitosan-gelatin hydrogels via in situ precipitation. Bi et al.[35] produced chitosan/collagen scaffolds for cartilage tissue engineering crosslinked with genipin and observed similar morphology, including pore wall with paper-like morphology.

3.2.4 Swelling Test

The average values and standard deviation of hydrogels swelling degree are in Figure 7. Comparing values of C and C/N samples, it is observed that the zinc nitrate addition decreased about 41% the swelling degree (p<0.05). This result is in accordance with the rheological analyses (figures 2 and 3), which showed that the addition of zinc nitrate increased the crosslinking degree of chitosan hydrogels. It may also be related to the reduction in porosity. However, there were no statistical differences between CNC samples (2, 4 and 6 wt%) and the swelling degree remained around 63% for these samples. The increase of crosslinking degree decreases the water absorption capacity due to the reduction of free OH groups present in chitosan and CNC, and the formation of a more rigid structure.

The addition of CNC caused a decrease in the crosslinking degree, verified by the viscosity decrease for hydrogels with CNC. Decreasing crosslinking degree would increase the swelling degree. However, increasing CNC concentration would increase the stiffness on hydrogel walls, which would decrease swelling ability. Thus, due to the coexistence of conflicting effects, there was no significant difference in the swelling degree, as expected.

3.2.5 Uniaxial Compression Test

The average values and standard deviation of hydrogels maximum strain and stress are shown in Figure 8.

The average values for maximum strain and stress were 27.4% and 28.0 kPa, respectively. Statistical analysis did not show significant differences between the samples. It is concluded that zinc nitrate and CNC addition did not influence the mechanical properties of the chitosan hydrogels. As observed by microtomography, the porosity suffered minor changes with the introduction of zinc nitrate and CNC, meaning that the general structure of the hydrogels was not changed. The maintenance of general structure could also be observed in SEM images. For this reason, mechanical properties were expected not to suffer great changes; so the values of maximum strain and stress showed above are in accordance to the previous results reported.

Yue et al.[36] reported significant improve on compression stress for sodium alginate-polyvinyl alcohol hydrogels reinforced with cellulose nanofibers (CNF). The enhancement in mechanical compression property was attributed to well dispersed CNF into the hydrogel structure.

Herein, what could have happened is an insufficient dispersion of CNC into the matrix, due to the high CNC concentration used. We may assume that CNC did not introduce imperfections in the hydrogel structure; however, it was not sufficiently well dispersed to improve mechanical properties.

The absence of improvements in mechanical properties due to inefficient filler dispersion was also reported by Jiang et al.[37]. When CNC is not satisfactorily dispersed, the material obtained behaves like composites reinforced with micron-sized fillers, and no significant differences are observed in the final mechanical property of the material.

3.2.6 In Vitro Degradation

The results of hydrogels degradation are shown in Figure 9, which shows the average values and standard deviation of remaining mass (%) as a function of time.

It can be observed in the curves from Figure 7 that the zinc nitrate and CNC addition did not influence the chitosan hydrogels degradation (P > 0.05). All hydrogels degradation curves had similar behavior and after 56 days of testing, the mass remained at around 22% of the initial mass. An accelerated mass loss was observed in the first 30 days.
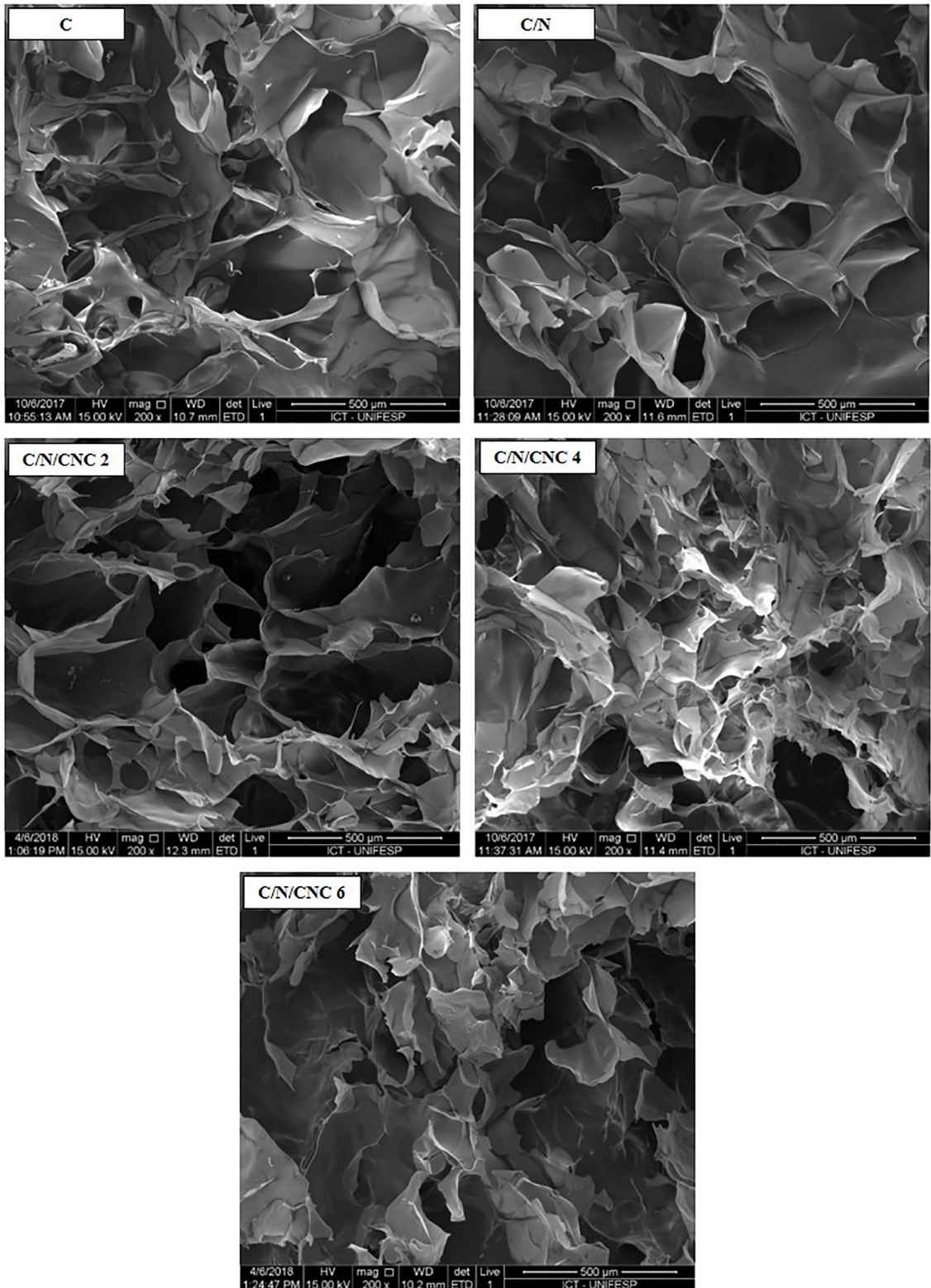
Figure 6. Fracture surface micrographs of chitosan hydrogels. Magnification of 200x.
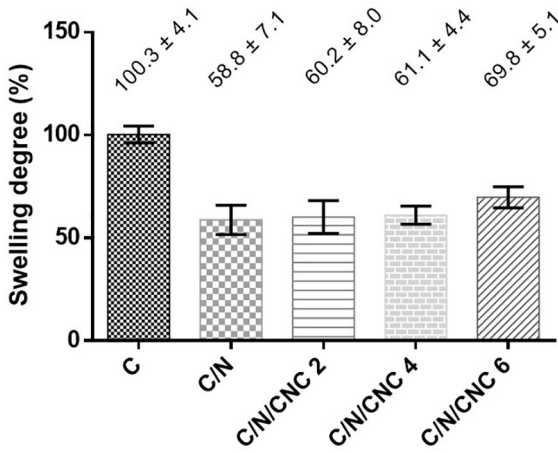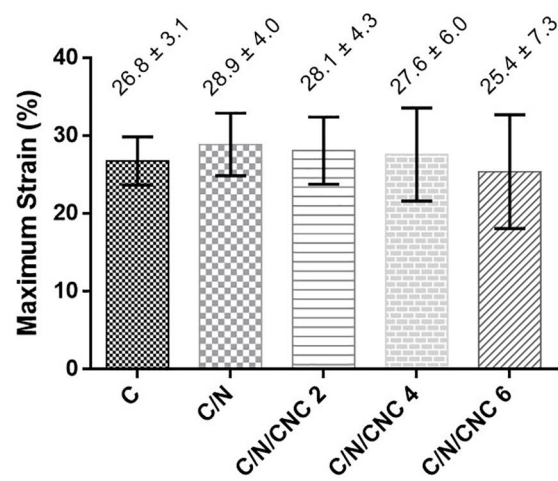
Figure 7. Swelling degree of hydrogels.

Figure 9. In vitro degradation curve of hydrogels.

Figure 8. Maximum strain (%) and maximum stress (%) of hydrogels.
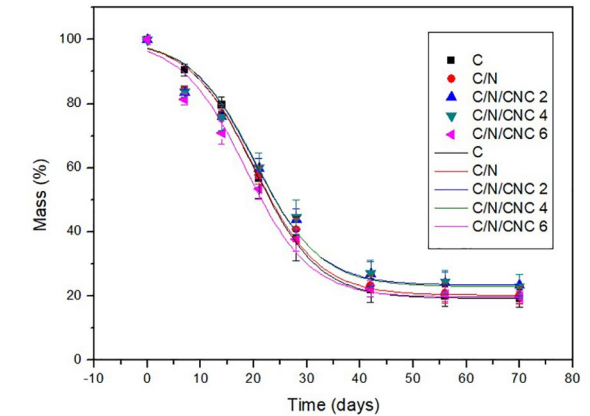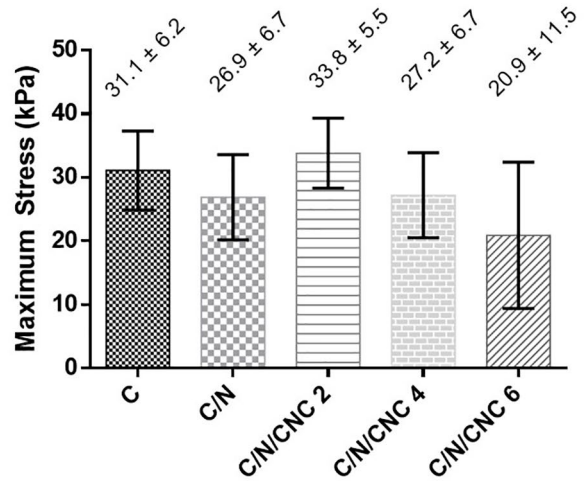
The behavior of the degradation curves can be described by Equation 3. This equation was taken from the curve of hydrogel C, however, as there is no significant differences between the degradation curves, that equation describes all curves behavior.

$$Y = \frac{100}{1 + (10^{(1,4359 + 0,0714X)})} \qquad (3)$$

Since it was already demonstrated that chitosan degrade *in vivo* in a period of 6 months[38,39], it is possible to extrapolate the equation taken from de degradation of hydrogel C and find the value of 5×10^{-13} % mass for 180 days for the *in vitro* studies. This result allows supposing that chitosan hydrogels will properly degrade *in vivo* within 180 days.

4. Conclusion

Chitosan hydrogels were crosslinked with glutaraldehyde, and zinc nitrate hexahydrate was used as a catalyst in the crosslinking reaction. CNC was chosen as nanofillers. Rheology analysis showed that the addition of zinc nitrate increases viscosity and crosslinking degree of chitosan hydrogel, while CNC addition oppositely affects both properties. However, in both cases, the porosity is reduced. Meanwhile, the morphology, evaluated by SEM and microtomography, did not suffer significant changes for all evaluated conditions, showing uniform construction. Due to the coexistence of conflicting effects, swelling degree was not changed after the introduction of CNC; however, this value was reduced about 41% after the addition of zinc nitrate to neat chitosan. Compressive mechanical properties remained constant, presumably because of an inefficient dispersion of CNC within the samples. As already expected, being a result of similar morphology, in vitro degradation test showed that all hydrogels degradation curves had similar behavior. Thus, the presence of zinc nitrate increased crosslinking degree proving that it is not only a catalyst but also participates in the crosslinking reaction of chitosan hydrogels. Accordingly,

using zinc nitrate, it may be possible to achieve satisfactory crosslinking degrees with reduced amounts of glutaraldehyde, which has high toxicity.

5. Acknowledgments

6. References

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