Structure and Properties of Nanocrystalline Chitosan

Abstract
Chitosan and its derivatives are polymers with excellent properties to be used in regenerative medicine because they guarantee efficiency in the healing process. This polymer has a great potential for the development of a new generation of biomaterials that can be used in regenerative medicine and tissue engineering. The nanocrystalline chitosan (nCh) is a modified form of chitosan prepared by the method of obtaining chitosan salts. It is characterized by having the same special properties of the precursor chitosan as biocompatibility, bioactivity, being non-toxic and biodegradable. The aim of this study was to develop a new method of obtaining nanocrystalline chitosan according to their chemical and physical characterization. The material was characterized by Absorption Spectroscopy in the Infrared Region - with the Fourier transform (FTIR-ATR), scanning electron microscopy, SEM, Nuclear Magnetic Resonance, NMR, Diffraction of X-rays, particle size analysis and the potential Zeta. The results indicated that the process of obtaining nanocrystalline chitosan did not change the structure of the precursor chitosan. The analysis in the FTIR showed the same functional groups of the precursor chitosan. The 1H-NMR spectroscopy was helpful in the analysis of the chitosan samples in a wide range of values to determine the degree of deacetylation (GD). The morphology indicates the homogeneity of the structure and the surface. The X-ray diffraction shows the reduction of crystallinity of QNC, which corresponds to the amorphous structure thereof. The value of the zeta potential of the chitosan acetate (AQ) in acid media (pH 4.43) was 43.6 mV, while the value of QNC (pH 7.3) was 15.4 mV due to its high polydispersity. The variation in particle size of samples, and AQ using QNC 0.450 μm mesh filter, indicated the average particle size of 55.52 and 266.0 nm, respectively.

Keywords: Chitosan nanocrystalline; Regenerative medicine; Tissue engineering

Introduction
Biomaterials are defined as naturally occurring materials or made by man and are used directly as a supplement and/or substituent of the functions of the living human body tissues. Two important parameters that need to have a biomaterial are biocompatibility and biofunctionality [1-3].

Polysaccharides are a class of materials that have generally been underutilized in the field of biomaterials. The recognition of the usefulness and potential of this class of materials, however, is growing and the biomaterials field to the polysaccharide base, is about to experience rapid growth. Three factors specifically contributed to this growing recognition of biomaterials polysaccharides base. The recent development of new synthesis techniques using biologically active oligosaccharides, which will allow to expand and explore new research in the area of tissue engineering and the associated, need to create new biomaterials and bio-composites with specific properties, controllable biological activity and biodegradability [4].

Chitosan (poly β-(1,4)-D-glucosamine) and its derivatives are characterized by their excellent biostimulant properties that facilitate the reconstruction and vascularization of damaged tissue, may also address the weaknesses of cellular components, wire forming little scarring. The cationic properties of chitosan tissue, may also address the weaknesses of cellular components, facilitate the reconstruction and vascularization of damaged are characterized by their excellent biostimulant properties that allow to expand and explore new research in the area of tissue engineering and the associated, need to create new biomaterials and bio-composites with specific properties, controllable biological activity and biodegradability [4].

Chitosan has a variety of physicochemical properties and biological properties, such as low toxicity and allergenicity. Biocompatibility and bioactivity make it a very attractive substance for various applications in fields, such as pharmaceutical and medicine. Its non-toxic, hydrophilic with extraordinary behaviour are great to create films. Chitosan is very suitable for medical applications, especially dressings and drug delivery system [6-9].

In the treatment of wounds or burns, chitosan can be used in the form of coating films or membranes, colloidal solutions or sponges. Nowadays a large number of research groups have been dedicated to the production of new and better healing through synthesis and modification of biocompatible materials. Strategic chains are also focusing on wound repair acceleration through systematically designed dressings. In particular, efforts are pointing to the use of organic materials so that chitin and derivatives are capable of accelerating the repair process in molecular, cellular and systemic levels [10-14].

Chemical derivations of chitosan provide to be good materials to promotion of new biological activity and modification of the mechanical properties. The primary amino groups in the molecule are reactive and provide mechanisms for lateral fixing of groups using a variety of reaction conditions. These additions in the side chain can disrupt the crystalline structure of the material and consequently increase the amorphous fraction. These changes produce a material with lower stiffness and often changed their solubility. Changes in chemical and biological properties depend on the nature of the side group. Furthermore, the characteristics
of chitosan, such as cationic, hemostatic and insoluble at high pH, can be completely reversed, which can make the anionic watersoluble molecule, and also presenting anticoagulants properties [15-20].

Chitosan and its derivatives are polymers with excellent properties to be used in regenerative medicine, as they ensure efficiency in healing processes. The biocompatibility, non-toxicity and antibacterial activity is excellent properties for development of new biomaterials, on the other hand, shows a strong resource sustainability, originating from biomass, or to be in the food industry, which further reinforce potential and the applicability of these polymers in health area [15].

The preparation of chitosan waste bark, for example, shrimp (Pandalus borealis), is economically viable, environmentally friendly due to the large amount of waste available now as a product or as waste from the food industry [7]. This article aims to develop a new method of obtaining nanocrystalline chitosan, evaluate chemical, structural and morphological properties in order to explore the potential of this class of materials and the importance of investing in the development of a multi science and interdisciplinary with profitable translational opportunities to generate new products helping the management of public health more diversified range of new low-cost products.

Materials and Methods

Materials

Chitosan with a degree of deacetylation of 95% and 12.4% moisture content, colour of yellow powder cream, density 0.31 g / ml, data provided by Polyarsen Science and Nutrition S/A (Fortress EC), acetic acid 99.7% pa (Dynamic) and sodium hydroxide (Sigma-Aldrich). All chemicals were used as received without further purification.

Methods

Synthesis of acetate 95% deacetylated chitosan: The used chitosan was dissolved in acetic acid and were prepared four subsequently solutions containing 1:0.4; 2:0.8; 3: 0.9 to 2:2 polymer content and acetic acid respectively, as shown in (Table 1). The dissolution occurred under agitation for propeller mixer at room temperature for a period of 2 hours at a speed 1,000 rpm to obtain one homogeneous and transparent solution. Twenty-four hours after the dissolution, each solution was filtered and the samples placed in open form polystyrene containers Petry card type to its drying at room temperature.

Table 1: Chitosan acetate solutions at different concentrations of acetic acid and polymer content.

| Solution | Acetic acid Concentration (%) | Polymer content (%) |
|----------|-------------------------------|---------------------|
| A        | 0.4                           | 1                   |
| B        | 0.8                           | 2                   |
| C        | 0.9                           | 3                   |
| D        | 2                             | 2                   |

Obtention method of nanocrystalline chitosan:

a) Synthesis of nanocrystalline chitosan from the chitosan acetate solution: The four solutions of chitosan acetate with about 2 liters, prepared above, with the following concentrations of 1:0.4; 2:0.8; 3:0.9 and 2:2 polymer content and acetic acid respectively referred to as A-I, B-II, C- and D III-IV. These solutions were under constant stirring at a rotation of 1,000 rpm by a propeller mixer at room temperature for 30 minutes, then was added amounts of glycerol as a plasticizer: 5 ml; 7.5 mL; 10 ml and 10 ml respectively, which are relating to 0.4% of polymer content. Then the respective solutions were added gradually sodium hydroxide (NaOH) solution, with constant stirring, with the respective concentrations of acetic acid to obtain complete neutralization of the acid in question, as shown in Table 2. After standing for twenty-four hours packed under cooling at constant temperature of 5 °C, the solution was filtered and washed with Büncher funnel with three liters of distilled and deionized water to remove the salt, residual sodium acetate which was formed in the neutralization reaction of acetic acid with sodium hydroxide. The samples were placed in polystyrene containers open type Petry plate until its drying at room temperature.

Table 2: Chitosan acetate solutions at different concentrations, polymer content and amount of glycerin.

| Solution | Acetic acid Concentration (%) a) | Polymer content (%) | Quantity of Glycerine (mL) | Solution of Na (OH) (%) b) |
|----------|---------------------------------|---------------------|----------------------------|---------------------------|
| A        | 0,4                             | 1                   | 0,5                        | 0,4                       |
| B        | 0,8                             | 2                   | 0,75                       | 0,8                       |
| C        | 0,9                             | 3                   | 10,00                      | 0,9                       |
| D        | 2                               | 2                   | 10,00                      | 2                         |

Analytical methods for the characterization of chitosan acetate and nanocrystalline chitosan

a) Absorption Spectroscopy in the Infrared Region Fourier Transform - (FTIR - ATR): FTIR spectra in the infrared region were recorded on a Perkin Elmer model Spectrum One, the region in the spectral range 4000-650 cm⁻¹, number 8 scans and resolution of 4 cm⁻¹. The measurements were performed in Reflectance Total Attenuated mode.

b) Scanning Electron Microscopy with Chemical Analysis by Energy Dispersive (SEM- EDS): The morphology of the chitosan samples was analysed in an electron microscope digital scan-brand JEO-L-JSM-6010LA model. To perform the analysis SEM-EDS part of the sample was bonded to the support (stub) with a tape of carbon and these were coated with a thin carbon layer by evaporation, to make them more conductive. It selected a region for evaluation by EDS. The images form obtained by detector Electron Secondary (SEI), 15 kV, under high vacuum, Working Distance: 10 mm Spot Size:30

c) X-Ray Diffraction (XRD): The diffraction data X-ray were obtained using Rigaku X-Ray equipment Diffractometer (XRD). Chitosan samples were analyzed by measurements of
X-ray diffraction with copper tube (λ=1.54Å), using voltage 40kV and 40mA current. The measurements were performed in the range 3°<2θ and graphite monochromator, normal incidence at room temperature. The results of the analysis were obtained by indexing the crystallographic forms.

d) Nuclear Magnetic Resonance Hydrogen (1H - NMR): The 1H-NMR spectra were obtained on a Varian Unity Plus 400 MHz spectrometer. In order to improve the resolution of the peaks the following conditions were set: 16 scans accumulated pulse and the LB 0.30 Hz spectral width, and points was 5000 Hz and 64K, respectively. The spectra were calibrated from the signal due to HOD. Initially, a solution acidified 1% (v/v) was prepared by adding 0.05 mL of concentrated HCl 4.95 mL of D2O. About 20 mg of sample were added to 5 mL of this solution and maintained under constant agitation for 24 hours at room temperature. HCl was added to the solvent in sufficient quantity (D2O/HCl 100:1 v/v) to promote solubilizing chitosan (essential for the neutralized form of chitosan), and in order to displace interfering signals in the region examined to determine the level of acetylation. An aliquot of this viscous solution was placed in quartz tubes of 5 mm diameter. To reduce the interference of the solvent signal (HDO) with the sample peaks, the experiment was conducted at a temperature of 70°C. NMR signals and integration of the peaks were assigned with the aid of MestReNova program.

e) Zeta Potential and particle size: The zeta potential and particle size of the chitosan acetate solution and nanocrystalline chitosan were measured using the ZETASIZER NANO ZS90 apparatus (Malvern Instruments).

Results and Discussion

Study spectroscopy in the infrared Fourier transform spectroscopy (FTIR - ATR)

The characteristic spectra in the infrared region of the precursor chitosan and the method of obtaining chitosan acetate salt (AQ) were confirmed by FTIR technique-TRS. That is illustrated in (Figure 3). Spectra were observed in the presence of bands in the region of 3200 cm⁻¹ relating to the stretching vibrations of the OH and NH of chitosan and also with regard to water absorption, the band 2800-2880 cm⁻¹ assigned to CH (symmetric) stretching the band in the region 1631 cm⁻¹ corresponding to stretching C=O of amide I characteristic of chitosan is not fully deacetylated and 1540 cm⁻¹ band assigned to deformation NH2. The bands at 1150 cm⁻¹ and 899 cm⁻¹ refer to the chemical group COC (glucose-β-1-4) [21].

Analysing the spectra of the infrared nanocrystalline chitosan region (QNC), it was observed the similarity to chitosan acetate (AQ), varying only the intensities of the bands that are proportional to polymer concentration. This confirms that after the aggregation method macromolecule structure glucosamine and purification process shown in (Figure 2), nanocrystalline chitosan shown with the main precursor of chitosan identifying peaks as illustrated in (Figure 4).
Morphology

Morphology of the films of chitosan acetate: The technique of scanning electron microscopy was used in order to evaluate the morphology of the surfaces of samples of ethyl Chitosan (Figure 5). are observed in micrographs of the samples of the chitosan acetate films at different concentrations as shown in (Table 1), a smooth, homogeneous and well dispersed, i.e. without formation of agglomerates, but with a texture characteristic (Figures A & B). The sample obtained the best results in the morphology study to continue the studies was 1:0.4 related to polymer concentration and acetic acid respectively, and this sample illustrated in the Figure. Figure 5C illustrates the compositional range of the region where EDS analysis was done.

The energy dispersive spectroscopy (EDS) was used to identify constituents of the sample and semi-quantitative results of the elements present in the chitosan acetate film 1:0.4, presented as in Figure 5c and Table 3.

Table 3: Results of semi-quantitative elements present in samples of dry chitosan acetate at room temperature obtained by the EDS.

| Chemical elements | AQ1:0.4 (% massa) |
|-------------------|------------------|
| C                 | 52.72            |
| N                 | 4.90             |
| O                 | 41.81            |
| Na                | 0.20             |
| Si                | 0.11             |

(Table 3) Results of semi-quantitative elements present in samples of dry chitosan acetate at room temperature obtained by the EDS. The traces in mass percent of the study sample 1:0.4 for the silicon and sodium suggest waste impurities arising from the process of obtaining initial chitosan used in this research. Carbon, Nitrogen and Oxygen confirm the organic and structural character of the polysaccharide confirmed by FTIR item 3.1.

Figure 5: Images from the scanning electron microscope samples of chitosan acetate films, dried at room temperature (A and B) AQ 1:0.4 in polymer content and acetic acid concentration with magnifying (A) 200 and (B) x 1.000. (C) the compositional spectrum of the region where it was made EDS analysis for the image with magnification 200x.

Morphology of chitosan nanocrystalline

As Figures 6A & 6B, there is a sample obtained by nanocrystalline Chitosan Chitosan acetate solution 1: 0.4 for polymer content and acetic acid respectively. From SEM micrographs, it was observed that samples of the same nanocrystalline hold chitosan chitosan acetate behavior showing a homogeneous surface, easy to obtain films, but with a different texture of chitosan acetate films. The agglomerates appearance suggests a higher water retention also evidenced by FTIR with increased band 3200 cm⁻¹ (Figure 4), this increase is related to the increased hydrophilicity of the polymer (amorphous part) due to dissolution of the macromolecule glucosamine and subsequent recrystallisation of the same. (Figure 6C) illustrates the constituents present in the chitosan sample nanocrystalline 1:0.4. The semi-quantitative results of the elements present in the chitosan sample nanocrystalline 1:0.4 are shown according to (Table 4).

Regarding traits in percentage by mass of the sample in study 1:0.4 for the silicon and sodium, silicon remained without a significant increase, unlike the sodium concentration increased significantly attributed to the agglomeration process of macromolecule glucosamine using sodium hydroxide. The purification process for removal of residual sodium acetate was not effective, being necessary to wash longer and with a greater amount of distilled water. Carbon, Nitrogen and Oxygen confirm the organic and structural character of the polysaccharide confirmed by FTIR item 3.1.

Table 4: Semi-quantitative results of the elements present in the samples analyzed nanocrystalline chitosan, dried at room temperature obtained by the EDS.

| Chemical Elements | QNC 1:0.4 (% massa) |
|-------------------|--------------------|
| C                 | 53.01              |
| N                 | 4.56               |
| O                 | 41.41              |
| Na                | 0.91               |
| Si                | 0.11               |

Diffractogram X-ray

The use of X-ray diffraction also allows distinguishing clearly the starting precursor chitosan (QP), chitosan acetate (AQ) and its derivative chitosan the nanocrystalline (QNC). The diffractogram of commercial chitosan sample showed more intense and/
or better peak, defined as that observed in the XRD patterns of chitosan after the reactions with acid and neutralization with sodium hydroxide (Figure 7), which is attributed to the existence of crystalline domains larger and more in the case of commercial chitosan. According Samuels [22], the peaks at 10° and 20° are characteristic of crystallinity of the chitosan membrane. Samples AQ 1:0.4 (broader band) have much less intense signals and are typical of amorphous chitosan due to the dissolution of the macromolecule of glucosamine by acetic acid. The lack of land to be attributed to the presence of positive charges (due to protonation of the amino groups) in the chain of samples and their respective counter ions (Figure 7), however the QNC samples show that they are similar in crystallinity. Suggesting a reduction of the crystal size, consequently the crystalline region and the amorphous region increases significantly, resulting in a structural change of the leaving polysaccharide polymer more reactive and less structural characteristics.

**Nuclear magnetic resonance of hydrogen (1H - NMR)**

The degree of deacetylation (GD) is considered one of the main parameters in the characterization of chitosan. It is defined as the number of free amine groups to the number of groups of the polymer chain. The assignment of the signal observed in the spectra of Figure 8 correspond to the signals, between 3 and 4 ppm of the hydrogens attached to carbons 2-6 and glycosaminoglycans ring. The singlet at 1.96 ppm region corresponds to the hydrogen from the methyl acetamido group. The reduction of AQ GD 1:0.4 refers to the dissolution of the macromolecule of glucosamine with addition of acetic acid, favouring the creation of new acetyl groups, as shown in (Figure 8A). The increased GD QNC 1:0.4 recrystallization is related to the glucosamine molecule with addition of sodium hydroxide, favouring the creation of new amino groups, due to sodium ions bind more easily to the acetyl group, freeing again free amine groups as shown in (Figure 8B). The GD AQ and QNC determined from the 1 H NMR spectrum of chitosan was 64 and 84%, respectively. That is, 64% of the monomers of the AQ are deacetylated and 36% are acetylated (Figure 3). In QNC, only 16% of the monomers were acetylated.

According to the technical information provided by enterprise Polymar, commercial chitosan used in this work has a GD of approximately 95%. The determination of NMR GD is recommended for samples with high chitosan deacetylation degree and has been the method most used in these cases [23-25].

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**Figures 6:** Images from the scanning electron microscope sample of chitosan nanocrystalline film (A and B) 1:0.4 in polymer content and concentration of acetic acid, magnification (A) 200 and (B) 1000x. (C) The compositional spectrum of the region where it was made EDS analysis for the image with magnification 200x.

**Figure 7:** XRD pattern of the precursor chitosan acetate AQ (A) 1:0.4 and (D) 2:2% polymer concentration and acid concentration and the QNC (AII) 1:0.4 and (C-III) 3:0.9% polymer concentration and acetic acid concentration.
Zeta Potential and particle size

The particle size and zeta potential analyses were performed in two different ways. The analyses of the samples chitosan acetate (AQ) 2:0.8 and nanocrystalline chitosan (QNC) 2:2 were made with and without the use of filters, as shown in Figure 9 & Table 5. These samples were used due to high content of polymer and high acetic acid content to know the influences of these two parameters in the outcome. Figure 9a & 9b shows the distribution of the particle size of the chitosan acetate (AQ) and nanocrystalline chitosan (QNC), without a filter, indicating the average particle size of 490.4 nm and 142.5, respectively. This shows that QNC has a more reactive, hydrophilic and more susceptible to particulate agglomeration. Figure 9c & 9d illustrates the variation in particle size of the samples, and AQ QNC using mesh 0.450 μM filters, indicating the average particle size of 266.0 nm and 55.52, respectively, showing the decreased polydispersity QNC the sample.

The high reactivity of QNC induces agglomeration of the chitosan nanoparticles which reflect in increased polydispersity, which is a characteristic of most amorphous polymers character [7]. Table 5 illustrates the difference in stability between the two solutions. It is observed that due to the high polydispersity related to high variation of molecules of various sizes and molecular weights, it was not possible to measure zeta potential of AQ and QNC solutions without the use of filters. The use of mesh 0.450 μM filter intended to reduce the polydispersity of the solutions to reduce the variation in molecular size, which is illustrated in Figure 9 & Table 5.

The measured zeta potential of the medium conducted in order to study the stability of the dispersion and the chitosan acetate solutions compared to the nanocrystalline chitosan. It is observed that the value of the zeta potential of AQ in acid medium (pH 4.43) is 43.6 mV, due to the amino groups are protonated (NH$_3^+$). According to Figueiredo [26] when the pH is at the pKa of chitosan (~6) amine groups start to deprotonate (NH$_2$) and zeta potential starts to decrease, which means that the load of chitosan tends to be nil. Therefore, the value of the zeta potential of QNC (pH 7.3) was 15.4 mV, i.e. nanocrystalline chitosan behaviour resembles that of the precursor chitosan.

| Samples                  | Particle Size (nm) | Number (%) | Standard Deviation (nm) | Zeta Potential (mV) |
|--------------------------|-------------------|------------|-------------------------|---------------------|
| AQ 2:0.8                 | 293.6             | 100        | 57.32                   | -                   |
| QNC 2:2                  | 142.5             | 100        | 14.79                   | -                   |
| AQ 2:0.8 with filter 0.450 | 266.0            | 100        | 45.01                   | 43.6                |
| QNC 2:2 with filter 0.450 | 55.52             | 96.3       | 10.34                   | 15.4                |

Table 5: Use of QNC obtaining method got a 55.52 nm particle size.

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Conclusion

This research was developed a new method for obtaining nanocrystalline chitosan, chitosan acetate derived. The FTIR results show the same chemical structure of the precursor of chitosan acetate and chitosan nanoparticulate not form observed changes in chemical structure and secondary products from the method of obtaining the nanocrystalline chitosan.

The study of morphology and EDS showed homogeneity on the surface of the obtained films and silicon and sodium traces in samples of chitosan acetate and increase in sodium traces on nanocrystalline chitosan due to agglomeration of the macromolecule glucosamine process. The analysis of X-ray diffraction showed the characteristic peaks of crystallinity precursor of chitosan, a decrease in the crystallinity of the chitosan acetate due to the dissolution of the macromolecule of glucosamine, increasing its amorphous character. This effect was more pronounced in nanocrystalline chitosan decreasing the size of the crystal, micro to nano also observed by particle size analysis, thus increasing its amorphous character, hydrophilic and consequently the reactivity of the polymer.

The magnetic resonance analysis also illustrates this structural change, adding to the variation of the index deacetylation, chitosan acetate both as the nanocrystalline chitosan. In the chitosan acetate was reduced deacetylation of the index, which indicates that the polymer dissolution process with acetic acid there were racemilados by adding acetic acid. It was possible to observe an opposite behavior recrystallization macromolecule glucosamine in the method for obtaining nanocrystalline chitosan (agglomeration process).

Addition of sodium hydroxide caused a increase in free amino groups to form sodium acetate, thus increasing the deacetylation index.

The value of the zeta potential of the AQ 2:0.8 in acid medium (pH 4.43) was 43.6 mV. While the value of QNC 2:2 (pH 7.3) was 15.4 mV. Thus, the behavior of nanocrystalline chitosan did not become stable due to the high polydispersity. The variation of the particle size of the samples AQ 2:0.8 and QNC 2:2 using 0.450 uM mesh filter, indicated the average particle size of 55.52 and 266.0 nm, respectively.

The nanocrystalline chitosan prepared by this method remained the same structure of the precursor chitosan therefore indicates to have the same properties of the initial chitosan, with a large increase in its hydrophilic character and extraordinary behaviour for direct training films and creating a particulate nano structure. This derivative of chitosan has a great potential for medical and pharmaceutical applications, creating a generation of biomaterials nano particles and to foster the creation of new complexes for regenerative medicine and tissue engineering.

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References

1. Misiek DJ, Kent JM, Carr RF (1984) Soft tissue responses to hydroxyapatite particles of different shapes. J Oral Maxillofac Surg 42(3): 150-160.

2. Muzzarelli RA, Muzzarelli RA (2002) Natural and artificial chitosan-inorganic composites. Journal of Inorganic Biochemistry 92(2): 89-94.

3. Oktay Y (2004) Preparation and characterization of chitosan/calcium phosphate based composite biomaterials, Master of Science Dissertation, Izmir Institute of Technology, Izmir, Turkey.

4. JK Francis Suh, Howard WT Matthew (2000) Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: a review. Biomaterials 21(24): 2589-2598.

5. M Rinaudo (2006) Chitin and Chitosan: Properties and Applications. Progress in Polymer Science 31(7): 603-632.

6. M Kucharska A, L Niekraszewicz, M Wisniewska Wrona, E Wesolowska, H Struszczyk (2003) Dressing Sponges Made of Chitosan and Chitosan-Alginate Fibres. Progress on Chemistry and Application of Chitin and Its Derivatives, PTChit 9: 69-72.

7. Pighinelli Luciano, Kucharska Magdalena, Wawro Dariusz (2012) Preparation of Microcrystalline chitosan: (MCh0/tricalcium phosphate complex with Hydroxyapatite in sponge and fibre from for hard tissue regeneration p. 19.

8. RAA Muzzarelli (2011) Chitosan Composites with Inorganics, Morphogenetic Proteins and Stem Cells, for Bone Regeneration. Carbohydrate Polymers 83(4): 1433-1445.

9. MNVR Kumar (2000) A Review of Chitin and Chitosan Applications. Reactive & Functional Polymers 46(1): 1-27.

10. Qiao xia Li, Bao zhen Song, Zhen qiu Yang, Hong lei Fan (2006) Electrolytic conductivity behaviors and solution conformations of chitosan in different acid solutions. Carbohydr Polymers 63(2): 272-282.

11. M Mohy Eklín, EA Soliman, AH Hashem, TM Tamir (2008) Antibacterial Activity of Chitosan Chemically Modified with New Technique. Trends Biomater Artif Organs 22(3): 125-137.

12. Jianyun Wang, Dongming Zhang, Fei Liu, Xixun Yu, Changsheng Zhao, et al. (2010) Structure and properties of chitosan derivatives modified calcium phosphate scaffolds. Polymer Degradation and Stability 95(7): 1205-1210.

13. Juan Peña, Isabel Inquiendo Barba, Alvaro Martínez, María Valet-Regi (2006) New method to obtain chitosan/apatite materials at room temperature. Solid State Sciences 8(5): 513-519.

14. S Green, M Roldo, D Dounoumis, N Bouroupoulos, D Lamprou, et al. (2009) Chitosan derivatives alter release profiles of model compounds from calcium phosphate implants. Carbohydr Res 344(7): 901-907.

15. Melina Handine, Marie Claude Heuzey, Andre Begin (2005) Effect of organic and inorganic acids on concentrated chitosan solutions and gels. Int J Biol Macromol 37(3): 134-142.

16. KV Harish Prashanth, RN Tharanathan (2007) Chitin/chitosan: modifications and their unlimited application potential-an overview. Trends in Food Science & Technology 18(3): 117-131.

17. Barbosa MA, PL Granja, CC Barrias, IF Amaral (2005) Polysaccharides as scaffolds for bone regeneration. ITBM-RBM 26(3): 212-217.

18. Skrtic D, JM Antonucci, ED Eanes (2003) Amorphous Calcium Phosphate– Based Biodegradable Composites for Mineralized Tissue Regeneration. J Res Natl Inst Stand Technol 110(3): 167-182.

19. Marino Lavorgna, Filomena Piscitelli, Pasqualina Mangiacapra, Giovanna G, Buonocore (2010) Study of the combined effect of both clay and glycerol plasticizer on the properties of chitosan films. Carbohydrate Polymers 82(2): 291-298.

20. Henryk Pospieszny, Wojciech Folkman (2004) Factors modifying a biological activity of chitin derivatives. Progress on Chemistry and Application of Chitin and its Derivatives. PTChit. 10: 07-12.

21. Costa Jr E, Mansur HS (2008) Preparação e caracterização de blendas de quitosana/poli(ácio vinílico) reticuladas quimicamente com glutaraldeído para aplicação em engenharia de tecidos. Química Nova 31(6): 1460-1466.

22. Wasina Thakhide, Sakamon Devahastin, Somchart Soponronnarit (2009) Chitosan derivatives alter release profiles of model compounds from calcium phosphate implants. Carbohydr Res 344(7): 901-907.

23. Hirai A, Odani, H Nakajima A (1991) Determination of degree of deacetylation of chitosan by 1H NMR spectroscopy. Polymer Bulletin 26(1): 87-94.

24. Iamazaki E, Douglas de Britto, Carla C Schmitt, Sergio P Campana Filho, Miguel G Neumann, et al. (2004) Preparation of substituted ionic carbohydrate polymers and their interactions with ionic surfactants. Colloid and Polymer Science 283(1): 33-40.

25. Lavoita M, Z Xia, AN Serreqi, M Berrada, A Rodrigues, et al. (2003) A validated 1H NMR method for the determination of degree of deacetylation of chitosan. Journal of Pharmaceutical and Biomedical Analysis 32(6): 1149-1158.

26. Henryk Pospieszny, Wojciech Folkman (2004) Factors modifying a biological activity of chitin derivatives. Ed. Progress on Chemistry and Application of Chitin and its Derivatives, PTChit 10: 07-12.