BINARY CHITOSAN/QUATERNIZED CHITOSAN *VIA* ELECTROSPINNING. MORPHOLOGY AND ANTIMICROBIAL ACTIVITY

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The paper reports about binary chitosan/quaternized chitosan nanofibers obtained by direct electrospinning of their solution, without using co-spinning polymers. Both quaternary salts, *N,N,N*-trimethyl chitosan chloride and *N*-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride, were used in the electrospinning process and provided nanofibers with a mean diameter lower than 100 nm. A morphological evaluation of the nanofibers prepared with quaternized chitosan and chitosan of different molecular weights indicated that chitosan of lower molecular weight yielded fibers of higher diameter, due to the necessity to increase the concentration of the electrospinning solution in order to reach chain entanglement. Polarized light microscopy suggested that the fibers were semicrystalline in nature, in line with the ability of the macromolecular chains to align in an electrical field. Furthermore, the investigation of the antimicrobial and antifungal activities against relevant gram-positive and gram-negative bacteria, as well as yeast strains, revealed the strong effects of the nanofibers, improved by the presence of quaternary chitosan and the lower diameter of the fibers.

Keywords: quaternized chitosan, electrospinning, antimicrobial activity

INTRODUCTION

Over the years, the electrospinning technique proved its versatility and efficiency in producing functional ultrathin fibers addressing a broad realm of applications.[1–3] Along with synthetic polymers, naturally originating ones have been successfully electrospun to yield nanofibers, especially suitable for biomedical applications, such as tissue engineering, wound dressings, and drug delivery.[4–6] Chitosan is in the top of researchers' preferences in designing nanofibrous biomaterials for such applications. This is because the intrinsic properties of chitosan, *i.e.* biocompatibility, biodegradability, hemostatic characteristic and lack of toxicity, are enriched *via* electrospinning, leading to increased active surface, similarity with the extracellular matrix architecture of the skin and good conformability.[6–8] However, the preparation of neat chitosan nanofibers remains a difficult task, because of its polycationic character, which hinders the attaining of the critical entanglement necessary for the formation of the jet during the electrospinning process.[7] Consequently, alternative methods to reach chitosan-based nanofibers were considered, mainly by using a synthetic polymer as co-spinning agent. To enrich the nanofibers' activity with specific properties, different bioactive agents, such as drugs, inorganic nanoparticles, or plant extracts were used as fillers.[7,9–14]

Quaternized chitosan, a chitosan derivative bearing quaternary ammonium groups either directly connected on the backbone or *via* a flexible chain, has enhanced properties compared to chitosan, in terms of bioadhesiveness, antimicrobial and antioxidant activity and ability to open the tight junctions on the cell membrane, enhancing the drugs' transport across epithelia.[15,16] Quaternized chitosan-based nanofibers were also prepared by electrospinning, either using synthetic polymers as co-spinning agents, or by surface modification of synthetic or natural fibers.[17,18] Research has demonstrated their high potential for application, especially in drug delivery, as hemostatic materials, antiviral filters, or hygienic textiles.[19–21]

Our literature survey shows that both kinds of biopolymers are prepared as hybrid nanofibers, usually using synthetic polymers, which limits the *in vivo* application, especially as biodegradable materials. It can also be remarked that nano-fillers are necessary in order to enrich the chitosan nanofibers with specific properties, such as antimicrobial activity, while quaternized chitosan-based nanofibers have high antimicrobial activity, but also a cytotoxicity degree, which is a consequence of the high content of quaternary ammonium groups.[17]

Considering this, the objective of the present study was to prepare chitosan/quaternized chitosan nanofibers *via* electrospinning. It was envisaged that by mixing chitosan and quaternized chitosan, fibers with high antimicrobial effect and low cytotoxicity will be achieved by a "dilution" effect of the quaternary units into the chitosan matrix. To reach the difficult goal of electrospinning of two polycationic polymers, different conditions were tried, varying the molecular weight of chitosan, and the type of quaternized chitosan, *N,N,N*-trimethyl chitosan chloride (TMC) or *N*-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride (HTCC). The influence of the electrospinning conditions on nanofibers' formation, their morphology and antimicrobial effect was investigated and discussed.

EXPERIMENTAL

Materials

Chitosan (197 kDa, DD = 84%) was purchased from Sigma Aldrich. In order to investigate the influence of the molecular weight of chitosan on electrospinning, chitosan of lower molecular weight was prepared by alkaline hydrolysis for 24 h, 48 h and 72 h, to yield chitosan of 126, 119, 109 kDa, DD = 99%. Polyethylene oxide (1000 kDa), iodomethane (99%), acetic acid (99.89%), potassium hydroxide (95%), potassium iodide (≥99%), glycidyltrimethylammonium chloride (≥90%), silver nitrate (≥99%), acetone (≥99.5%) were purchased from Sigma Aldrich and used as received. *N*-methyl-2-pyrrolidone (≥9.8%) was purchased from Roth, and formic acid was purchased from Chemical Company and used as received. Ethanol (98.89%) was purchased from Sigma Aldrich and dried on molecular sieves before use.

Synthesis

The synthesis of *N,N,N*-trimethyl chitosan (TMC) (with a quaternization degree of 41.38%, a dimethylation degree of 42.43%, and *O*-methylation degree of 30.48%, determined by ^{1}H-NMR spectroscopy, Scheme 1b) was carried out in two steps *via* the Eschweiler-Clarke reaction, as described by Verheul.[22] Briefly, in the first step, chitosan (1 g) was mixed with 2.4 g potassium iodide, 5.5 mL potassium hydroxide (15%) and 6 mL iodomethane, and suspended in 40 mL *N*-methyl-2-pyrrolidone (NMP), under magnetic stirring for one hour at 70 ºC. The reaction mixture was precipitated in ethanol, filtered and washed with diethyl ether, and dried at 40 ºC. 0.5 g of the obtained compound was dispersed in 20 mL of NMP at 60 ºC, and 1.2 g of KI, 2.7 mL of KOH (15%) and 1.75 mL of iodomethane were added under magnetic stirring. After 30 minutes, an excess of 0.8 mL of iodomethane and 0.2 g of solid KOH were added. The reaction took place for an hour, and was further precipitated in ethanol, centrifuged and the solid was washed with diethyl ether. The iodide counterion was replaced with chloride by dissolving the compound in 10% sodium chloride for 24 hours. The final compound was obtained by precipitation in ethanol, centrifugation, washing with diethyl ether and drying at 40 ºC.

The synthesis of *N*-(2-hydroxy)propyl-3-trimethyl ammonium chitosan chloride (HTCC) (quaternization degree 52.9% determined by conductometric titration) was achieved by reacting chitosan with glycidyltrimethylammonium chloride (GTMAC) in a heterogeneous system in water, according to a slightly modified procedure.[15,23] Briefly, chitosan (1 g) was dispersed in 60 mL of MilliQ water and heated to 85 °C under magnetic stirring for 3 hours. 2.2 mL of GTMAC were slowly added dropwise, in 3 equal portions, at one-hour time interval, and maintained under heating for 24 hours. The reaction medium was precipitated in cold acetone and kept in the fridge overnight. The formed solid was washed with a mixture of acetone/ethanol (1/1, v/v), dried, solubilized in water, filtered to remove any traces of unreacted chitosan, and lyophilized. The successful quaternization was confirmed by NMR spectroscopy (Scheme 1c).

Equipment

Electrospinning of chitosan was done using a Tong Li Tech TL-PRO electrospinning machine. Electrospinning of chitosan/quaternized chitosan fibers was realized with an Inovenso starter kit electrospinning device, with a rotary drum collector whose speed was controlled by a microcontroller.

FT-IR spectra of the fibers were collected with a Bruker VERTEX 70 FT-IR Spectrophotometer (Billerica, MA, USA) using an ATR Module.

Polarized optical microscopy images were acquired with a Zeiss Axio Imager M2 microscope (Wetzlar, Germany) in the reflection mode.

The morphology was studied using a ThermoScientific Verios G4 UC Field Emission

Scanning Electron Microscope (SEM) at a 5 kV acceleration voltage. The average diameter of the fibers was calculated according to Hotaling *et al*.,[24] using the Image J program and the Diameter J plug-in.

Electrospinning

Electrospinning of chitosan was realized by varying the electrospinning parameters for solutions with concentration from 3 to 7% in acetic acid from 50 to 90%, until the Taylor cone was formed. In this way, it was established that 90% acetic acid is the most suitable solvent for attaining continuous fibers and the electrospinning parameters were set up at: voltage of -8 +18 kV, needle-collector distance of 10 cm, flow rate of 0.4 mL/h.

Similarly, binary chitosan/quaternized chitosan fibers were prepared from blend solutions by varying the electrospinning parameters applied to the binary solutions with different mass ratios in the range of 50–90%, using chitosan of different molecular weight, in acetic acid. The electrospinning parameters for each fiber specimen were given in Table 1.

Table 1
Composition, codes and electrospinning parameters of the studied nanofibers

Code	Composition (weight ratio)	Mw (kDa)	C_{sol} (%)	C_{AcOH} (%)	Voltage (kV)	NCD (cm)	Flow (mL/h)	Speed (RPM)
C1	CS	126	5	90	-8+18	10	0.4	800
C2	CS	126	6	90	-8+18	10	0.4	800
C3	CS	126	7	90	-8+18	10	0.4	800
CT1	CS/TMC 75/25	126	3	80	+28	10	0.4	800
CT2	CS/TMC 80/20	109	3	80	+17	12	0.08	800
CH1	CS/HTCC 75/25	119	2	80	+14	10	0.08	800
CH2	CS/HTCC 75/25	109	3.5	80	+21	12	0.08	800

Mw: molecular weight of chitosan determined by viscosimetry; C_{sol}: concentration of the solution; C_{AcOH}: concentration of acetic acid; NCD: needle to collector distance; Speed: collector's speed expressed in RPM

Antimicrobial and antifungal activity

The antimicrobial efficiency of the samples was investigated *via* a modified Japanese industrial standard JIS Z2801:2000,[25] against three different reference strains: a gram-positive one represented by *Staphylococcus aureus* ATCC25923 (*S. aureus*), a gram-negative one represented by *Escherichia coli* ATCC25922 (*E. coli*), and a yeast represented by *Candida albicans* ATCC90028 (*C. albicans*). The bacterial strains were refreshed in nutrient broth (NB), and the yeast strain was refreshed in Sabouraud dextrose broth (SDB), all of them for 24 h at 37 ºC.

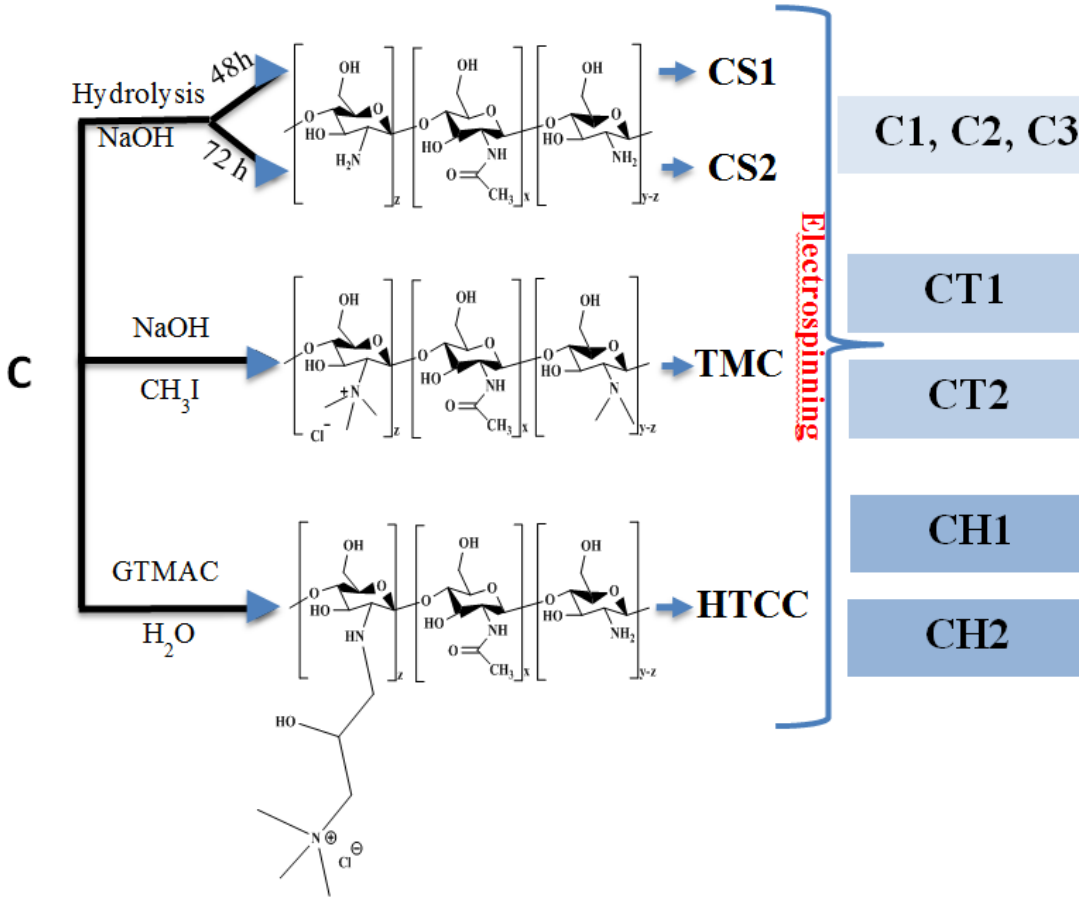
The test surfaces were prepared as follows: each sample of 10 mm was placed in a sterile Petri dish and the bacterial inoculum was adjusted to standard 0.5 McFerland. Then, 100 µL of the inoculum was placed on the sample's surface and incubated for 24 h at 37 ºC. After incubation, the samples were rinsed repeatedly, and the resulting suspension was transferred into 96-well plates. The antimicrobial activity of samples after incubation with the microorganisms was assessed by MTS assay using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI USA), according to the manufacturer's instructions. After 23 hours, the samples were removed from the plates and MTS reagent was added 1 h prior to absorbance readings. After the formation of formazan, the final reading was performed at 490 nm on a FLUOstar® Omega microplate reader (BMG Labtech, Ortenberg, Germany). Experiments were done in triplicate and treated cell viability was expressed as percentage of control cells' viability. Graphical data were expressed as mean ± standard error of the mean.

RESULTS AND DISCUSSION

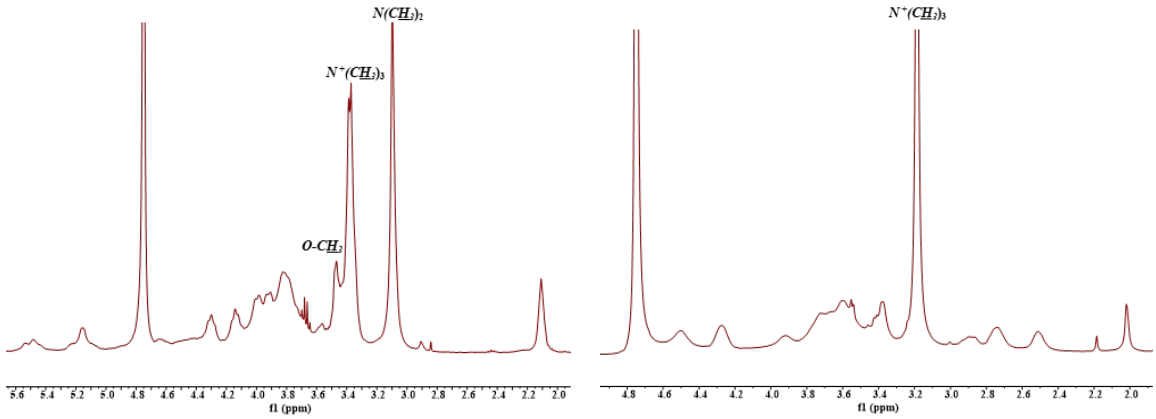
By varying the electrospinning conditions, chitosan/quaternized chitosan nanofibers were successfully attained for four different compositions, when the molecular weight of chitosan was slightly varied from 109 to 126 kDa, and the quaternized chitosan was either trimethyl chitosan chloride (TMC), for which the quaternary ammonium group was directly bonded to the chitosan backbone, or *N*-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride (HTCC), for which the quaternary ammonium group was bonded to the chitosan backbone *via* a flexible spacer (Scheme 1). To understand the impact of the quaternized chitosan on the morphology and properties of the nanofibers, chitosan nanofibers were also prepared under similar conditions.

By varying the concentration of chitosan from 3 to 7%, the Taylor cone was formed for solutions of high concentrations, from 5 to 7% (Fig. 1). SEM images confirmed the fibrous morphology with progressively rarer beads as the

concentration increased. The fibers' diameter was very low, around 40 nm, with no relevant statistical differences among the samples. Compared to literature data, which reports increasing fiber diameter with solution concentration,[7,26,27] the lack of relationship between these parameters indicates that the high concentration of acetic acid used for solutions' preparation led to insignificant variations of viscosity at high concentrations, and thus lack of influence on the fibers' diameter.



a)



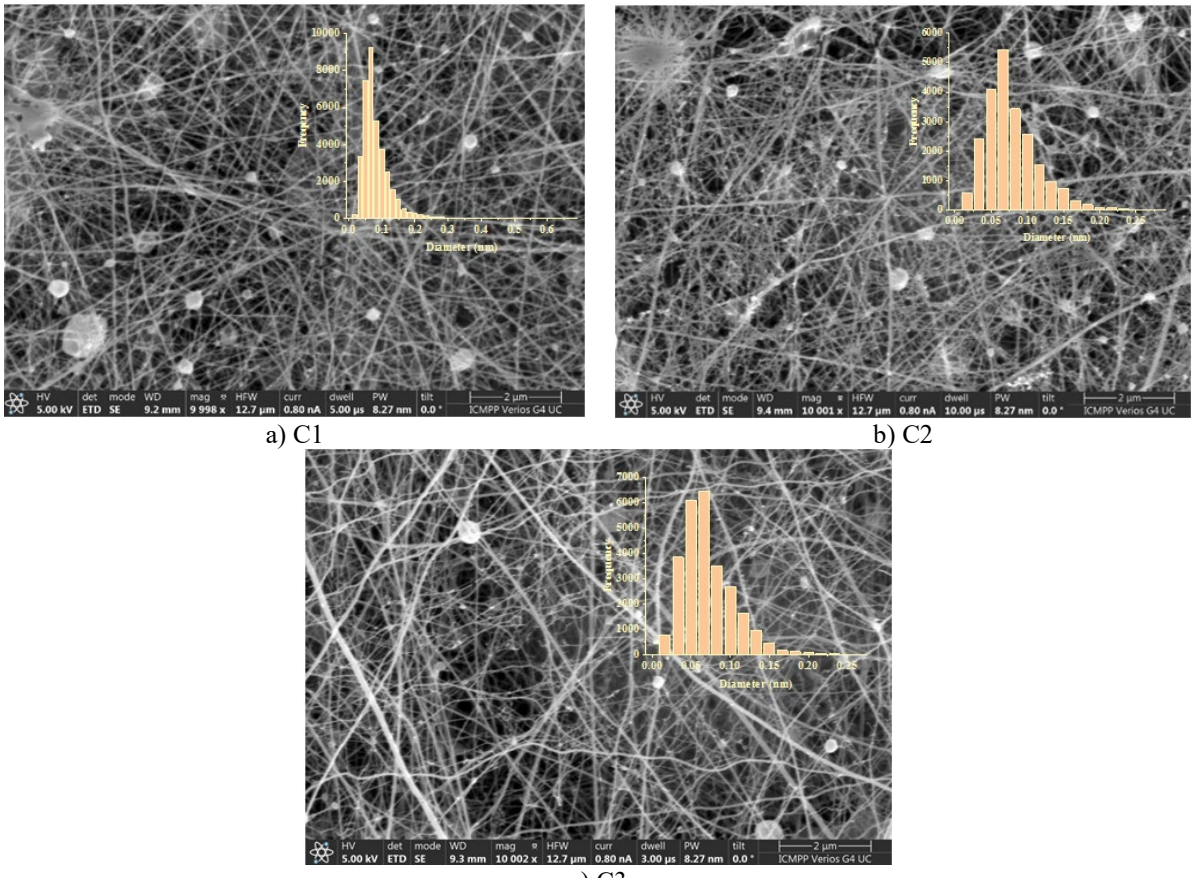
b) c)

Scheme 1: a) Synthesis routes of the reagents used for electrospinning, the compositions and codes of the obtained nanofibers; ^{1}H-NMR spectra of b) TMC and c) HTCC reagents, with annotation of the signals of distinctive protons confirming successful synthesis

Regarding the electrospinning of the quaternized chitosan-based solutions, some interesting conclusions were drawn. First, (i) the Taylor cone was formed when the solutions were obtained in 80% acetic acid for (ii) chitosan/quaternized chitosan blends, 80/20 or 75/25%, respectively. (iii) The electrospinning was favored when chitosan of lower molecular weight (109-126 kDa) was used instead of higher molecular weight (197 kDa) and (iv) the electrospinning process proceeded better in the case of HTCC compared with TMC, (v) for the solution concentration of 3%. The Taylor cone was formed for different electrospinning parameters (flow rate, voltage, needle-to-collector distance) for each blend solution, which were adapted depending on the solution's viscosity and type of quaternized chitosan. It was remarked that HTCC based fibers were obtained when significantly lower voltage was applied (14 and 21V, respectively) compared with those based on TMC, whose electrospinning required higher voltage (17 and 28V, respectively).

The analysis of SEM images of the quaternized chitosan-based nanofibers revealed that, even slight differences in the molecular weight of chitosan, 126 kDa *vs.* 109 kDa, influenced the quality of the fibers, those based on higher molecular weight chitosan presenting more pronounced bead defects, compared to those prepared from lower molecular weight chitosan (109 kDa), which were smooth, with almost no defects (Fig. 2).

However, the electrospinning yield was low, a large amount of solution being lost as drops during electrospinning. An interesting aspect is related to the fibers' diameter. Against the belief that the presence of quaternized chitosan leads to a decrease in fiber diameter,[17] in this case, the opposite appeared to be true, the fibers containing HTCC and TMC showed a statistically significant increase in fiber diameter to almost double (79 and 91 nm), compared to chitosan nanofibers (~40 nm) (Fig. 3).



a) C1 b) C2

c) C3

Figure 1: SEM images and statistical distribution of fiber diameter of samples obtained by electrospinning of chitosan solutions of a) 5%; b) 6%, c) 7%

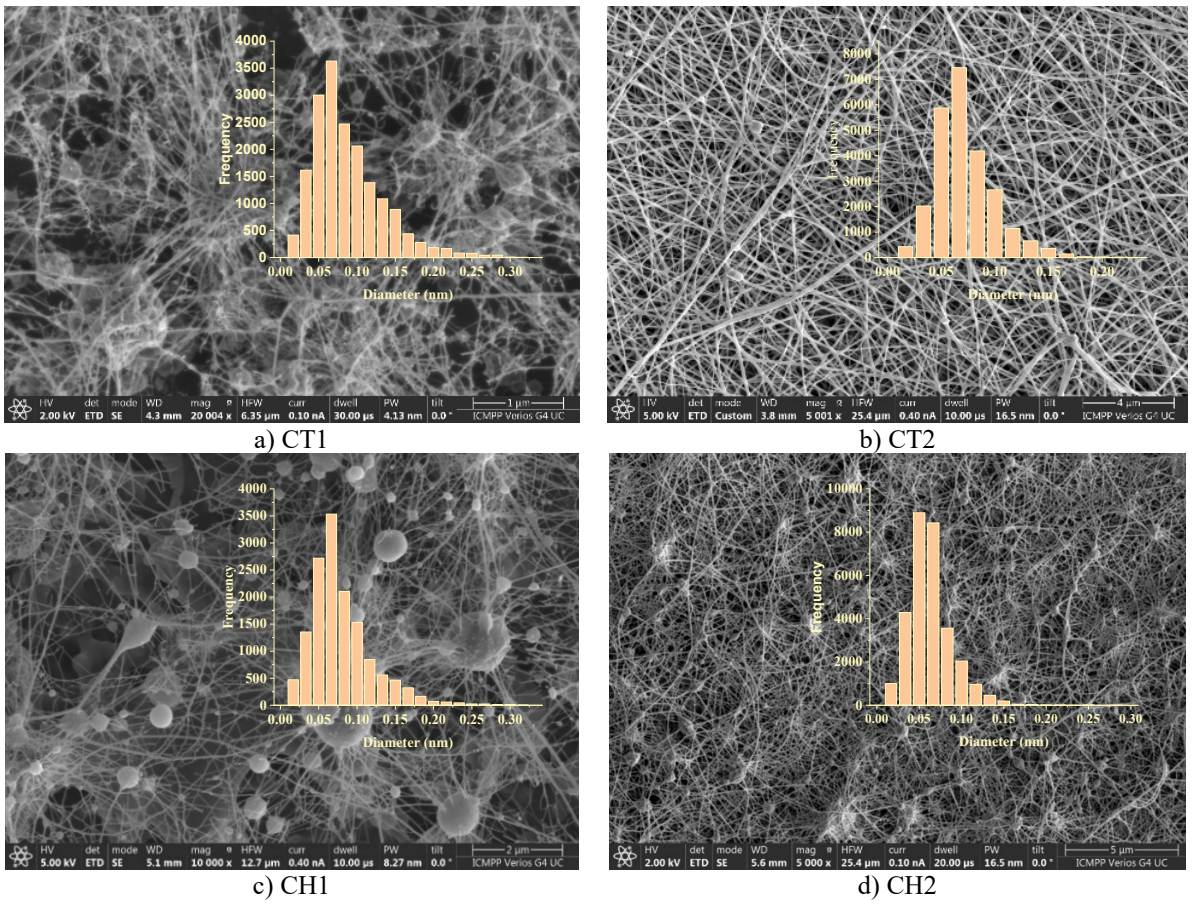


Figure 2: SEM images of chitosan/quaternized chitosan fibers and graphical representation of the average diameter of the studied fibers as insert

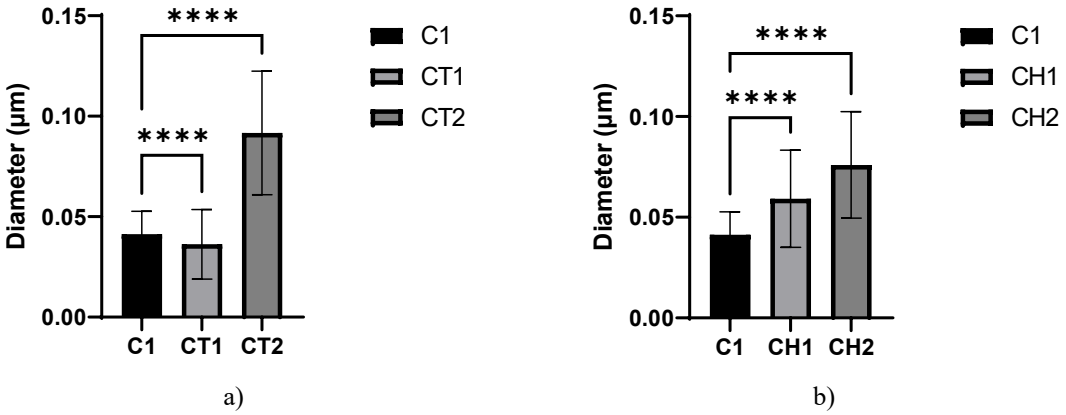


Figure 3: Statistical comparison of fiber diameters

This was not correlated with an increase in the solution's viscosity, and most probably, was inflicted by the decrease in the flow rate, necessary to form the Taylor cone. The observation of the fibers under polarized light showed more intense birefringence for the chitosan and chitosan/TMC nanofibers, compared to the chitosan/HTCC ones (Fig. 4), suggesting better entanglement of the macromolecular chains, which impede their alignment during

electrospinning and explain the better electrospinning process in the case of HTCC based fibers compared to TMC ones.

The successful obtention of the binary fibers was supported by FTIR spectra (Fig. 5). Thus, the presence of chitosan was confirmed by the absorption bands of the main groups, such as amine units at 1571 cm^{-1}, ether bonds around 1060 cm^{-1}, C-H bonds of the -CH_2- units around 2907 and 2860 cm^{-1}, and amine and hydroxyl groups, as well as the H-bonds formed by them, in the 3500-3000 cm^{-1} spectral domain. Besides, a broad absorption band of low intensity appeared around 1461 cm^{-1}, characteristic of the vibration of the C-H bonds of the -$N(CH_3)_3$, confirming their presence. The low intensity and slight shift of this band are in line with the low percentage of quaternary units and with their ability to form hydrogen bonds, as also acknowledged by other authors.[23,28,29]

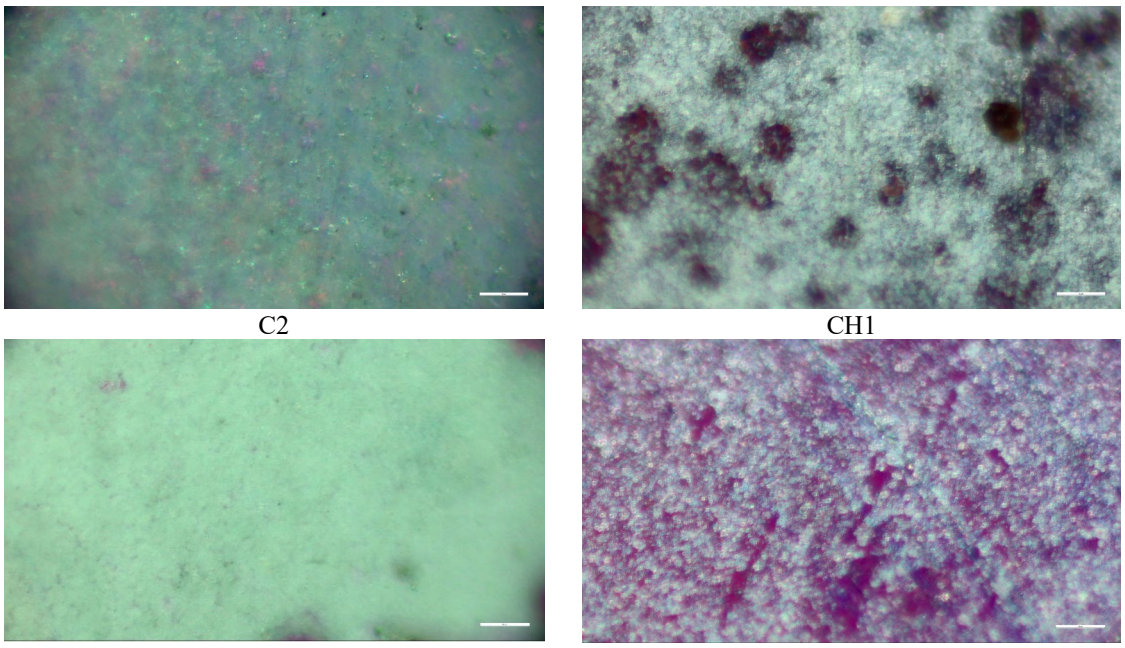


Figure 4: POM images of representative fibers studied

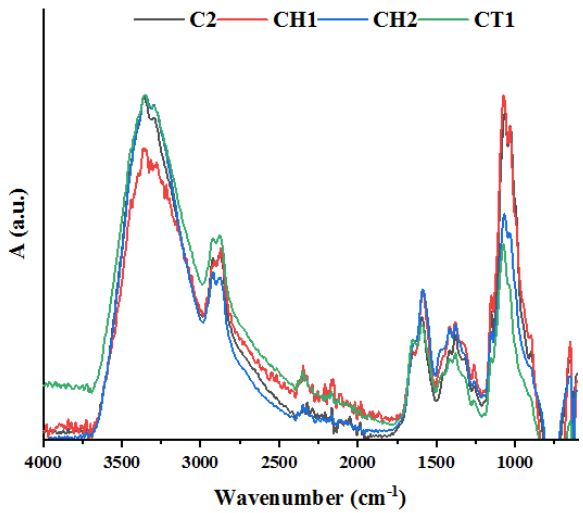


Figure 5: FTIR spectra of studied fibers

It is well known that the quaternary units improve the antimicrobial activity of chitosan due to the presence of the permanent positive charge, making it more prone to develop intermolecular

forces with the negatively charged bacterial membrane, compared to the amine units of chitosan.[15,30,31] This is especially true of nanofibers, which have an increased area-to-volume ratio, augmenting their specific surface and thus their properties.[7,17,32,33] All the tested samples, containing or not quaternary chitosan, showed very high antibacterial activity against the gram-positive bacterial strain *S. aureus*, with more than 90% bacterial inhibition. Moreover, the samples containing quaternized chitosan revealed slightly lower bacterial viability, compared with that based on chitosan, in line with its higher activity. The influence of quaternary chitosan was more evident in the case of gram-negative bacteria represented by *E. coli*, for which the samples containing quaternary units reduced the bacterial cells viability by more than 80%, while chitosan fibers were less efficient, reducing it by a little over 60%. Interestingly enough, in the case of the yeast strain represented by *C. albicans*, the efficiency of sample CH2 was lower than that of the other samples, with a bacterial cell viability around 20%, compared to 10%. Analyzing the morphological data, it can be seen that this lower activity correlated well with the higher mean diameter of the fibers, and consequently with the narrower active surface. It can be concluded that the antimicrobial activity is related to the presence of quaternary groups, but also to the specific surface of the fibers, which allows a corresponding improvement of the contact between bacteria and fibers. Overall, the fibers were very active, considering that the pathogen inoculum was enormously higher, compared with those from real life.

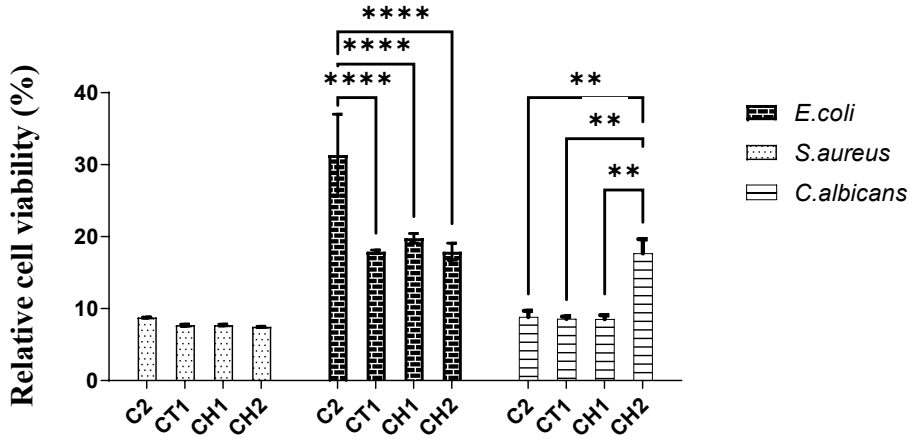


Figure 6: Antimicrobial activity of the sample against the reference strains

CONCLUSION

Quaternized chitosan-based nanofibers were prepared for the first time by electrospinning chitosan/quaternized chitosan solutions, without using a synthetic co-spinning agent. Fibers of good quality, with a diameter below 100 nm, were obtained when *N,N,N*-trimethyl chitosan chloride and *N*-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride were mixed with chitosan of low molecular weight, *i.e*., 109 kDa. The fibers presented high antimicrobial and antifungal activity against *S. aureus*, *E. coli* and *C. albicans*, proving that the inclusion of quaternized chitosan into nanofibers significantly improved the antimicrobial activity against gram-negative bacteria, while the activity against yeasts was dependent on the active surface of the sample given by the fibers' diameter. On the other hand, the electrospinning yield of the fibers was low, further optimization investigations being necessary for practical applications.

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