

CHITOSAN OLIGOMERS – SYNTHESIS, CHARACTERIZATION AND PROPERTIES

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The paper presents the synthesis of chitosan oligomers with different polymerization degrees (PD) by chitosan depolymerization in the presence of nitrous acid, aiming to study the correlation between their polymerization degree and their properties. Seven oligomers were synthesized and purified. The oligomers were characterized from the structural point of view by FTIR, NMR and UV-Vis spectroscopy, revealing the formation of shorter chains with an ending unit of 2.5-D-anhydromannofuranose. The morphology evaluation at micro and nano levels by SEM and AFM revealed the granular morphology of the oligomer films, while WXR and POM demonstrated their higher degree of ordering, in comparison with the parent chitosan. The antimicrobial tests showed the potential of the synthesized compounds to be used for biomedical purposes, the oligomers presenting antifungal activity, which increased with the decrease in their polymerization degree, especially against *Aspergillus niger*.

Keywords: chitosan oligomers, antimicrobial properties, granular morphology

INTRODUCTION

Chitosan, a polycationic biopolymer obtained from chitin has attracted a huge interest in the last years.¹⁻³ It presents many advantages, which include availability, low cost, high biocompatibility, biodegradability and ease of chemical modification. Chitosan presents great biological properties, including promotion of wound healing, hemostatic (chitosan bandages are used by paramedics to stop bleeding instantaneously), hypolipidemic and antimicrobial activity, immune enhancement and mucoadhesion. However, in order to benefit from all these biological activities, it is very important to know the metabolism of chitosan in physiological medium.⁴ Studies have demonstrated that this metabolism is significantly influenced by the molecular weight of the polymer. That is why, the researchers have turned their attention to chitosan oligomers, which have the same or even better biological characteristics, without presenting the weaknesses of chitosan. The production of well-defined oligomer mixtures, or even pure oligomers, is of great interest, but it is not straightforward.⁵

Chitosan oligomers, usually named chitoooligosaccharides (CHOSs) are obtained by chitosan depolymerization, which can occur chemically or enzymatically, by acid hydrolysis or by enzymatic hydrolysis.⁶

Enzymatic depolymerization of chitosan involves chitinases and chitosanases, which are able to hydrolyze the glycoside bonds from the chitosan structure. Both enzymes are found in fungi, bacteria and plants,⁷ and the most important difference between them is the fact that chitinases preferentially attack polymers with a high N-acetyl content.^{8,9} When the enzymatic method is used for chitosan depolymerization, the price of the resulted products increases and their applicability at a larger scale is not feasible. That is why, new physical methods, such as microwave,¹⁰ hydrothermal,¹¹ ultrasonication¹² and gamma-rays assisted methods,¹³ have been developed.

Further, in 1957 Horowitz *et al.* prepared CHOSs using a chemical method.^{14,15} They showed that acid hydrolysis of chitosan with concentrated hydrochloric acid is possible and leads to chitosan oligomers with low values of the polymerization degree.¹⁴ After this first success, other depolymerization agents were used, such as nitrous acid, phosphoric acid and hydrofluoric acid.¹⁷

Chitosan depolymerization by nitrous acid is a homogeneous reaction where the number of broken glycosidic bonds is stoichiometric to the amount of nitrous acid used, which makes the reaction easy to control.^{16,17} Even though the reaction has been known for a long time, in the literature there is no exhaustive study from the structural, morphological and supramolecular points of view regarding the

dependence between the glucosamine/nitrous acid molar ratio and the properties of the resulted oligomers. Therefore, the present study aims to bring some light in the chitosan depolymerization reaction by nitrous acid, from these points of view.

EXPERIMENTAL

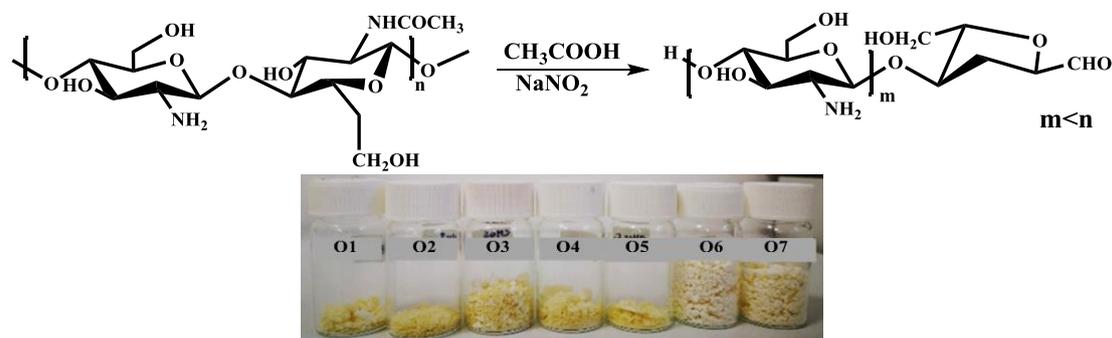
Materials

Low molecular weight chitosan, with a molecular weight of 147 kDa, measured by viscosimetry, and a deacetylation degree of 82%, calculated from NMR, as well as acetic acid, ethanol, sodium nitrite and ammonium hydroxide were purchased from Aldrich and used as received.

Synthesis of chitosan oligomers

Seven chitosan oligomers (O1-O7) were synthesized by chitosan depolymerization with nitrous acid (Scheme 1), by changing the molar ratios between the reagents.^{18,19}

The experimental procedure for obtaining the oligomers was as follows: to a 0.15% chitosan solution in a mixture of water and acetic acid (50 mL water + 2.5 mL CH₃COOH), a certain volume of NaNO₂ solution in water (1.22%) was added in order to start the depolymerization process (Table 1). The reaction time was 18 h (at r.t.). The reaction was stopped by the addition of 5 mL of ammonium hydroxide 25%. The solid product was separated by centrifugation (4 cycles, each of 30 min at 6000 r/min). After each centrifugation step, the liquid phase was removed and the solid was washed with double-distilled water, until pH 7. In order to obtain the oligomers in the form of powder, the products were lyophilized.



Scheme 1: Synthesis of chitosan oligomers and picture of the obtained samples

Table 1
Synthesis of chitosan oligomers

Sample	Chitosan (mg)	H ₂ O (mL)	Acetic acid (mL)	Sol _{NaNO₂} (μL)	Yield (%)	PD*	Observation
O1	750	50	2.5	2500	53	11	Precipitated with 5 mL NH ₄ OH and 5 mL of ethanol
O2	750	50	2.5	1250	68	14	Precipitated with 5 mL NH ₄ OH
O3	750	50	2.5	830	75	18	Precipitated with 5 mL NH ₄ OH
O4	750	50	2.5	625	84	21	Precipitated with 5 mL NH ₄ OH
O5	750	50	2.5	500	86	26	Precipitated with 5 mL NH ₄ OH
O6	750	50	2.5	375	96	34	Precipitated with 5 mL NH ₄ OH
O7	750	50	2.5	250	98	51	Precipitated with 5 mL NH ₄ OH

* from NMR data

Equipment and methods

The oligomer powder was obtained by lyophilization at -54 °C and 1.510 mbar, for 24 h, using a LABCONCO FreeZone FreezeDry System (Canada).

The NMR spectra were recorded using a Bruker Avance DRX 400 MHz Spectrometer (Bruker, Germany), equipped with a 5 mm QNP direct detection probe and z-gradients. For the recording of the NMR spectra, 10-12 mg of oligomer powder was dissolved in a mixture of 0.8 mL D₂O and 5 μL HCl.

The ATR-FTIR spectra of the oligomer powder were recorded on a Bruker Vertex 70 Spectrophotometer (Ettlingen, Germany), with a ZnSe single reflection ATR accessory.

UV-Vis absorption spectra were recorded on an Agilent Cary 60 UV-Vis spectrophotometer in acetic acid aqueous solutions.

The morphology was evaluated using a Scanning Electron Microscope SEM EDAX – Quanta 200 (Eindhoven, Germany), with a CBS detector on oligomer films obtained by casting from a 2% solution.

Atomic force microscopy (AFM) was performed on an Ntegra Spectra Instrument (NT-MDT, Russia) operated in the semicontact mode, using a silicon cantilever. The images were recorded on samples prepared by drop-casting oligomer solutions (2%) on freshly cleaved mica, and dried in air at room temperature.

X-ray diffraction analysis was performed on a Rigaku Miniflex 600 diffractometer on oligomer films using CuK α -emission in the angular range 2–40° (2 θ) with a scanning step of 0.0025° and a recording rate of 2 °/min.

The supramolecular architecture of the oligomers was evaluated by polarized optical microscopy (POM) using a Leica DM2500 microscope, (Hamburg, Germany).

The antimicrobial activity screening of the samples was determined by the disk diffusion assay²⁰ against five different reference strains: *Staphylococcus aureus* ATCC25923, *Klebsiella pneumoniae* ATCC10031, *Penicillium chrysogenum* ATCC10106, *Cladosporium cladosporioides* ATCC16022 and *Aspergillus brasiliensis* ATCC9642. All microorganisms were stored at -80 °C in 20% glycerol. The bacterial strains were refreshed on trypticase soy agar (TSA) at 37 °C, and the fungal strains were refreshed on Potato Dextrose Agar (PDA) at 25 °C. Microbial suspensions were prepared with these cultures in sterile solution to obtain turbidity optically comparable to that of 0.5 McFarland standards. Volumes of 0.2 mL from each inoculum were spread onto TSA/PDA plates and then the sterilized paper disks (6 mm) with an aliquot (20 μ L) of the samples were added.

To evaluate the antimicrobial properties, the growth inhibition was measured under standard conditions after 24 hours of incubation at 37 °C for the bacterial strains and after 72 hours at 25 °C for the fungal ones. All tests were carried out in triplicate to verify the results. After incubation, the samples were analysed with SCAN1200®, version 8.6.10.0 (Interscience) and were expressed as the mean \pm standard deviation (SD) performed with XLSTAT Ecology version 2019.4.1 software.

RESULTS AND DISCUSSION

Synthesis and structural characterization

Seven chitosan oligomers (O1-O7) were synthesized by chitosan depolymerization in the presence of nitrous acid, obtained *in situ* from sodium nitrite and acetic acid (Scheme 1).^{18,19} The obtained oligomers were characterized from the structural point of view by NMR, FTIR and UV-Vis spectroscopy. The ¹H-NMR spectra of the oligomers present the characteristic signals of the four protons from the end unit of 2,5-anhydro-D-mannofuranose, at 4.11, 4.21, 4.43 and 5.1 ppm, confirming the success of the depolymerization. The other signals from the NMR spectra of the oligomers are due to the protons from the D-glucosamine units from the starting chitosan, as follows: H3-H6 in the 3.5-4 ppm region and at 3.1 ppm the proton H2 (Fig. 1a,b).¹⁸

On the other hand, NMR spectroscopy allowed determining the polymerization degree of the oligomers by using the following equation:

$$DP = I(D)/I(M) + 1 \quad (1)$$

where I(D) is the value of the integral of H2 proton from the D-glucosamine unit (~3.1 ppm), while I(M) is the value of the integral of one proton from the mannofuranose unit (*e.g.* the one at ~4.45 ppm) (Fig. 1).

Further, a complementary structural characterization of the oligomers was done by FTIR spectroscopy (Fig. 2), in comparison with the chitosan used for their preparation. The FTIR spectra of the oligomers are quite similar with the spectrum of chitosan in both peak positions and shapes. In the FTIR spectra of the oligomers, there appeared a vibration band between 3700 and 3000 cm⁻¹, corresponding to the amine and hydroxyl groups involved in intra- and inter-molecular hydrogen bonds.²¹ In comparison with chitosan, a shifting of the bands maxima towards higher wavenumbers was noticed, attributed to the changes that occurred in the hydrogen bonds environment, due to the decrease in the polymerization degree of the oligomers. Another proof of this fact lies in the changes that occurred in the 1080-1020 cm⁻¹ spectral region.

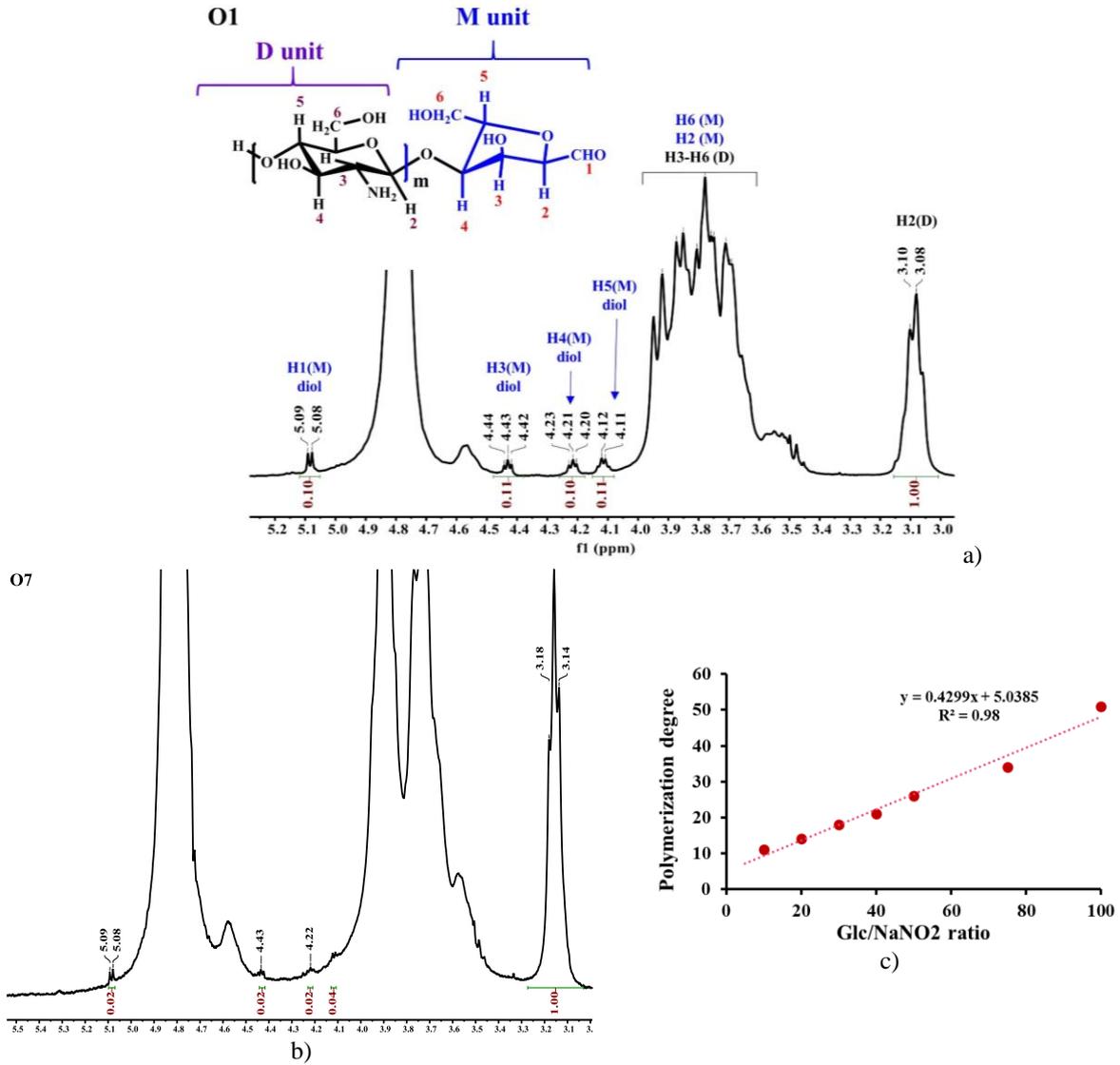


Figure 1: NMR spectra of two representative oligomers (D₂O+HCl) (a, b) and the correlation between the polymerization degree with Glc/NaNO₂ molar ratio (c)

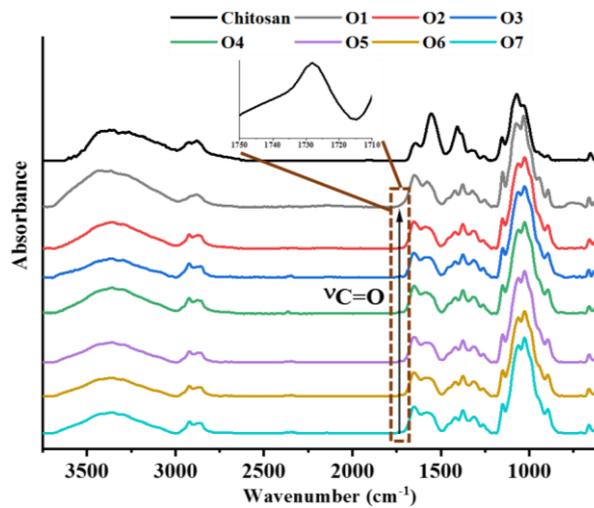


Figure 2: FTIR spectra of chitosan and the obtained oligomers

In the chitosan spectrum, the band is formed from two maxima – a first one – intense, at 1072 cm^{-1} and a second one – less intense, at 1022 cm^{-1} .²² On the contrary, in the spectra of the oligomers, the intensity of these two bands switched, the second one becoming more intense, while the first one decreased in intensity. The bands in discussion correspond to the vibrations of the C-O-C and C-OH, and are highly dependent on the hydrogen bonds environment.²² Moreover, the appearance of a new absorption band, at 1726 cm^{-1} , in the spectra of the oligomers, corresponding to the C=O from the aldehyde group proved the formation of 2,5-anhydro-D-mannofuranose end-group.²²

The structural changes after chitosan depolymerization were also monitored by UV-VIS spectroscopy. As expected, chitosan didn't show any considerable absorption peak between 200-400 nm, which is in agreement with the literature (Fig. 3).²³ By chitosan depolymerization, shorter saccharide chains with a 2,5-D-mannofuranose end groups are obtained, as previously demonstrated by FTIR and NMR spectroscopy. This is easily observed in the UV-VIS spectra of the oligomers, by the presence of an absorption peak at 268 nm, which corresponds to π - π^* transition from the aldehyde chromophores.²⁴ Moreover, the oligomer solutions and films emitted white-bluish light when illuminated with a UV lamp, the intensity of the emitted light increasing with the decrease of the oligomers PD.

Therefore, the structural characterization of the obtained products, by NMR, FTIR and UV-VIS spectroscopy, confirmed the formation of shorter saccharide chains, with an end group of 2,5-anhydro-D-mannofuranose.

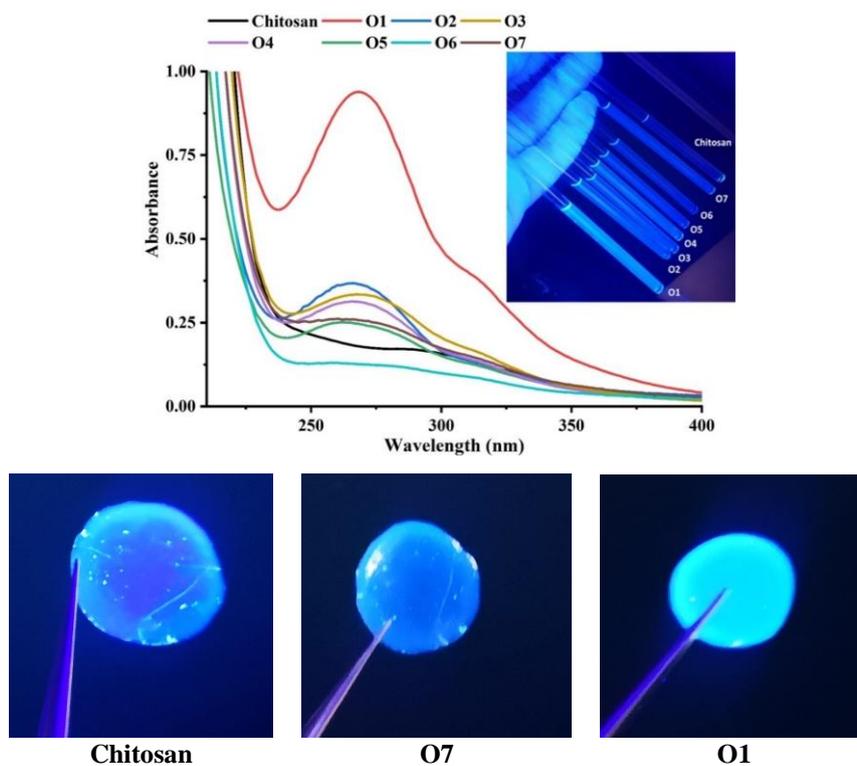


Figure 3: UV-VIS spectra and images of the oligomer solutions under UV lamp illumination

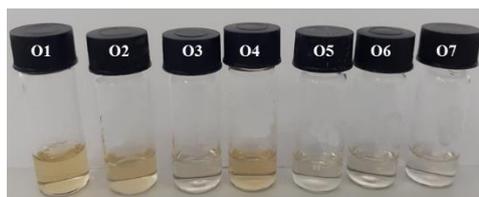


Figure 4: Oligomers in acetic acid aqueous solutions

Solubility tests of the oligomers

It should be expected that a decrease in the polymerization degree of the chains would lead to an increase in the solubility of the compounds. This behavior was evaluated by studying the amount of acetic acid required for the complete dissolution of the oligomers. For the solubilization of chitosan used as starting material, a solution of 0.7% acetic acid in water was needed. In the case of the oligomers, the concentration of the acetic acid was lower and depended on their PD. Therefore, for the O7 oligomer, the one characterized by the highest value of PD, the concentration was 0.6%, while for the lowest PD oligomer, O1, a concentration of 0.1% was sufficient to form a homogenous solution (Fig. 4).

Supramolecular architecture by WXR and POM

Chitosan is known as being a semicrystalline polymer due to its regular microstructure, its diffractogram presenting a broad band with two maxima: at ~ 12 and ~ 20 two theta degree, corresponding to 7.4 and 4.5 Å, due to the presence of numerous hydrogen bonds forming less hydrated crystalline zones, which are dispersed into an amorphous mass.^{2,25} Depending on its processing (temperature, water content), chitosan may present slight differences in its diffractogram regarding the shape of the two maxima – more or less intense.²⁵ By decreasing the polymerization degree of chitosan, it is expected the linearity of the chains in solution to increase and thus their self-assembling ability would also increase.² Indeed, in their diffractograms, four sharper maxima at ~ 9 , 12, 19.7 and 23.5 appeared, more evident for O1-O3 oligomers, corresponding to the distances calculated with Bragg's law: 9.8, 7.4, 4.5 and 3.8 Å (Fig. 5). Moreover, an increase of the diffraction peak from 12 degrees, the one corresponding to intramolecular hydrogen bonds was observed, due to the linearization of the shorter oligosaccharides chains. These data indicate a more ordered supramolecular architecture, with a narrower polydispersity of the intermolecular distances and shorter intramolecular distances, in accordance with the increased linearity of the oligomers' chains, which allow their higher packing.

In order to visually evaluate the changes in terms of supramolecular architecture after chitosan depolymerization, polarized optical microscopy images were acquired for chitosan and the synthesized oligomer films. All the samples presented birefringence under polarized light, including chitosan (Fig. 6). The birefringence is much more intense in the case of the oligomers, especially for the ones characterized by lower values of PD. This can be explained if we take into consideration the conformation of these oligomers in solution. The shorter the chains, the higher their mobility in solution and the linearity of the oligomeric chain.² That is why, the oligomers with lower PD led to higher degrees of ordering and further increased birefringence under polarized light. These data which correlate very well with the XRD findings.

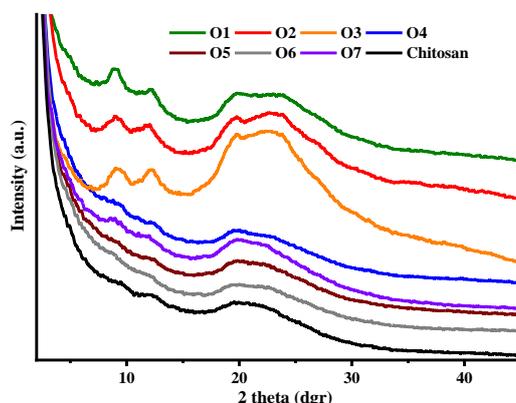


Figure 5: WXR diffractograms of the synthesized samples and chitosan

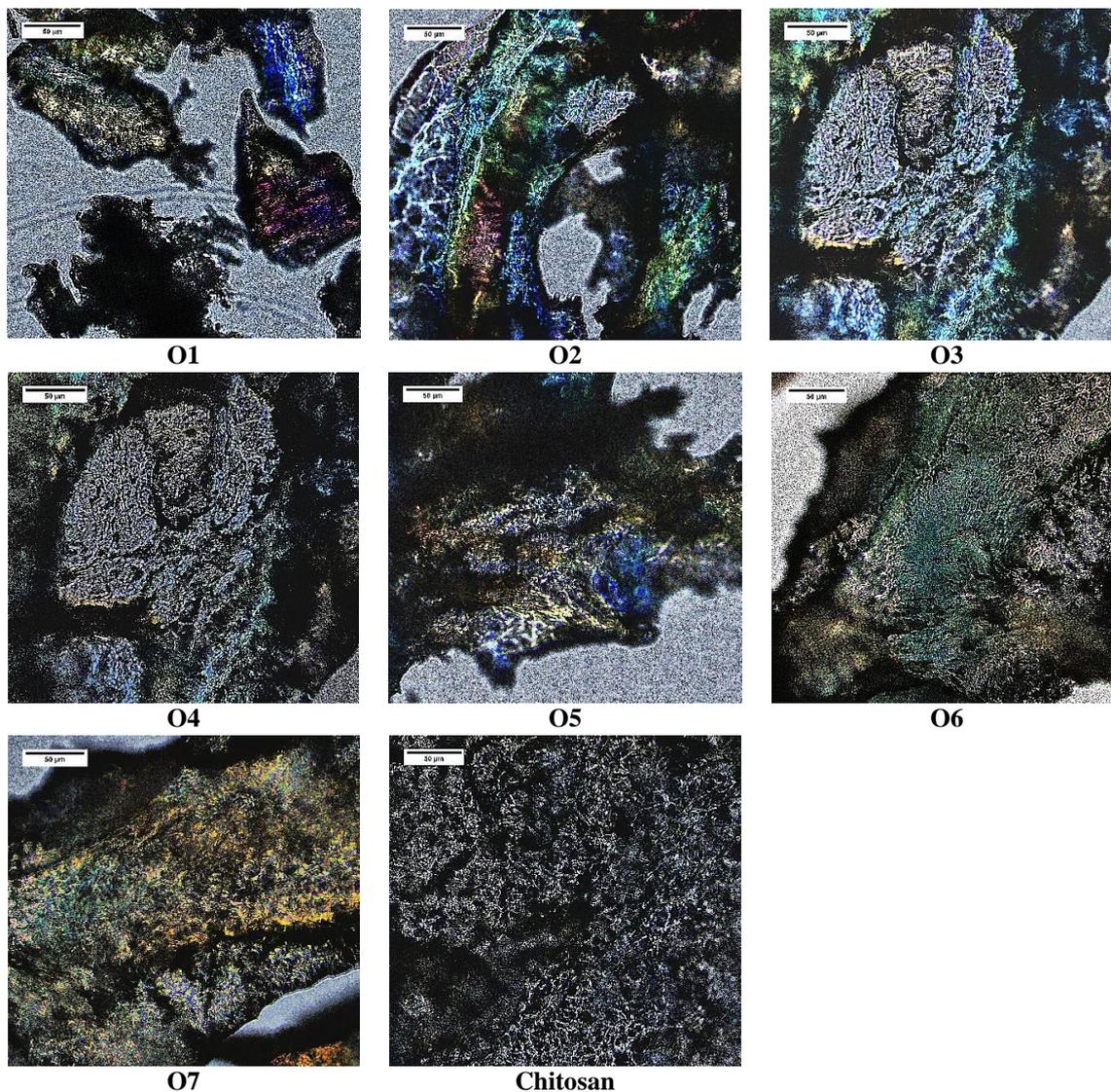
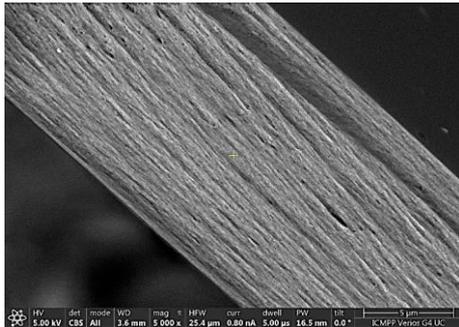


Figure 6: Polarized optical microscopy images of chitosan and the obtained oligomers

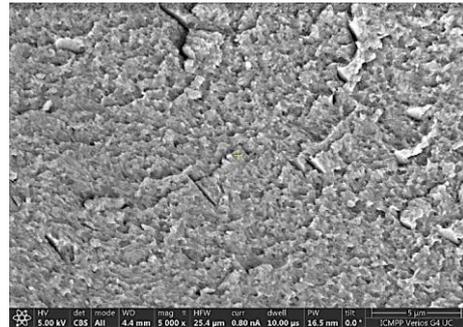
Morphological characterization by SEM and AFM

The samples' morphology at a micrometric scale was evaluated by scanning electron microscopy on thin films. All the samples presented granular morphology, due to their semicrystalline nature (Fig. 7). As could be observed from the SEM microphotographs, the films presented different roughness, depending on the PD of the oligomers.

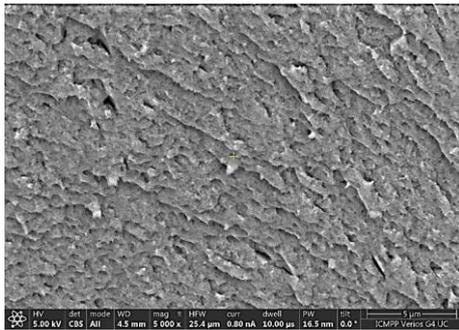
Considering that SEM images did not bring conclusive information regarding the morphology of the samples, a deeper analysis, at a nanometric level was performed by AFM (Fig. 8). All the samples presented granular morphology, with different values of average roughness (Ra), depending on the PD of the oligomers. Thus, the chitosan film presented the smoothest surface, with a Ra of 3.1 nm, while the shortest oligomer, O1 led to films that presented the highest roughness, of 9.1 nm. The other samples presented intermediate values of the Ra parameter, the oligomers with the highest PD reaching values of the Ra close to the one of chitosan (O6 with Ra of 4.1 nm and O7 with Ra of 3.9 nm).



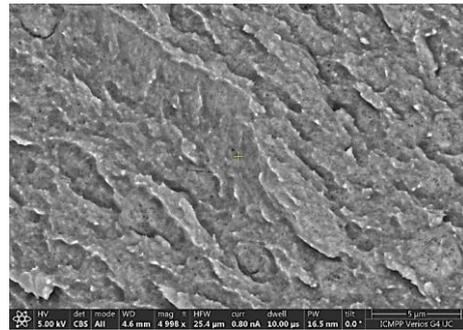
Chitosan



O1

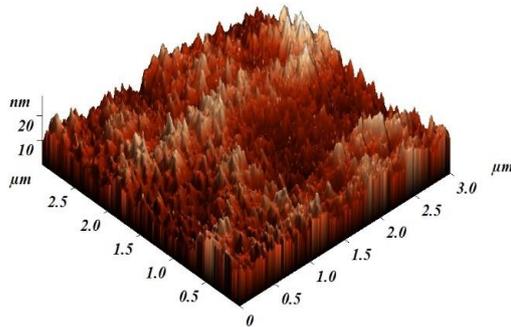


O4

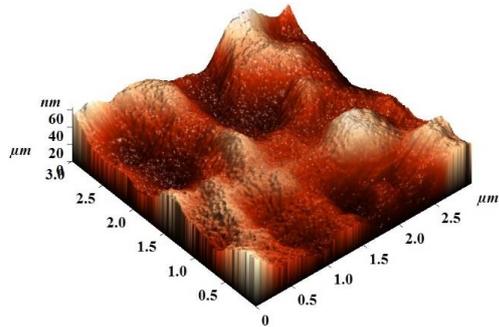


O7

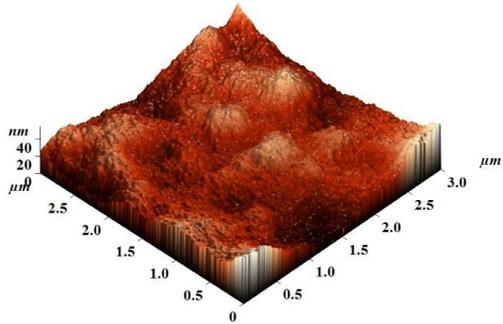
Figure 7: SEM images of chitosan and of representative oligomers



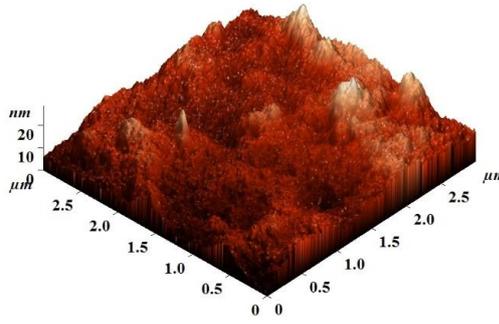
Chitosan (Ra=3.1 nm)



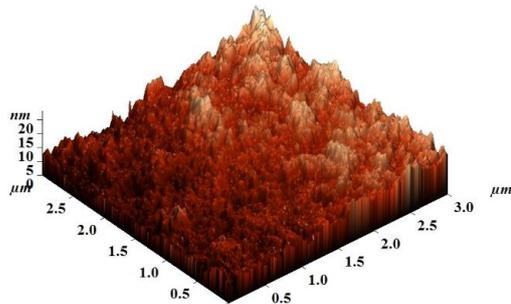
O1 (Ra=9.1 nm)



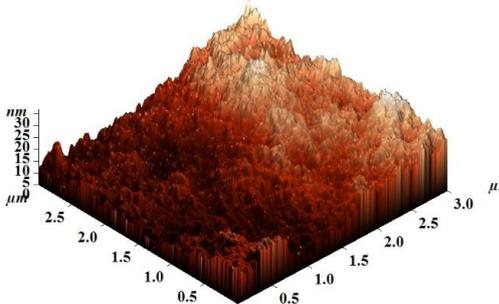
O2 (Ra=5.4 nm)



O3 (Ra=5.7 nm)



O4 (Ra=6.6 nm)



O5 (Ra=6.7 nm)

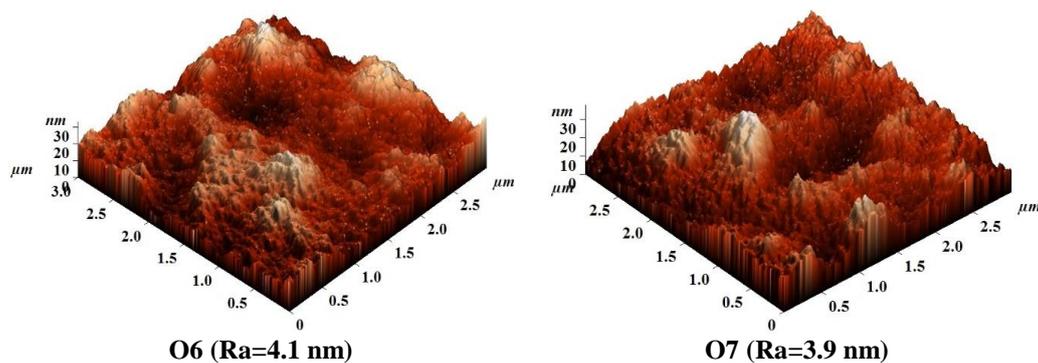


Figure 8: AFM images of the synthesized samples and chitosan

Antimicrobial activity

The antimicrobial activity of the oligomers and the chitosan used as starting material was evaluated on representative microorganisms: *Staphylococcus aureus* – a gram-positive bacterial strain, *Klebsiella pneumoniae* – a gram-negative bacterial strain and three fungal strains: *Penicillium chrysogenum*, *Cladosporium cladosporioides* and *Aspergillus brasiliensis* (Table 2). Even though chitosan was reported in the literature to have antifungal and antibacterial properties, it was considered necessary to confirm this in the present study, as it is known that its antimicrobial properties depend on its molecular weight, as well as on its deacetylation degree.^{1,2}

It has been found from the antibacterial study of the oligomers that the samples presented only antifungal properties. None of the samples were efficient against the bacterial strains (Table 2). As expected, the antifungal activity of the oligomers was higher with a decrease in their PD. Samples O1 up to O4 were very efficient against *A. brasiliensis*, especially sample O1 (up to ~37 mm of inhibition zone) (Fig. 9).

Samples O1-O3 presented moderate efficiency against *C. cladosporioides*, while sample O1 had the same activity against *P. chrysogenum*. Data on the diameters of the inhibition zones of each tested compound are presented in Table 2.

Table 2
Antimicrobial activity (mm) of the samples against the reference strains

Sample	<i>S. aureus</i> ATCC25923	<i>K. pneumoniae</i> ATCC10031	<i>P. chrysogenum</i> ATCC10106	<i>C. cladosporioides</i> ATCC16022	<i>A. brasiliensis</i> ATCC9642
Chitosan	-	-	-	-	-
O1	-	-	16.28 ± 0.18	16.88 ± 0.54	36.8 ± 0.89
O2	-	-	-	10.75 ± 0.24	33.68 ± 0.22
O3	-	-	-	8.50 ± 0.21	27.9 ± 0.45
O4	-	-	-	-	15.84 ± 0.37
O5	-	-	-	-	-
O6	-	-	-	-	-
O7	-	-	-	-	-

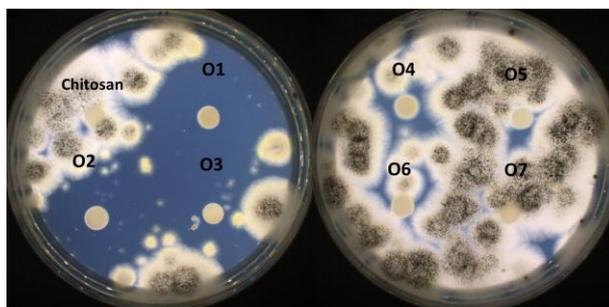


Figure 9: Antimicrobial activity of the samples against *A. brasiliensis*

CONCLUSION

Chitosan oligomers with polymerization degrees from 11 to 51 were synthesized by chitosan nitrous depolymerization in a homogeneous system. NMR, FTIR and UV-Vis spectroscopy demonstrated the success of the depolymerization reaction, while their granular morphology at micro- and nanoscale was assessed by SEM and AFM. WXR and POM analyses demonstrated the higher degree of ordering at supramolecular level for the obtained oligomers in comparison with that of chitosan. The antimicrobial tests revealed that the synthesized oligomers present potential for being used as antifungal agents, especially for the treatment of Aspergillosis.

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