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„Petru Poni" Institute of Macromolecular Chemistry

WATER SOLUBLE CHITOSAN DERIVATIVES FOR BIOMEDICAL APPLICATIONS

Summary of doctoral thesis

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We inform you than on 20th of October 2023, at 11:00, in the **Conference Hall of „Petru Poni" Institute of Macromolecular Chemistry**, Iasi, will take place the defense of the thesis entitles "Water soluble chitosan derivatives for biomedical applications", author Bianca-Iustina Andreica, to acquire the PhD degree.

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According to the Regulations on the organization and PhD defense within the Romanian Academy, please find enclosed a summary of the PhD thesis. Your comments and appreciations will be highly acknowledged. You are kindly invited to attend the public presentation of the PhD thesis.

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Introduction

Chitosan, whose history dates to 1859, is a natural biopolymer derived from chitin, main component of crustaceans, exoskeleton of insects and some types of fungi. Chitosan attracted special attention in the biomedical domain, due to its unique properties and potential benefits generated by its chemical structure. The biocompatibility, biodegradability, reduced toxicity, and antimicrobial properties recommend it as an attractive candidate for a large realm of medical applications, with accent on wound healing, drug delivery systems, tissue engineering and gene therapy. Despite all of these, chitosan presents a drawback, namely its limited solubility in media of neutral or basic pH, an aspect which can limit its efficiency for some bioapplications. This drawback has led to the development of *water soluble derivatives*, with the goal of preserving the beneficial properties of chitosan, while overcoming the barrier induced by the restricted solubility, only to acid media.

The limited water solubility of chitosan derives from its chemical structure, due to the degree of crystallinity, a consequence of intra- and inter-molecular hydrogen bonds. Water soluble chitosan derivatives are designed to overcome this problem, by chemically modifying the structure of chitosan without affecting the main chain. These modifications involve processes such as grafting, carboxyalkylation or quaternization, techniques which introduce groups that impart solubility in water or perturb the overall charge distribution of the chitosan macromolecule.

Water soluble chitosan derivatives offer several distinct advantages for biomedical applications. First, they can be easily incorporated into aqueous solutions, facilitating their integration into formulations for drug delivery systems, wound dressings, and hydrogels with multiple functionalities. Their improved solubility facilitates the controlled release of bioactive compounds, leading to improved bioavailability and efficacy. In addition, these derivatives can improve interactions with biological molecules, such as proteins and nucleic acids, which is beneficial for applications such as gene therapy and tissue engineering. Their water soluble nature also promotes their compatibility with biological systems, reducing the risk of adverse reactions and improving biocompatibility.

In this context, the thesis had the main goal of obtaining of a series of water soluble chitosan derivatives and of materials based on them, targeting their applicability in the biomedical field. The achievement of this goal was followed in strong correlation with the specific objectives settled at the beginning of the stage: **O1** – Obtaining of quaternized chitosan, with an optimum quaternization degree, to assure a balance between solubility and properties, considering especially the biocompatibility; **O2** –

Designing and obtaining of materials based on quaternized chitosan, with various biomedical applications, in accordance with the contemporary necessities, and **O3** – Developing of a new method for the chemical modification of chitosan, to obtain water soluble derivatives with applicability perspectives.

Therefore, the thesis entitled "**Water soluble chitosan derivatives for biomedical applications**" was conceived and conducted in the following manner: (***i***) Synthesis and characterization of hydrogels based on quaternized chitosan and a natural monoaldehyde and the evaluation of the potential of these materials to act as biocidal disinfectant hydrogels; (***ii***) Obtaining and characterization of binary chitosan/quaternized chitosan nanofibers *via* electrospinning technique, with potential role in regenerative medicine and tissue engineering; (***iii***) Obtaining and characterization of composite nanofibers based on imino-chitosan/quaternized chitosan and investigation of their potential use as hemostatic bandages able to contribute to wound healing and (***iv***) Synthesis and characterization of a new class of chitosan derivatives by grafting poly(trimethylene carbonate) chains on the chitosan backbone and investigation of the essential properties for biomedical applications.

The thesis is structured in two main parts which comprise five chapters: **Part I** includes **Chapter I**, in which are briefly presented literature data relevant for the studied topic, and **Part II** which comprises **Chapters II, III, IV**, in which are exposed the main results obtained for the personal research and **Chapter V** in which are presented the materials, techniques, equipment and supplementary experimental data.

Chapter I is designated to the presentation of the literature data about the general techniques used for the obtaining of water soluble chitosan derivatives, with accent on the obtaining of quaternized chitosan. Also, information about the state of the art on the topic of hydrogels and nanofibers based on quaternized chitosan, used in the biomedical field, were presented.

In **Chapter II** are presented data about the obtaining and characterization of quaternized chitosan, further used in the obtaining of hydrogels using salicylaldehyde, designed as biocidal disinfectant hydrogels. The hydrogels were completely characterized from the structural, supramolecular and morphological points of view, and their properties relevant for the envisaged application were also evaluated, in terms of bioadhesiveness, antibacterial activity, biodegradability and *in vivo* biocompatibility.

Chapter III is divided into two subchapters; The first subchapter describes the obtaining of quaternized chitosan/chitosan nanofibers, and the second one aims to expand the design, by including bioactive vanillin. The first subchapter highlights the potential of obtaining defect-free binary nanofibers, starting from an electrospinning mixture containing poly(ethylene oxide) as a co-spinning agent, followed by its selective

and total removal from the fibers. The fibers were fully characterized from the structural, morphological, and thermal points of view and their aqueous behavior, antibacterial activity, muco- and bioadhesion, biodegradability, as well as their *in vitro* and *in vivo* biocompatibility were evaluated. The second subchapter describes the obtaining of fibers encapsulated *in situ* with vanillin, by the imitation reaction with both chitosan and its quaternized derivative. Complete characterization of fibers with the above-mentioned techniques has also been carried out. The application for these fibers was as hemostatic dressings with role in wound healing, and in this regard, the kinetics of vanillin release, the antioxidant activity of fibers, and their capacity for retention and clotting of blood, as well as adhesion of red blood cells were evaluated.

In **Chapter IV** is presented the obtaining of a new water soluble chitosan derivative, by grafting poly(trimethylene carbonate) chains on the chitosan backbone. The grafting method was based on the ability of chitosan's hydroxyl groups to act as initiators in the Ring-Opening Polymerization reaction of trimethylene carbonate. Different reaction conditions were evaluated, in relationship with the properties of the obtained derivatives, in order to obtain an optimal solubility / properties balance. The compounds that were water soluble were subjected to structural, morphological, and surface characterization, and their biodegradability as well as *in vitro* biocompatibility were assessed.

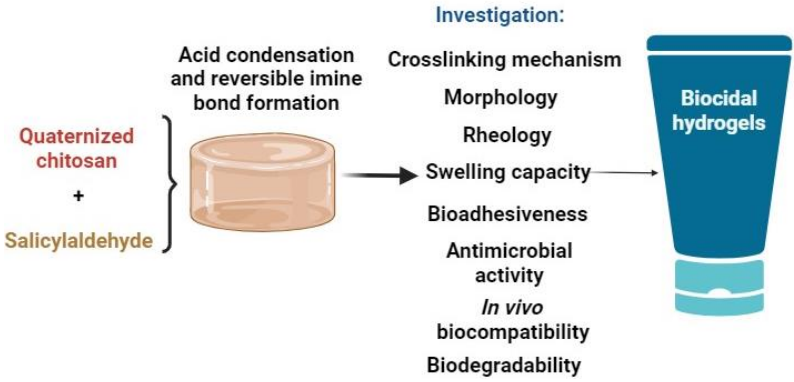
Chapter V is designated to the experimental part, which covers the materials, equipment and techniques used during the studies. Also, the synthesis and protocols are described, as well as supplementary information complementary to those presented in the previous chapters, such as spectra, graphs and images.

The thesis ends with the presentation of the dissemination activity carried out during the three years of doctoral studies, the financial support that contributed to the studies, the mobilities performed and the bibliography consulted in order to acquire new information, as well as to correctly interpretate the personal results.

Chapter II
Hydrogels based on quaternized chitosan

II.1. Introduction

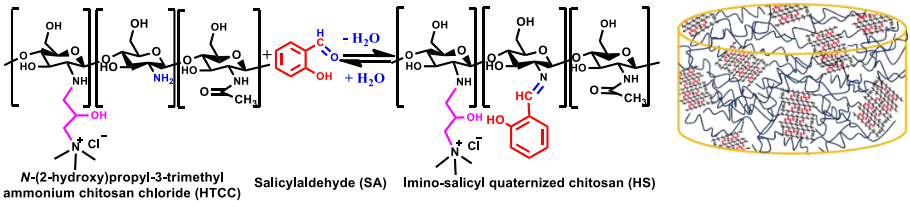
Recent global events, such as COVID-19 pandemic, have brought to light the critical importance of proper hygiene practices, and the need for efficient disinfectants to prevent infections with bacteria and viruses. Quaternary ammonium compounds are well-known ingredients of alcohol-free disinfectants, being able to interact with the membranes of bacteria, but there are some concerns regarding their cycle in the environment, as well as the limited sustainability in their obtaining [1]. A promising alternative is the use of quaternary ammonium salts of chitosan (QCS), which have the advantages of being naturally originated, bio- and mucoadhesive, biodegradable and biocompatible, while also possessing broad-spectrum antibacterial activity [2]. Moreover, recent studies reported the antiviral activity of *N*-[(2-hydroxy)propyl-3-trimethyl ammonium] chitosan chloride (HTCC) against coronaviruses, including SARS-CoV-2 [3]. Taking all these data into consideration, the goal of the present study was the obtaining of a series of biocompatible, biodegradable, and bactericidal hydrogels, composed of quaternized chitosan (HTCC) crosslinked with salicylaldehyde (SA) *via* reversible imine linkages. The choice of salicylaldehyde was based on its natural origin, lack of toxicity on healthy cells and its ability to form imine bonds with chitosan [4]. The obtained hydrogels were completely characterized from the structural, morphological, supramolecular, and rheological points of view. For the targeted application, their time-dependent antibacterial activity was evaluated, as well as the *in vivo* biocompatibility and soil degradability (Scheme II.1).



Scheme II.1. Experimental plan for the obtaining and characterization of the hydrogels.

II.2. Results and discussions

Derivatization of chitosan with 43% quaternary ammonium groups endows chitosan with water solubility, allowing thus to prepare hydrogels in media of neutral pH, more prone for *in vivo* applications. By using the strategy of acid condensation reaction of the free amino groups of HTCC with the formyl functionality of salicylaldehyde, a large series of 15 hydrogels was attained, when the molar ratios varied from 1/1 to 15/1. The hydrogels were coded with HS, followed by a number describing the molar ratio between the components. Previous studies demonstrated that chitosan's crosslinking with aldehydes proceeds by the occurrence of three concomitant processes, i.e. *(i)* reaction of amine groups of chitosan with formyl group of aldehydes to form imine units (Scheme II.2b); *(ii)* self-assembling of the imine units belonging to different chitosan chains into clusters (Scheme II.2c) and *(iii)* development of new intermolecular forces [4,5]. To verify if the same general strategy applies to quaternary salts of chitosan, the obtained hydrogels were analyzed by means of specific methods, to monitor the occurrence of the above-mentioned processes.



b) **c)**

Scheme II.2. b) Imination reaction of QCS with SA and c) Schematic representation of the self-assembling processes governing the hydrogelation.

^{1}H-NMR and FTIR spectroscopy on a hand, and X-ray diffraction and POM images on the other hand, indicated that the hydrogelation of quaternized chitosan with salicylaldehyde occurred due to imination reaction concomitant with the self-assemble of the imine units into layered hydrophobic clusters, which play the role of crosslinking nodes. Moreover, the chemical structure of HTCC and SA favored the occurrence of strong intermolecular forces, aiding the hydrogelation even for low amounts of SA.

The rheological investigation of the gelation kinetics of the salicyl-imine-HTCC indicated that it proceeded in two steps. During the first 15 min, the storage modulus (G') increased rapidly; the amplitude of this increase depending on the imination degree. Then, a linear evolution of the modulus was observed, indicating a slow reorganization of the hydrogels. Furthermore, the plateau modulus values (G_0) of imino-HTCC barely

changed upon heating, contrary to that of HTCC, showing that the intermolecular interactions present in the precursor weaken as the temperature increases.

Further, the self-healing behavior of salicyl-imine-HTCC was investigated by dynamic rheometry, but also visually, by injecting two hydrogels into a vial, one blank composed only with HTCC and SA, and another one containing Rhodamine B, for a better contrast (Fig. II.4). The inverted tube test allowed to visually observe that the two hydrogels connected, forming a hydrogel which held its integrity. Further, the hydrogel was able to completely reshape after spreading it on the walls of the vial and passed the inverted tube test. Thus, this test demonstrated the self-healing ability of salicyl-imine-HTCC hydrogels, and their ability to be easily injectable, important characteristics when topical bactericidal materials are envisaged, because they could be easily stored in a tube and applied on the skin.

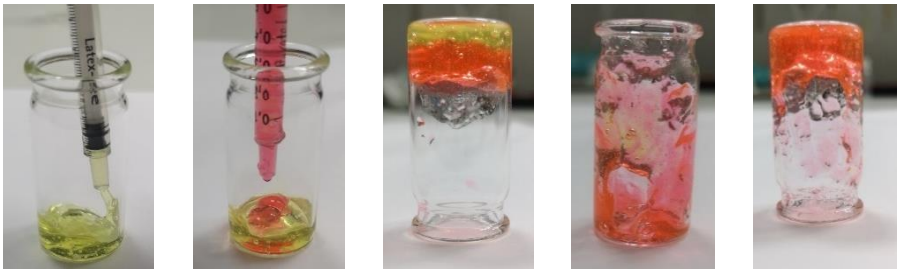
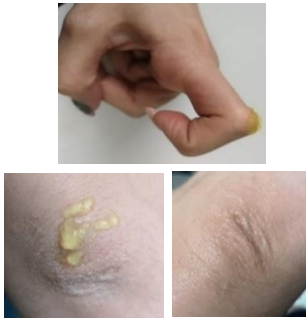
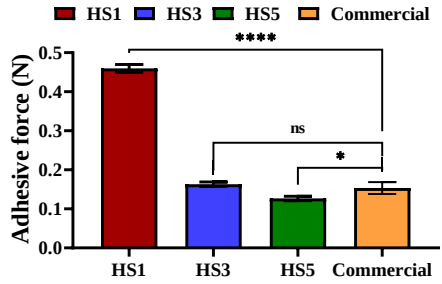


Figure II.4. Images revealing the self-healing ability of the investigated hydrogels (represented for HS3).

One of the vital improvements given by grafting quaternary ammonium units on the chitosan backbone is the enhancement of the adhesive properties [2]. The adhesive force of the hydrogels was measured on chicken skin as a model tissue, in comparison with a commercially available hygienic hand gel (based on alcohol and carbomer) (Fig. II.7b). The highest adhesive force (0.46 N) was obtained for the hydrogel with the highest imination degree, and the values decreased as the imination degree decreased, reaching 0.13 N for HS5 sample. It appeared that the salicyl units were favorable for improving the bioadhesiveness, most probably due to the presence of hydrophilic hydroxyl units. Compared to the commercial antibacterial hydrogel, HS1 was statistically significant more adhesive ($p<0.0001$), HS3 did not lead to statistically significance and HS5 was less adhesive ($p<0.5$). These data recommend the studied hydrogels as suitable disinfectants, having the skin adherence similar to the gels already used on a large scale by consumers.

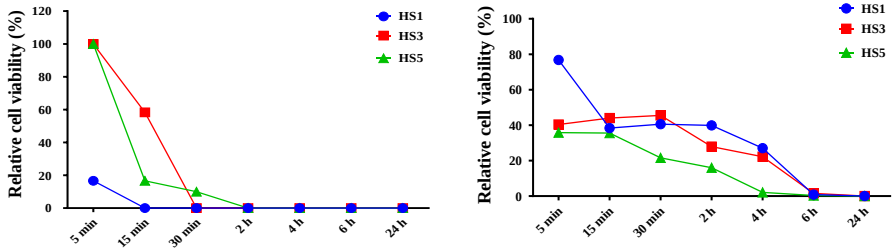




a) **b)**

Figure II.6. a) Adhesiveness of the QS3 hydrogel, investigated on glass, steel and human skin and b) Adhesive force measured on chicken skin as model tissue (**** $p<0.0001$; * $p<0.5$).

The most relevant property for the envisaged application is the antimicrobial activity of the hydrogels. In the case of the Gram-positive bacterial strain represented by *S. aureus*, the sample with the highest imination degree (HS1) was the most effective, completely suppressing the bacteria after 15 minutes, with less than 20% bacteria viability within 5 minutes. (Fig. II.7a). In the case of the Gram-negative bacterial colonies represented by *E. coli*, all the hydrogels were able to suppress the bacteria cells more than 60 % in the first 15 minutes, and the killing effect continued up to the cell's viability ±1% after 6 hours and no colonies were detected after 24 hours (Fig. II.7b).



a) **b)**

Figure II.7. Assessment of the antimicrobial activity of the hydrogels against a) *S. aureus* and b) *E. coli.*

The hemocompatibility of the studied hydrogels was investigated *ex vivo* by measuring the hemolysis percent when the samples were in contact with mice blood, compared with Triton X-100 and physiological serum (PS) as positive and negative control, respectively [6]. No statistically significant deviation was noticed between the value obtained for PS and those for the hydrogels (~2.75%), which suggests that hydrogelation with SA did not inflict any destructive effect on red blood cells.

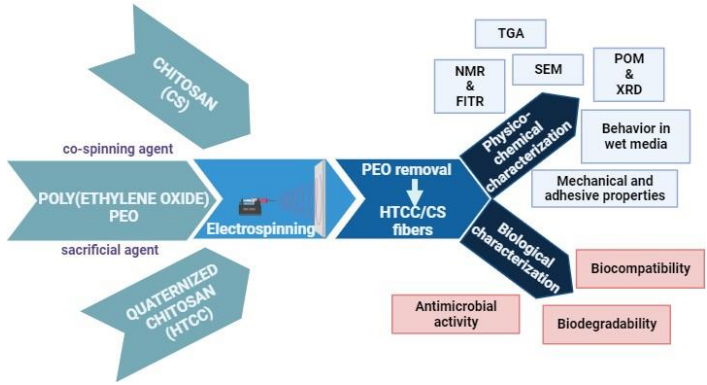
The acute and systemic toxicity of the hydrogels was locally investigated, after placing sterile patches impregnated with hydrogels on the back of mice, using physiological serum (PS) as negative control. The use of hydrogels HS1, HS3 and HS5 did not produce significant hematological, biochemical, and immunological changes, and did not obviously influence some specific parameters of oxidative stress, compared to the use of patches impregnated with saline. These data indicate that the investigated hydrogels have a good *in vivo* biocompatibility and they do not present any potential risks in view of biomedical applications, either in direct contact with the skin, or for disinfection of surfaces.

Chapter III
Nanofibers based on quaternized chitosan

III.1. Binary nanofibers based on chitosan/quaternized chitosan

III.1.1. Introduction

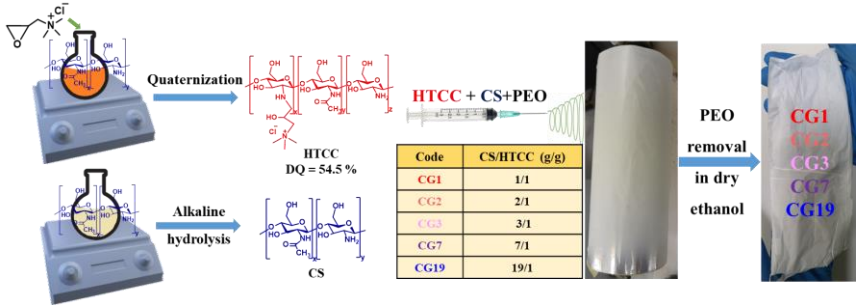
Chitosan nanofibers are of special interest, due to their specific morphology mimicking 3D scaffolds of natural tissues, being therefore suitable for a plethora of biomedical applications. [7,8]. Even though they present numerous advantages, neat chitosan nanofibers are difficult to be obtained by electrospinning, because of the polycationic character of chitosan, and to overcome this limitation, the technique of using co-spinning agents such as poly(ethylene oxide) or poly(vinyl alcohol) was chosen. [7]. The disadvantage of this technique is the use of synthetic polymers, which are not biodegradable, limiting therefore the *in vivo* use. In this context, the aim of this study was the obtaining of chitosan (CS) and quaternized chitosan (HTCC) nanofibers, with potential biomedical applications, such as tissue engineering, regenerative medicine, wound healing, but not only. To reach this goal, a ternary mixture was used for electrospinning, using poly(ethylene oxide) as co-spinning agent, which was then removed from the fibers by selective washing in a non-solvent for the other components (Scheme III.1)



Scheme III.1. Experimental plan for the obtaining and characterization of the nanofibers.

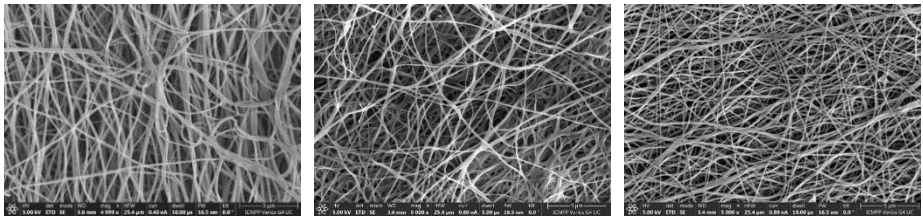
III.1.2. Results and discussions

Binary chitosan/quaternized chitosan (CS/HTCC) fibers were prepared by electrospinning of CS/HTCC/PEO solutions with different weight ratios, while keeping constant the amount of PEO (CS-HTCC/PEO = 4/1, m/m), and the removal of PEO was achieved by washing the fibers with dry ethanol, previously activated on molecular sieves (Scheme III.2) [9]. The samples were coded with CG, followed by a number which represents the mass ratio between CS and HTCC.



Scheme III.2. Schematic representation of the obtaining of CS/HTCC binary electrospun mats.

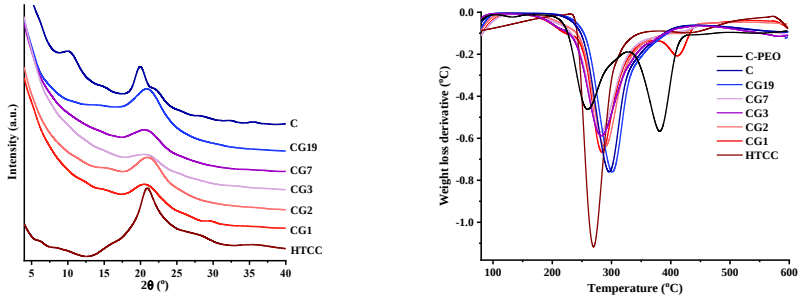
The strategy was a successful one, because after the removal of PEO, the morphology of the fibers was a smooth one, with no defects even for high amounts of HTCC, allowing the formation of a three-dimensional network with inter-fiber pores with the dimensions under 2 μm (Fig. III.1).



CG1 **CG3** **CG19**

Figure III.1. SEM microphotographs of the CS/HTCC fibers

The supramolecular characterization of the binary CS/HTCC nanofibers was achieved using Wide Angle X-ray Diffraction. The diffractograms indicated the semicrystalline nature of the fibers, due to the alignment of the macromolecular chains during the electrospinning process (Fig. III.3a). This diffraction pattern, somehow similar to that of bulk HTCC, suggests the disruption of inter- and intra-molecular bonds amongst the chitosan chains, in line with the strong repulsive forces developed by the ammonium units of HTCC.



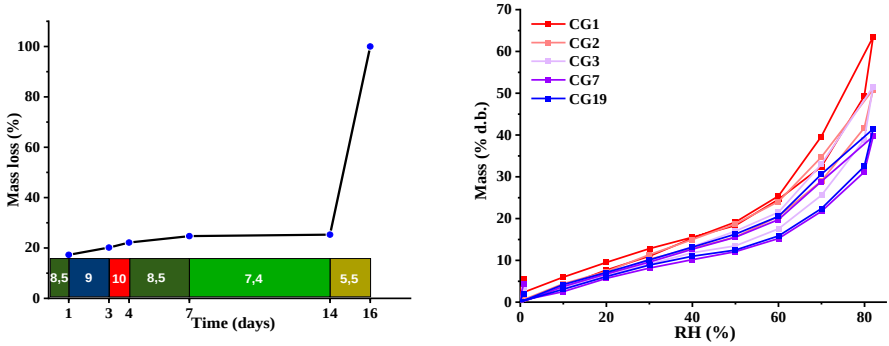
a) **d)**

Figure III.3. a) X-ray diffraction patterns and d) TGA derivatives (C-PEO – represent chitosan/PEO fibers).

The thermogravimetric analysis (TGA) was used to confirm the total removal of PEO from the fibers, but also to assess the thermal stability of the fibers. The TGA curves and their derivatives of the binary fibers present two degradation steps, with the maximum at 100 °, attributed to the evaporation of water traces, and the second one at 270 °, which corresponds to the lysis of the σ bonds from chitosan (Fig. III.3d). The degradation step corresponding to PEO, in the interval 300-400 °C [10], is missing in the case of the binary fibers, confirming therefore its selective and total removal.

To simulate the behavior of the nanofibers in media of different pH, similar to that of living organisms, a biodegradation study was conducted, by varying the pH of

the media in a similar manner with its evolution during the wound healing process [11]. At basic pH of 8.5, most encountered in the first day after a wound/burn, the mass loss of the fibers reached values of almost 18%, then increased slowly till 25% during the next 14 days, and reached complete degradation in media of pH 5.5, corresponding to the dermis (healthy tissue) (Fig. III.4d). Therefore, the binary CS/HTCC nanofibers are ideal candidates to be used as resorbable bandages for the treatment of wounds, by completely degrading only after total healing of the wounds, when the pH reaches normal values.



d) **e)**

Figure III.4. Mass loss of the samples in d) lysozyme solution of different pH, represented for sample CG7 and e) DVS curves.

To evaluate the ability of the fibers to retain humidity, dynamic water vaper sorption (DVS) experiments were performed. The fibers with the highest amount of HTCC (CG1) reached values of maximum moisture uptake capacity of 63%, in a media of relative humidity (RH%) of 85%, values which decreased with the decrease in the HTCC content, till 41% for CG19 sample, and only 35% for neat chitosan fibers [12]. Another important characteristic observed during performing the DVS experiments was the ability of the fibers to completely desorb the retained humidity, during the cycle of RH decrease, evidencing the good breathability of the fibers, property relevant for the use of the fibers as bandages.

The fibers' mechanical properties were evaluated by analyzing the stress-strain curves. Neat chitosan fibers withstand to a maximum tensile stress of 33.7 MPa before breakage [13]. This value greatly increased to 49.39 MPa for CG3 and decreased to 29.9 and 24.1 MPa for CG7 and CG19, respectively. The lack of precise correspondence between the tensile strength (TS) and HTCC content can reflect the random orientation of the fibers within the nanofibrous mat and can also be due to the variation of the inter- and intra-fiber pores created after the removal of PEO. The fibers resisted to changes of

shape without crack, with elongation at break (EB) values from 5 to 9.14 %. Once again, the lowest value was recorded for the CG19 sample, and there is no clear EB/HTCC content relationship, pointing to the same irregular distribution of the fibers. On the other, a clear increase of the Young's modulus from 8 N/m^2 for C to 19 N/m^2 for CG3, suggested HTCC as an elasticity enhancer, possible do to its influence on the crystallinity degree, as the X-ray diffraction indicated.

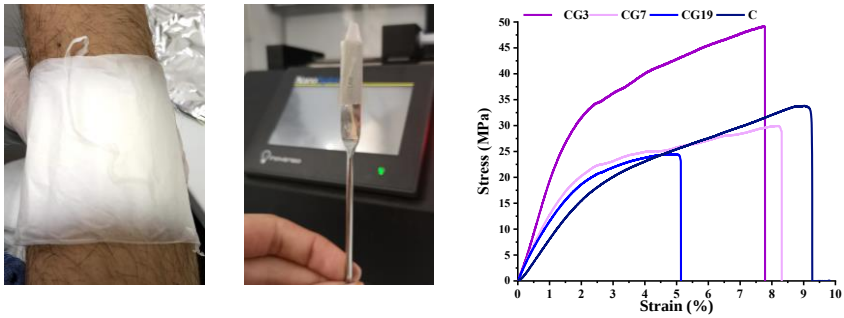


Figure III.7. a) Images of the fibers proving their mechanical and adhesion properties; b) Stress-strain curves of the samples.

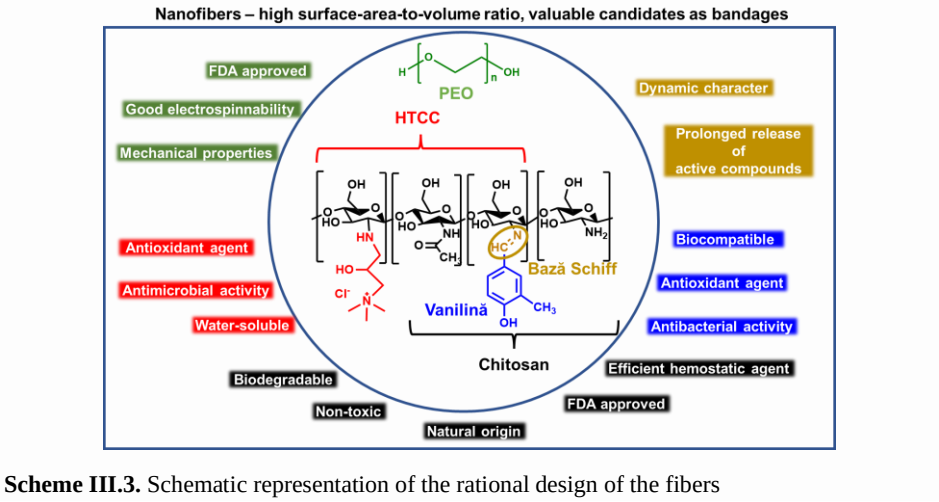
Regarding the antimicrobial activity of the binary fibers, small quantities of samples were able to inhibit the growth of *E. coli* and *S. aureus* in less than 6 hours, and 24, respectively, while the neat chitosan fibers presented a limited effect. Tested on Normal Human Dermal Fibroblasts (NHDF), the samples presented cytocompatibility adequate for biomedical devices, while their *in vivo* biocompatibility evaluation on rats, by subcutaneous implantation, revealed the lack of toxicity on blood, liver and kidneys, and also lack of allergic effect on the immune system.

III.2. Composite nanofibers based on imino-chitosan/quaternized chitosan

III.2.1. Introduction

Uncontrollable bleedings represent one of the main causes of deaths worldwide, being responsible for more than 5 million deaths annually, at an estimated cost of 518 milliards dollars globally [14]. Consequently, the reduction of blood loss is of utmost importance, driving to the development of various hemostatic materials for first-aid treatment in civilian traumatic situations or on the [15]. Supplementary to the hemostatic effect, choosing the right components of the nanofibers can lead to materials able to improve wound healing. Amongst materials, nanofibers present the advantage of high porosity and high surface-area-to-volume ratio and conformability, and they are easy

and simple to apply. All these advantages justify the interest manifested in the obtaining of hemostatic bandages based on composite chitosan nanofibers, domain which is in an ascending trend. In this context, the goal of this study was the obtaining of composite nanofibers that possess fast hemostatic effect, which can also facilitate rapid and normal wound healing. To achieve this, the components of the nanofibers were chosen considering their properties, namely: *chitosan (CS)*, *quaternized chitosan (HTCC)*, *vanillin (V)* and *poly(ethylene oxide)(PEO)* (Scheme III.3).



Scheme III.3. Schematic representation of the rational design of the fibers

III.2.2. Results and discussions

The composite nanofibers were obtained based on the protocol established in the previous subchapter, by varying the mass ratio between CS and HTCC, while keeping the amount of PEO constant. Vanillin was added in the electrospinning solutions in a 4/1 molar ratio between the amine functionalities of chitosan and the aldehyde functionality of vanillin.

In accordance with the multicomponent nature of the fibers, FTIR spectra were complex, with many superposed bands. However, the successful reaction of the amine groups of CS and HTCC with vanillin was confirmed by the occurrence of a sharp band at 1640 cm^{-1}, characteristic to the stretching vibration of imine bonds (Fig. III.13a) [16]. Deconvolution of the 1700-1620 cm^{-1} spectral domain indicated the presence of another two superposed bands, around 1660 and 1672 cm^{-1}, attributed to the amide group of chitosan/HTCC and traces of unreacted vanillin, respectively (Fig III.13b).

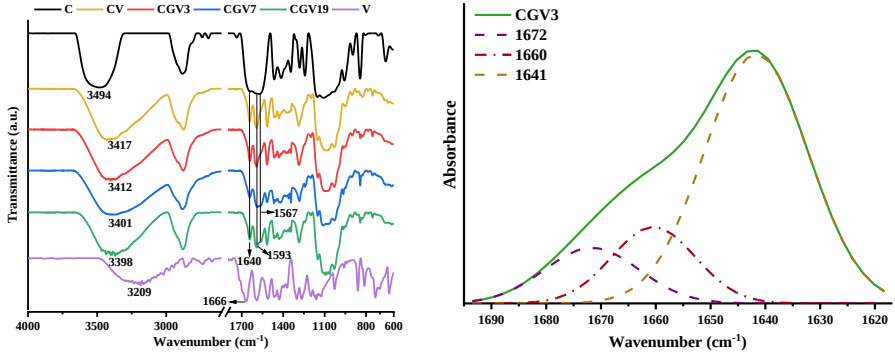


Figure III.13. Structural characterization of the nanofibers by a) FTIR Spectroscopy; b) Deconvolution of the 1700-1620 cm^{-1} spectral domain, for CGV3 sample.

The efficiency of the nanofibers to absorb blood was evaluated for the composite materials, but also for the binary CS/HTCC fibers (abbreviated CG) and for the ternary fibers CS/HTCC/PEO (abbreviated CGP), to assess the influence of each component. Also, for comparison, two materials were tested: gauze and a hemostatic sponge commercially available. The superporous nature of the hemostatic sponge led to the absorbance of 32 g/g blood, significantly higher value in comparison to gauze, which absorbed half this value. The composite fibers presented a retention capacity in the range of 20-30 g/g, similar to their water retention capacity. Surprisingly, the results indicate that the presence of PEO in the fibers had a negative effect regarding the blood retention. On the other hand, the data revealed that vanillin has an important role in the design of these materials, annihilating the negative impact of PEO.

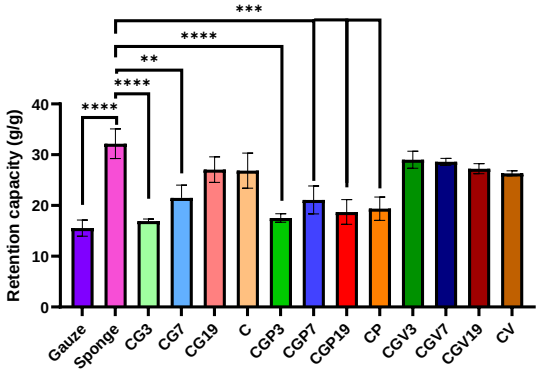


Figure III.16. Capacity of the fibers to retain c) blood.

Vanillin can prevent the risk of infections, associated with the inflammatory period, and can also contribute to a facile healing of the wounds, having a beneficial role in the reepithelization process [17]. Considering that vanillin is chemically bonded to chitosan and quaternized chitosan by reversible imine bonds, the vanillin's release

kinetic was evaluated in a manner controlled by its consumption, in ultrapure water and PBS of pH 7.4 [18]. The cumulative release was observed for 24 hours, after 12 successive cycles of removing/refreshing the media, at different time intervals. The samples presented a progressive release of vanillin in both media, with an inferior cumulative release in PBS (76%) in comparison with water (100%) (Fig. III.18).

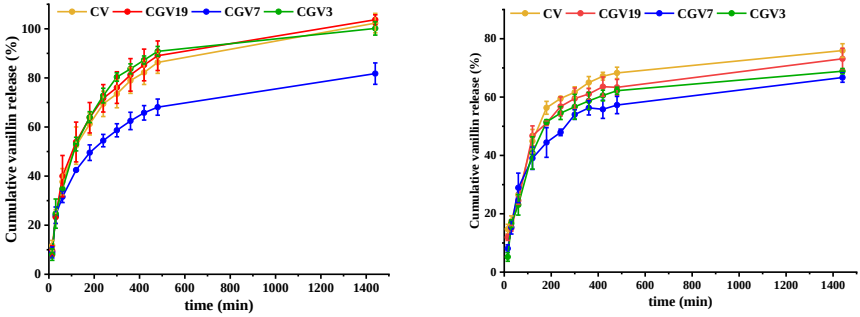
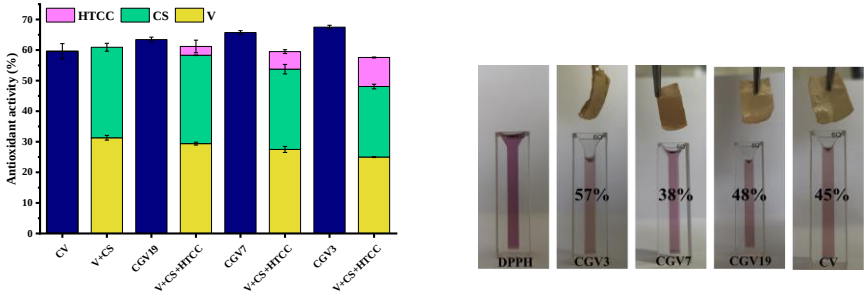


Figure III.18. Release kinetics of vanillin in media of a) ultrapure water and b) PBS 7.4.

The antioxidant activity of the composite fibers was assessed in two manners, by investigating the ability to bond DPPH radicals in (i) solution and (ii) solid state. In acidic aqueous solutions, the antioxidant activity increased with the increase in the HTCC content, from 60% for the fibers without HTCC (CV), to 68% for the fibers with the highest content of HTCC (CGV3) (Fig. III.19a). Moreover, the activity of the fibers was higher than the sum of the activities of neat components, the difference being more pronounced as the HTCC content was higher, pointing for its synergistic effect. (Fig. III.19a). The antioxidant activity manifested similarly when pieces of nanofibers were immersed in DPPH solutions. After one hour, the DPPH solution decolored, while the nanofibers preserved their aspect, in agreement with their antioxidant activity, reaching 57% for CGV3 sample (Fig. III.19b). This fact strengthens the hypothesis that the fibers are able to assure a physical barrier against free radicals, contributing to a normal healing of wounds.



a) **b)**

Figure III.19. Antioxidant activity of the fibers in a) solution and b) solid state (indicated as percent inserted on the images).

An important characteristic of the materials designed to promote wound healing is their ability to inhibit microbial growth. The fibers were extremely efficient in destroying Gram-negative bacteria (91%), regardless of their composition. On the contrary, the effect was dependent on the composition when the fibers were tested on Gram-positive bacteria and yeast (Fig. III.20).

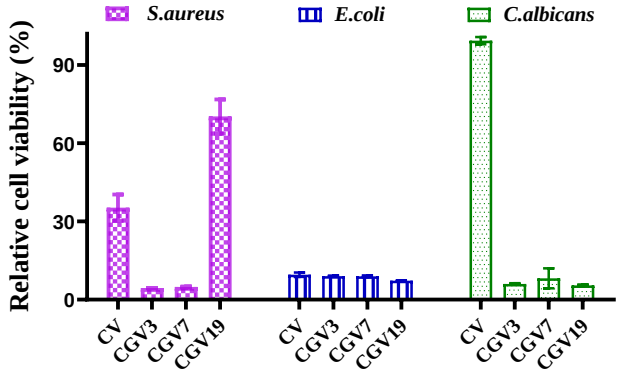
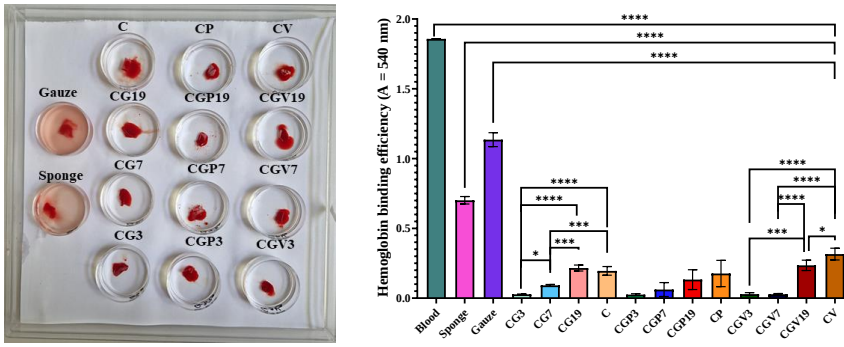


Figure III.20. Antimicrobial activity of the fibers investigated against *S. aureus*, *E. coli* and *C. albicans*.

The evaluation of the nanofibers' capacity to act as hemostatic bandages with rapid effect was assessed by investigating their blood-clotting capacity. After 10 minutes of contact, the unclotted blood was rinsed with PBS 7.4. As can be seen in Fig. III.23a, the solution with the strongest reddish color, associated with the lowest clotting efficiency, was obtained for gauze, followed by the commercial hemostatic sponge. On the contrary, all the solutions obtained for the nanofibers, regardless of their

composition, had a significantly higher hemostatic effect, all the solutions being colorless, only small differences between the samples being observed.



a) **b)**

Figure III.23. Investigation of the clotting efficiency of the fibers a) in dynamic regime, visually observed and b) by recording the absorbances of the washing solutions, at 540 nm (****p < 0.0001 for all samples *vs* blood, gauze or sponge).

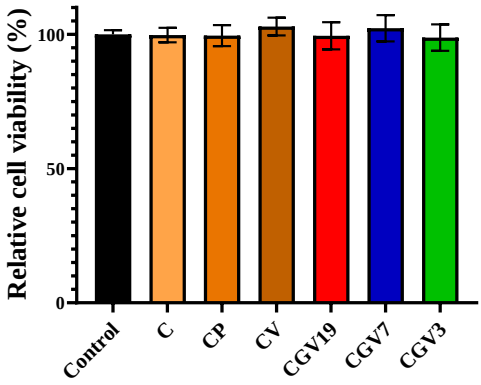


Figure III.25. Results of the *in vitro* biocompatibility tests for the composite nanofibers, obtained by MTS assay, represented as average ± D.S.

The biocompatibility of the composite nanofibers was assessed *in vitro* on NHDF cells, in agreement with ISO-10093 standard for biomedical devices [19], which states that the relative cell viability needs to be higher than 70% in order to consider a material biologically safe. All the investigated fibers respected this criterion (Fig. III.25), having a relative cell viability close to 100%. Therefore, it can be concluded that the fibers are safe in terms of biocompatibility, and can be used in direct contact with fibroblasts.

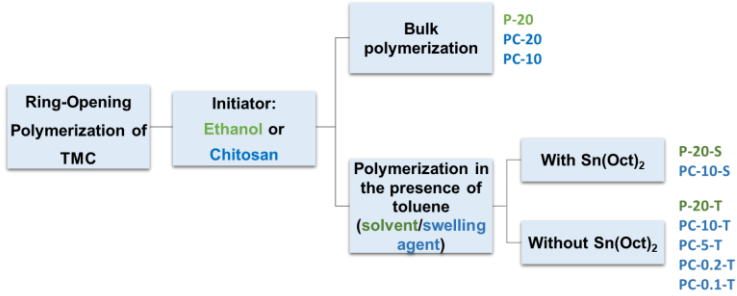
Chapter IV
Chitosan derivatives grafted with poly(trimethylene carbonate) aliphatic chains

IV.1. Introduction

Even though numerous classes of water soluble chitosan derivatives were synthesized and characterized, aiming to obtain compounds suitable for biomedical applications, the necessities of the contemporary life and the rapid adaptation of microorganisms to currently used compounds have encouraged researchers to investigate novel synthetic pathways. In this context, the goal of the study was the obtaining of new water soluble chitosan derivatives and the evaluation of the main properties required for their application in biomedicine. To this aim, chitosan was used as a hydrophilic macroinitiator to obtain poly(trimethylene carbonate) chains grafted on its backbone, by Ring-Opening Polymerization technique. It was hypothesized that in this way, the properties of chitosan can be enhanced, with accent on its solubility, targeting to obtain biocompatible copolymers, with potential to be used in the biomedical field.

IV.2 Results and discussions

To assess the optimum synthetic procedure for the obtaining of the desired compounds, various reaction conditions were varied, such as *(i)* the molar ratio between the glucosamine units of chitosan and trimethylene carbonate (TMC), *(ii)* the presence/absence of tin octanoate ($Sn(Oct)_2$) catalyst and *(iii)* the presence/absence of the solvent (toluene), which is a swelling agent for chitosan (Scheme IV.1). The synthesis of the PTMC homopolymer was also performed, in order to have a reference for the structural characterization of the chitosan derivatives. The copolymers were coded considering *(i)* the main structural constitutive units: chitosan (C) and poly(trimethylene carbonate) (P); *(ii)* the molar ratio between TMC and the initiator (ethanol or glucosamine in the case of chitosan (TMC/ethanol or TMC/glucosamine); *(iii)* the presence of $Sn(Oct)_2$ (S) catalyst or toluene (T) solvent.



Scheme IV.1. Schematic representation of the synthetic strategies and the corresponding codes of the obtained polymers grafted with PTMC.

The derivative PC-10-T, obtained in a molar ratio monomer/initiator of 10/1, in the presence of toluene, presented water solubility. It was characterized by ^{1}H-NMR spectroscopy (Fig. IV.1), and all the characteristic signals were observed, those corresponding to chitosan, H2-H6 in the range 3.2-3.8 ppm, and the protons corresponding to the acetyl group, at 1.96 ppm, and also the signals attributed to the formation of PTMC. The signal of the reference methylene group, which corresponds to 2 protons, overlapped with the H2-H6 protons from chitosan, hardening the determination of the polymerization degree. The integrals' ratio of the chemical shifts of the protons in the repeating units and the end groups allowed a rough estimation of the polymerization degree of 7. The integrals' ratio of the chemical shifts of the protons from the end groups and the protons of the -CH3 of the acetyl group of chitosan allowed the estimation of the substitution degree, which was 5%.

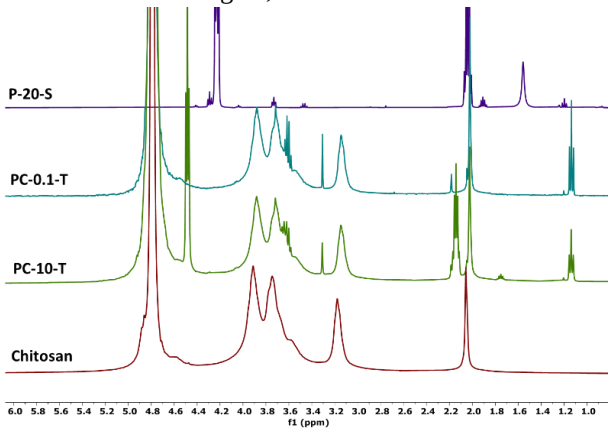
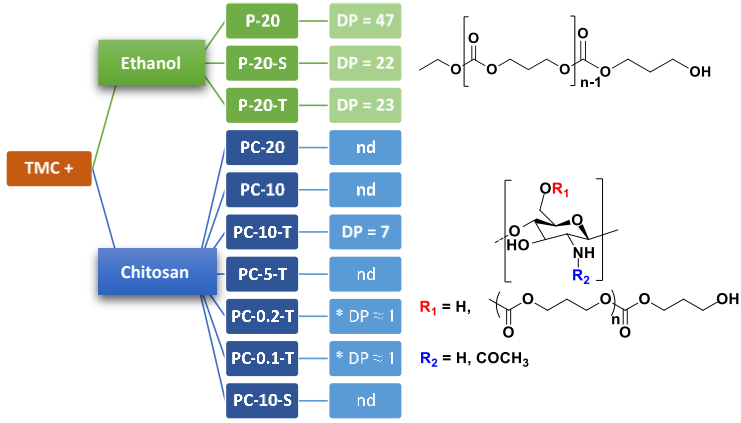


Figure IV.1. ^{1}H-NMR spectra of the derivatives PC-10-T and PC-0.1-T, the homopolymer P-20-S and of chitosan reference.

Therefore, by analyzing the data obtained for the structural characterization, it was observed that for the use of small quantities of monomer (PC-0.2-T, PC-0.1-T), the reaction takes place with the formation of only one unit of TMC bonded to chitosan, explaining the apparition of the multiplet signal in the NMR spectra. Moreover, the grafted chains can form non-classical hydrogen bonds with the neighbor hydroxyl groups, [20] causing the nuclei "deshielding" and consequently shifting the peak downfield[21]. A schematic representation of the synthetic routes and of the corresponding obtained derivatives was described in Scheme IV.2.



Scheme IV.2. Synthetic routes for the obtaining of chitosan grafted with poly(trimethylene carbonate) and of the reference homopolymers, and the degree of polymerization of each compound. *DP = degree of polymerization obtained based on ^{1}H-NMR spectra, nd – not determined due to the insolubility of the compounds.

The solubility tests by visual observation indicated that PC-10-T and PC-0.1-T samples have good water solubility for concentrations of 1% (Fig. IV.5). Moreover, PC-10-T also showed good solubility in DMSO, while PC-5-T and PC-0.1-T showed only partial solubility. Interesting, in the less polar DMF, the solubility increased along with decreasing the content of the grafted poly(trimethylene carbonate): PC-0.1-T showed partial solubility, PC-5-T swelled, while PC-10-T sample was completely insoluble. In the most polar solvent, methanol, all the samples were insoluble. It was concluded that the ratio of the hydrophilic chitosan/hydrophobic poly(trimethylene carbonate) controlled the solubility of the final product. In this light, PC-10-T which has good solubility in water and DMSO biodispersants appears to be the most promising for further use in bioapplications.

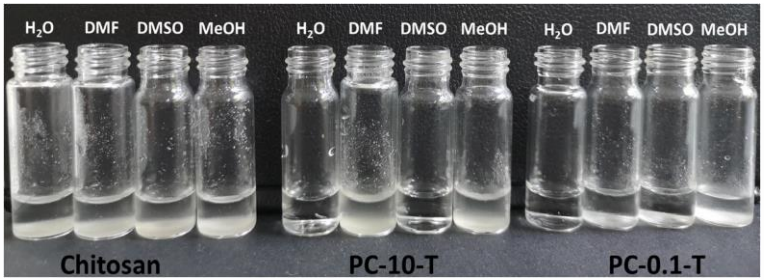


Figure IV.5. Visual assessment of the solubility of the chitosan-PTMC copolymers, in comparison to pristine chitosan.

For *in vivo* applications, of major importance is the ordering degree and the surface of materials, being well known that a nanostructured surface is beneficial for cell growing [22]. To evaluate the influence of grafting PTMC chains on the surface properties of chitosan, films casted from aqueous solutions of the grafted chitosan derivatives and pristine chitosan were observed by polarized optical microscopy (POM) and atomic force microscopy (AFM). POM images indicated strong birefringence with fine granular texture for chitosan (film and powder) (Fig. IV.7), in agreement with its semicrystalline nature [23]. On the contrary, images of poly(trimethylene carbonate) homopolymer revealed lack of birefringence, indicating its amorphous nature, due to the flexibility given by the linear chains. No significant alteration of this texture was observed for the grafted chitosan derivative PC-10-T (Fig. IV.7). However, a careful analysis of the images revealed richer details resulting in a clarification of the texture (see the figure inset in Fig. IV.7).

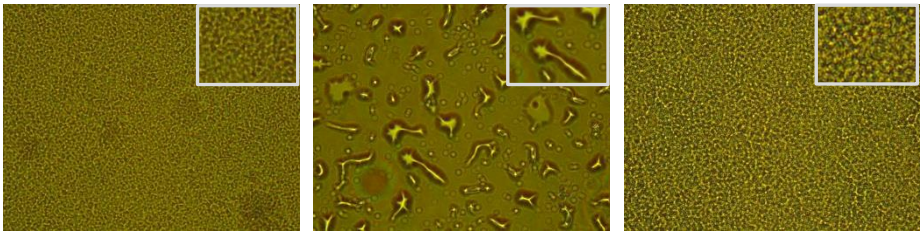


Figure IV.7. POM images of films based on a) chitosan, b) P-20-T and c) PC-10-T.

The modification of chitosan raises questions related to the possible alteration of its main properties, such as the biocompatibility. PC-10-T sample presented superior relative cell viability in comparison to pristine chitosan, at all the investigated concentrations. While chitosan solutions showed biocompatibility for concentrations lower than 200 µg/mL, PC-10-T displayed good biocompatibility for concentrations

higher than 300 µg/mL. Moreover, while for the 400 µg/mL solutions all the cells in contact with chitosan died, those in contact with PC-10-T showed a viability of almost 60 %. Also, the aspect of the cells treated with the investigated compounds is similar to those of the untreated cells used as control, for the samples with relative cell viability higher than 70% (Fig. IV.10).

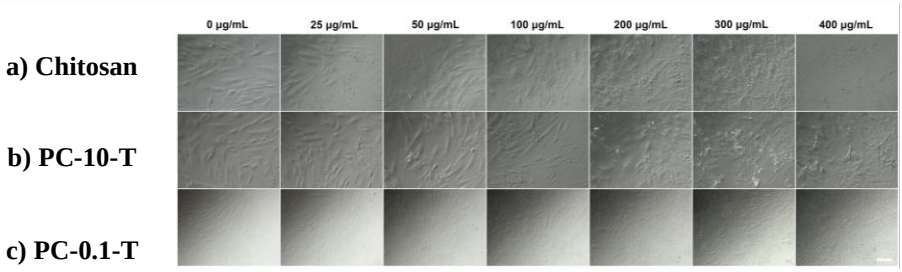


Figure IV.10. Aspect of the NHDF cells in contact with the tested solutions of a) Chitosan, b) PC-10-T and c) PC-0.1-T.

The biodegradability of the compounds was investigated in lysozyme buffer solution (pH=7.4), for 21 days. Pristine chitosan showed a mass loss of 66%, while PC-10-T derivative reached a mass loss of 81%. The more advanced biodegradation of PC-10-T was attributed to its higher solubility at physiological pH, induced by grafting PTMC side chains on chitosan's backbones. This hypothesis was supported by SEM images recorded on the samples before and after degradation. They indicated a surface erosion process of the samples on the out-in direction, reached by degradation/dissolution processes.

General conclusions

The PhD thesis entitled **„Water soluble chitosan derivatives for biomedical applications"** contains 272 pages divided into five chapters, which include 19 tables, 108 figures, 19 schemes and 446 bibliographic references. The thesis is structured in two parts: Part I (*Chapter I*) contains a literature study and Part II (*Chapters II-V*) contains the personal contributions, and the final part is dedicated to general conclusions.

The literature data presented in *Part I* reinforce the importance of the chosen topic of the thesis, by briefly presenting the most important information about the synthetic pathways for the obtaining of water soluble chitosan derivatives, with emphasis on the class of quaternary ammonium salts. Also, information on the advances

made in the development of materials based on these compounds are provided, especially nanofibers and hydrogels, with a role in biomedicine.

Part II presents the original results obtained during the doctoral studies, in accordance with the proposed objectives, as follows:

- Obtaining and characterization of hydrogels based on imino-quaternized chitosan.
- Obtaining, characterization and investigation of chitosan/quaternized chitosan-based nanofibers.
- Synthesis of water-soluble chitosan derivatives by grafting poly(trimethylene carbonate) chains and their characterization.

The general conclusions drawn from these studies are:

1. Quaternary ammonium salts of chitosan were synthesized using the technique of indirect binding of the quaternary group by means of an aliphatic spacer.

- The synthesis performed in neutral water, in heterogeneous media, led to selective substitution of amino groups of chitosan.
- By optimizing the reaction conditions (varying the molar ratio between the amino groups of chitosan and glycidyltrimethyl ammonium chloride (GTMAC), temperature and reaction time), quaternized derivatives of chitosan with different degrees of quaternization were obtained.
- Chitosan derivatives with good solubility in both neutral and basic pH media were obtained for a degree of quaternization higher than 43%.
- Structural characterization of the derivatives was performed by NMR and FTIR spectroscopy, confirming the presence of all the characteristic signals and bands. The degree of quaternization was determined by conductometric titration.
- Quaternized chitosan presents a semicrystalline nature, with a degree of crystallinity lower than the pristine chitosan, as evidenced by WXRD. Images recorded with a polarized light microscope revealed a high degree of ordering, by the presence of strong birefringence.
- Quaternized chitosan derivatives were *in vivo* biocompatible on experimental mice, confirming that the chosen degree of quaternization did not induce toxicity.

2. A series of 15 disinfectant biocidal hydrogels was synthesized by imination reaction in neutral aqueous solution, the condensation taking place between the free amino groups of quaternized chitosan and the formyl groups of salicylaldehyde, a natural monoaldehyde.

- The hydrogelation mechanism was based on the imination reaction, followed by self-assembly of the imine units into ordered clusters acting as cross-linking nodes.
- The mechanism was demonstrated by ^{1}H-NMR and FTIR spectroscopy, which revealed the formation of imine bonds, and by X-ray diffraction and POM microscopy, which demonstrated the presence of layered supramolecular architectures. Furthermore, the spectroscopic methods have indicated the reversible nature of imine bonds, which exist in a state of equilibrium with the reagents, as well as the major contribution of the quaternized chitosan to hydrogelation, through the formation of strong physical bonds.
- The hydrogel state, thixotropic behavior, self-healing ability as well as injectability of hydrogels were confirmed through rheological analysis.
- The hydrogels proved superabsorbent capacity, reaching a degree of swelling of up to 43000%.
- The dynamic nature of the imine bonds favored the biodegradation of hydrogels, both in wet environments and in soil, over five days.
- The hydrogels showed high adhesion on glass, steel, and skin surfaces, with adhesive force values comparable to or higher than commercial disinfectant gels. In addition, they have proven easy application and moisturizing ability of the skin.
- The antimicrobial activity of the hydrogels was high, leading to complete suppression of the viability of *S. aureus* and *E. coli* bacteria in a time- and composition-dependent manner.
- The obtained hydrogels proved hemocompatibility according to standards for medical devices intended for use in contact with blood, and their application on the skin of laboratory mice did not induce hepatotoxicity, nephrotoxicity, immunotoxicity or alteration of reactive oxygen species, indicating good *in vivo* biocompatibility.

3. A series of 5 binary nanofibers based on chitosan/quaternized chitosan was obtained by electrospinning technique, in different mass ratios of the reagents, presenting a potential role in tissue engineering and wound healing.

- Binary chitosan/quaternized chitosan nanofibers were obtained by electrospinning, using poly(ethylene oxide) as co-spinning agent, followed by its selective and total removal from the system.

- The success of the adopted strategy was proven based on FTIR and NMR spectroscopy techniques, which confirmed the presence of the two biopolymers in the fibers, and TGA analysis which proved the total removal of the synthetic polymer.
- SEM images indicated the obtaining of defect-free nanofibers with an average diameter of around 160 nm and inter-fiber pores of around 2 μm.
- X-ray diffraction and polarized light optical microscopy indicated the semicrystalline nature of the fibers, with a degree of crystallinity lower than pure chitosan fibers.
- Investigation of the swelling capacity of fibers in water and in the environment simulating physiological pH resulted in swelling degrees of 24 and 18 (g/g), respectively, similar to those of commercial dressings used to treat wounds.
- The nanofibers partially dissolved in water/PBS and biodegraded in the presence of lysozyme, with biodegradation rates controlled by the content of quaternized chitosan and pH of the environment. In an environment simulating exudate's evolution during the wound healing period, the fibers achieved complete biodegradation within 16 days.
- The binary fibers showed reversible water vapor sorption/desorption, indicating good breathability and maximum moisture absorption capacity of up to 63%, similar to that of commercial dressings.
- The mechanical properties had values comparable to those of some synthetic polymers-based fibers and human skin, with values up to 49 MPa for tensile strength, 9% for elongation at break, and 19 N/m^2 for Young's modulus.
- The adhesive force measured in contact with a tissue increased as the quaternized chitosan content in the fibers increased, reaching values of 1.36 N, pointing for a good bioadhesion capacity. Quaternization also had a positive impact on fiber-mucin interactions, resulting in improved mucoadhesiveness compared to pure chitosan fibers, demonstrated by UV-vis and Zeta potential measurements.
- The evaluation of antimicrobial activity demonstrated that the presence of quaternized chitosan substantially improved the fibers' ability to inhibit the growth of *E. coli* and *S. aureus*, even when testing very small sample amounts. The fibers totally inhibited the bacterial growth in less than 6 hours and 24 hours, respectively, while pure chitosan fibers showed limited antimicrobial effect.
- Tested on normal human dermal fibroblasts (NHDF), the samples showed adequate cytocompatibility for application as medical devices, while *in vivo* testing on rats by subcutaneous implantation demonstrated no toxicity to blood,

liver and kidneys, as well as tissues at the implantation site and no allergic effect on the immune system.

4. A series of 3 composite nanofibers based on imino-chitosan/quaternized chitosan/poly(ethylene oxide) was obtained by *in situ* encapsulation of vanillin, targeting the development of hemostatic dressings with a subsequent role in wound healing.

- FTIR and NMR spectroscopy demonstrated the covalent binding of vanillin to quaternized chitosan and chitosan by reversible imine bonds and the development of intermolecular forces between the components.
- X-ray diffraction and POM microscopy indicated the semicrystalline nature of the fibers, mainly due to phase segregation of PEO-rich crystalline domains, and SEM microscopy revealed the formation of entangled nanofibers with an average diameter of around 140 nm.
- The fibers showed improved thermal stability and good mechanical properties for the targeted application: tensile stress value of 3.8 MPa, elongation at break of 10.6%, and Young's modulus of 0.99 MPa.
- The fibrous nature allowed high moisture sorption (30 g/g), but also a high blood retention capacity (20-30 g/g). The fibers had a high degree of degradation over 21 days, expressed by a loss in mass of approx. 44% in PBS, 54% in the presence of lysozyme and 100% in soil after 7 weeks.
- The reversibility nature of imine bonds allowed a prolonged release of vanillin, controlled by its removal from the system, indicating the possibility of "on demand" release under the action of external stimuli, such as its consumption in the process of inhibition of pathogens.
- Quaternized chitosan improved the antioxidant activity of fibers up to 60% even in the solid state, through a synergistic effect of activating the hydroxyl groups of the other components.
- The fibers showed a high inhibiting effect of relevant pathogens, Gram-positive and Gram-negative bacteria, and yeast.
- The adhesiveness and water vapor transmission rate presented values comparable to other commercial hemostatic dressings.
- The evaluation of blood clotting capacity, assessed visually and by UV-Vis or SEM, revealed values superior to a commercially available hemostatic sponge, the fibers being able to absorb and coagulate in a short time high quantities of blood, with respect to their weight.

5. New water-soluble chitosan derivatives with potential for biomedical applications were obtained by grafting of poly(trimethylene carbonate) chains, using Ring-Opening Polymerization technique.

- The Ring-Opening reaction of the trimethylene carbonate cycle was initiated by the nucleophilic hydroxyl groups of chitosan, which can play the role of initiators.
- The reaction was successful in a heterogeneous environment, in dry toluene, in which chitosan has the ability to swell, ensuring good contact between the reagents.
- The reaction conditions, such as the molar ratio between the reagents, temperature, and reaction time, were optimized to obtain soluble derivatives, which allows their ease processing.
- Good solubility in water and DMSO biodispersants was achieved for a degree of substitution around 5%, with poly(trimethylene carbonate) side chains having a degree of polymerization of about 7.
- The water solubility allowed the avoiding of acids' use for the preparation of solutions, improving the *in vitro* biocompatibility of the obtained compounds, reaching a cell viability value of ~80% compared to less than 50% for the pristine chitosan, values recorded for solutions of 300 μg/mL concentration.
- The improved solubility of the newly synthesized chitosan derivatives also resulted in a higher rate of biodegradation, with a mass loss of 81% compared to 66% for the pristine chitosan, obtained after 21 days in lysozyme medium.
- Moreover, the functionalization proved to be beneficial for improving the hydrophilicity of films deposited from aqueous solutions, demonstrated by measurements of the contact angle with water.

A part of the results obtained during the doctoral studies were published in the form of scientific articles, in ISI international journals.

Papers published in ISI journals:

1. Bianca-Iustina Andreica, Daniela Ailincai, Andreea-Isabela Sandu, Luminita Marin, Amphiphilic chitosan-*g*-poly(trimethylene carbonate) – A new approach for biomaterials design, *International Journal of Biological Macromolecules*, (2021) 193. (**FI ISI: 8,2**)

2. Bianca-Iustina Andreica, Alexandru Anisiei, Irina Rosca, Andreea-Isabela Sandu, Aurelian Sorin Pasca, Liliana Mititelu-Tartau, Luminita Marin, Quaternized chitosan/chitosan nanofibrous mats: An approach toward bioactive materials for tissue

engineering and regenerative medicine, *Carbohydrate Polymers*, (2023) 302. (**FI ISI: 11,2**)

3. Luminita Marin, **Bianca-Iustina Andreica**, Alexandru Anisiei, Sandu Cibotaru, Maria Bardosova, Elsa Maria Materon, Osvaldo Novais Junior Oliveira, Quaternized chitosan (nano)fibers: A journey from preparation to high performance applications, *International Journal of Biological Macromolecules*, (2023) 242. (**FI ISI: 8,2**)

4. Bianca-Iustina Andreica, Alexandru Anisiei, Irina Rosca, Luminita Marin, Quaternized chitosan-based nanofibers with strong antibacterial and antioxidant activity designed as ecological active food packaging, *Food Packaging and Shelf Life*, (2023) 39. (**FI ISI: 8**)

5. Bianca-Iustina Andreica, Liliana Mititelu-Tartau, Irina Rosca, Irina Mihaela Pelin, Erwan Nicol, Luminita Marin, Biocompatible hydrogels based on quaternary ammonium salts of chitosan with high antimicrobial activity as biocidal agents for disinfection, *Carbohydrate Polymers, sent to publication*. (**FI ISI: 11,2**)

6. Bianca-Iustina Andreica, Alexandru Anisiei, Manuela-Maria Iftime, Razvan Vasile Ababei, Lacramioara Ochiuz, Decebal Vasincu, Ingrid-Andrada Vasilache, Constantin Volovat, Diana Boboc, Vladimir Poroch, Lucian Eva, Maricel Agop, Dragos-Viorel Scripcariu, Simona Ruxandra Volovat, Swelling and biodegradation profile of chitosan/quaternized chitosan nanofibers in media mimicking wound exudate. A theoretical model – experimental approach, *Pharmaceutics, under review*. **(FI ISI: 5,4)**

Papers published in ISI journals (*results which are not included in the thesis*)

1. Sandu Cibotaru, Daniela Ailincai, **Bianca-Iustina Andreica**, Xinjian Cheng, Luminita Marin, TEGylated Phenothiazine-Imine-Chitosan Materials as a Promising Framework for Mercury Recovery, *Gels*, (2022) 8. (**FI ISI: 4,6**)

2. Alexandru Anisiei, **Bianca-Iustina Andreica**, Liliana Mititelu-Tartau, Corneliu Coman, Rostyslav Bilyy, Galyna Bila, Irina Rosca, Andreea-Isabela Sandu, Evžen Amler, Luminita Marin, Biodegradable trimethyl chitosan nanofiber mats by electrospinning as bioabsorbable dressings for wound closure and healing, *International Journal of Biological Macromolecules*, (2023) 249. (**FI ISI: 8,2**)

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Patent requests:

1. „Netesuta de chitosan cu co-eliberare controlata de antibiotic si principii active", Luminita Marin, Alexandru Anisiei, Daniela Ailincai, Sandu Cibotaru, **Bianca Andreica**, Irina Rosca, No. CBI : A / 00478 / 08.08.2022.

2. „**Procedeu de electrofilare de nanofibre de chitosan și chitosan/chitosan cuaternizat**", Luminita Marin, Alexandru Anisiei, **Bianca Andreica**, Liliana Mititelu Tarțău, No. CBI : A / 00749 / 21.11.2022.

Mobilities carried out during the doctoral stage:

1. *Le Mans Institute of Molecules and Materials* (*IMMM*), Le Mans University, Le Mans, France, under the guidance of Dr. Erwan Nicol, 9 - 22.05.2022.

2. *Institute of Physics of Sao Carlos (IFSC),* Sao Paolo University, Sao Carlos, Brazil, under the guidance of Dr. Oliveira Osvaldo N. Jr., 10.01. - 9.04.2023.

Participation at national and international scientific conferences:

a) Oral communications

1. Bianca-Iustina Andreica, Daniela Ailincai, Luminita Marin, *Ring-Opening Polymerization as a technique for the obtaining of soluble chitosan derivatives*, ICMPP - open door to the future scientific communications of young researchers (MacroYouth'2020), 1st edition, Iasi, Romania, 19 November 2020.

2. Bianca-Iustina Andreica, Irina Rosca, Luminita Marin, *Synthesis and characterization of novel imine derivatives of quaternary ammonium salt of chitosan*, ICMPP - open door to the future scientific communications of young researchers (MacroYouth'2021), 2nd edition, Iasi, Romania, 19 November 2021.

3. Bianca-Iustina Andreica, Irina Rosca, Luminita Marin, *Development of biomaterials based on quaternized chitosan*, International Congress of "Apollonia" University of Iasi, 32nd edition, Iasi, Romania, 28 February – 2 March 2022.

4. Alexandru Anisiei, **Bianca-Iustina Andreica**, Luminita Marin, *Chitosan-based nanofibers for wound dressing applications*, International Congress of "Apollonia" University of Iasi, 32nd edition, Iasi, Romania, 28 February – 2 March 2022.

5. Bianca-Iustina Andreica, Irina Rosca, Luminita Marin, *Design and properties of newly developed imino-quaternized chitosan biomaterials*, International Conference on Materials Science and Engineering (BraMat 2022), 12nd edition, Brasov, Romania, 9-11 March 2022.

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7. Luminita Marin, Alexandru Anisiei, **Bianca-Iustina Andreica**, Liliana Mititelu-Tartau, Rostyslav Bilyy, Galyna Bila, Irina Rosca, Andreea-Isabela Sandu, Evžen Amler, *Quaternized chitosan based nanofibers as bioabsorbable wound dressings*, International Conference of the European Chitin Society (EUCHIS 2023), 14th edition, Siglufjörður, Iceland, 11– 14 September 2023.

8. Bianca-Iustina Andreica, Irina Rosca, Luminita Marin, *Composites nanofibers based on quaternized chitosan for food packaging*, 8th EPNOE International Polysaccharides Conference (EPNOE 2023), Graz, Austria, 18 – 22 September 2023.

9. Sandu Cibotaru, Daniela Ailincai, **Bianca-Iustina Andreica**, Luminita Marin, *TEGylated phenothiazine-chitosan based frameworks for mercury recovery*, 8th EPNOE International Polysaccharides Conference (EPNOE 2023), Graz, Austria, 18 – 22 September 2023.

10. Luminita Marin, Alexandru Anisiei, **Bianca-Iustina Andreica**, Liliana Mititelu-Tartau, Rostyslav Bilyy, Galyna Bila, Irina Rosca, Andreea-Isabela Sandu, Evžen Amler, *Nanofibers based on quaternized chitosan as bioabsorbable wound dressings*, 8th EPNOE International Polysaccharides Conference (EPNOE 2023), Graz, Austria, 18 – 22 September 2023.

b) Posters

1. Bianca-Iustina Andreica, Irina Rosca, Luminita Marin, *Biomaterials based on imino-quaternary ammonium salts of chitosan; synthesis and characterization*, EPF European Polymer Congress 2022, Prague, Czech Republic, 26 June - 1 July 2022.

2. Bianca-Iustina Andreica, Daniela Ailincai, Luminita Marin, *Synthesis of chitosan based derivatives with improved solubility in water, towards biomaterials' design*, EPF European Polymer Congress 2022, Prague, Czech Republic, 26 June - 1 July 2022.

3. Alexandru Anisiei, **Bianca-Iustina Andreica**, Luminita Marin, *Electrospinning of chitosan/quaternary salts of chitosan nanofibers for biomedical application*, EPF European Polymer Congress 2022, Prague, Czech Republic, 26 June - 1 July 2022.

4. Bianca-Iustina Andreica, Alexandru Anisiei, Irina Rosca, Andreea-Isabela Sandu, Liliana Mititelu-Tartau, Luminita Marin, *Binary chitosan/quaternized chitosan nanofibers – from design to possible applications*, Workshop do Instituto Nacional de Eletronica Organica (INEO) 2023, Nazare Paulista, Brazil, 2 – 6 April 2023.

5. Sandu Cibotaru, Daniela Ailincai, **Bianca-Iustina Andreica**, Luminita Marin, *Mercury recovery frameworks based on TEGylated phenothiazine-chitosan xerogels,*

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6. Bianca-Iustina Andreica, Alexandru Anisiei, Irina Rosca, Andreea-Isabela Sandu, Liliana Mititelu-Tartau, Luminita Marin, *Chitosan/quaternized chitosan nanofibers designed for biomedical applications*, 8th EPNOE International Polysaccharides Conference (EPNOE 2023), Graz, Austria, 18 – 22 September 2023.

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