CHITOSAN COMPOSITES PREPARED WITH HYDROXYAPATITE AND LACTIC ACID AS BONE SUBSTITUTE

GALO CARDENAS-TRIVIÑO [a*] *AND GUIDO CARRASCO-GARCIA* [b]

[a]*Facultad de Ingeniería, DIMAD, Escuela de Ingeniería Química, Universidad del Bío-Bío, Concepción, Chile.*
[b]*Facultad de Química, Universidad de Concepción, Concepción, Chile.*

ABSTRACT

The need to find new therapies to improve the functioning of the injured tissue has made emerge a multidisciplinary field of research. Field that is related to the problems that exist for transplant to a patient with defect in their tissues and nerves, these trasplants are one of the most serious and costly health problems. In biomaterials research has undergone a major expansion, generating a variety of stands of different chemical nature. Biocompatible materials for human where they serve the same function as the actual tissues and also exhibit a total degradation in the body without the need for any other post-operation to remove a device installed on the bone region. Biodegradables and biocompatible composites prepared were subjected to studies to test the best concentrations of components of these, for example Chitosan(CH), hydroxyapatite(HAP) and polylactic acid(PLA), with these is created a material that fulfills the requirements for a good result in bone regeneration. The results show different analyses of the composites that the proportion of Chitosan 4%, hydroxyapatite 3% and polylactic acid 1.5% is the more appropriate composition to produce a bone regeneration.

Keywords: Biomaterials, biocompatible, chitosan, polylactic acid, bone regeneration.

1. INTRODUCTION

Chitosan, main deacetylated derivative of chitin, second polysaccharide in nature after cellulose, is produced by N-deacetylation of chitin using a strong alkali [1]. Both chitin and chitosan are similar in structure to glycosaminoglycans, and are represented by a single structure, which corresponds to the copolymer composed of units of β-D-glucosamine and N-acetyl-β-D-glucosamine linked in the position (1->4), the relationship between monomers as defined as degree of deacetylation (DD) makes the difference between the two structures. Where the following figure shows the structure of biomaterials [2,3].

Structural unit of the poly-acetyl-Glucosamine.

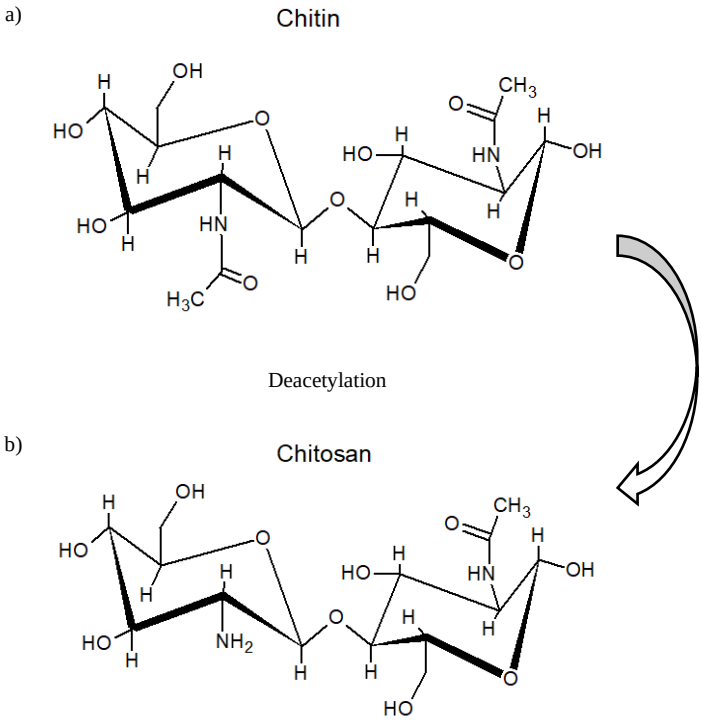

Figure 1. (a) Repetitive unit of chitin and (b) The repetitive unit of Chitosan.

Both chitin and chitosan have a wide range of applications; medical, pharmaceutical, agricultural and other areas of research that have been developed over the past 30 years [4].

Bone is a living tissue made up of living bone cells surrounded by an inert substance and hard [5]. The inorganic phase of bone is composed mainly by minerals such as hydroxyapatite (HAP) which represents 60-70% of the dry weight.

Bone provides support to the soft tissues of the body and protects the nervous system and hematopoietic tissue. The total or partial tissue damage and loss of function of an organ are among the most serious and costly health problems. Initially, these problems addressed by the transplantation of organs and incoming tissues, however, this option is limited by the low availability of donors [6]. As a result, each year died a large numbers of patients in list of waiting and, more serious still many others do not even manage to integrate them [7].

The need to find new therapies to improve the functioning of the injured tissue has made emerge a new multidisciplinary area of research. This new field which requires the integration of knowledge of cell biology, science of materials, engineering, mathematical and chemical physics, aims to build functional tissues that can be used for the maintenance, in the laboratory regeneration and replacement of damaged tissues [8].

The damaged bone is able to regenerate itself through the creation of an exactly equal to the original fabric. In this way, when continuity of solutions is presented, whether by fractures, tumors, or tissue defects, are immediately activated osteoformers mechanisms in order to restore the bone tissue [9] reconstruction of large bone defects and in cases of insufficient tissue regeneration treatment require grafts [10].

Optimal material for graft it is own bone tissue from the patient (autologous transplant), this type of grafts are characterized by osteoconductives, osteoinductives and osteogenic [11,12]. The alternative to bone grafts is the use of natural or synthetic materials that are able to reproduce the characteristics of the autologous material, these materials must be as already mentioned, osteoconductives, osteosubstitutes and biocompatible, of structure similar to bone, easy to use, biodegradable and affordable production [12].

Calcic phosphates have been widely used and marketed due to its chemical and structural similarity to the bone matrix and its biocompatibility, the most widely used calcium phosphate is hydroxyapatite for coated implants for filling defects [13]. Natural materials like artificial being used for bone regeneration, Chitosan already known is used for this purpose, as it is a polysaccharide biocompatible, non-toxic, and biodegradable by lysozyme [14].

**Corresponding author email: gcardenas@ubiobio.cl*

Chitosan promotes the adhesion, proliferation and induces differentiation of planted material progenitor cells, also causes a minimal reaction to be implanted. Chitosan stands are osteoconductives and promote the formation of bone tissue both *in vitro* and *in vivo*. However, this material is mechanically weak and unstable [15]. For this reason, is proposed the synthesis of hybrid materials or composites with Chitosan, hydroxyapatite and polylactic acid (PLA).

Among polyesters, poly alpha-hydroxyacids, including polylactic acid and its copolymer, are polymers used in bone tissue engineering. Polylactic acid is a semi-crystalline polymer that is synthesized by polymerization produced by the opening of the ring of the cyclic diester of lactic acid (Figure 2) this polymer has been widely studied in applications such as liberation controlled drugs, sutures biodegradable, different implant fixation sutures and as support for the growth of cells in tissue engineering[16] and recently, on the support of these polyesters cell culture has allowed the development of substitutes for bone and cartilage tissue [17].

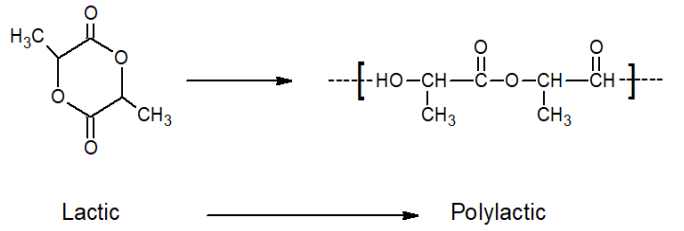

Figure 2. Synthesis of PLA.

PLA is one of the polymers FDA-approved since 1995, its main advantage and biodegradable.PLA is used in mechanical materials such as nails at the junction of ligaments, the repair of meniscus, sutures and screws in fixation of fractures and maxillofacial, cardiovascular surgery and drugs of slow release [18,19]. PLA is used in mechanical materials such as nails at the junction of ligaments, the repair of meniscus, sutures and screws in fixation of fractures and maxillofacial, cardiovascular surgery and drugs of slow release [18,19]. PLA is not sold commercially in plates for osteosynthesis in support of long bones by the high mechanical requirements, although biodegradable research tries to generate those with greater strength and rigidity in plates for osteosynthesis, with a polymer matrix reinforced with fibers or particles ceramic or vitreous or of the same polymer. Engineering of tissues therapy for the regeneration of skin cells, liver, cardiovascular and recently of cartilage and bone [20].

The criteria for selection of the PLA depends on their applications either greater mechanical strength as the amorphous (DL-PLA) or biodegradable in the long run as the semi-crystalline form (L-PLA). L/DL-PLA copolymers are used to preserve both the mechanical properties and the speed of biodegradability [21]. The biodegradability of plastics depends on the chemical structure of the material, the composition of the final product, not only commodity raw material employed. The "American Society of Testing and Materials" (ASTM) defines a biodegradable material as "that which mineralizes into CO_2, H_2O, inorganic components or biomass by microbial action" which can be measured by standard tests in a specific timeperiod, in standard conditions of deposit. PLA biocompatible as an intermediate in the metabolism of carbohydrates in human as shown in the diagram in Figure 3.

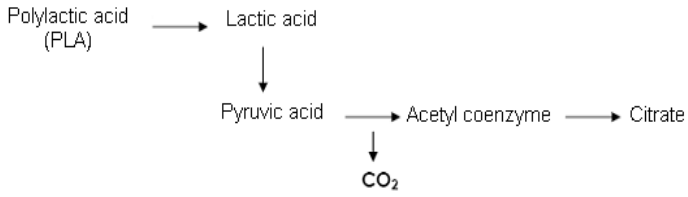

Figure 3. Diagram of the metabolism of polylactic acid in humans**.**

The metabolic pathway of polylactic acid begins with its conversion into pyruvate, lactate dehydrogenase enzyme, then generates Acetyl Coenzyme A molecule that enters the cycle of citric acid, mitochondrial level that produces ATP by oxidative decarboxylation by oxidative phosphorylation with H_2O and CO_2, eliminated in the breath, excreted by the kidneys [22].

2. EXPERIMENTAL

2.1 Determination of the degree of deacetylation (DD).

The degree of deacetylation (DD) is one of the chemical parameters to evaluate this type of material, because of its influence on the use of chitosan in many of its applications. For the determination of the degree of deacetylation a potentiometric titration will be carried out between 0.10 - 0.12 g weight of material by adding 25 mL of HCl 0.2 N and titrating with 0.1N NaOH. For the registration of changes in pH of the solutions versus the concentration of the titrate used a pH meter WTW pH 330 equipped with an electrode of glass combined WRW Sen Tix 41, pH 0-14/0 - 80 °C.

2.2 Infrared Spectroscopy (FT-IR)

The measurements are performed in a FT-IR Nicolet Magna 550 team with a detector of 4 cm^{-1} with the OMNIC software for data processing. Samples are prepared using 100 mg of KBr and 1 mg of corresponding sponges to produce a pill, which is prepared very carefully grinding the sample that will be crushed in a press for the shape.

2.3 Thermogravimetric analysis (TGA)

Thermal analysis (TGA) is used to determine the thermal stability of the samples. This technique measures the gain or loss in weight of a sample as a function of temperature and time in a gaseous medium. Thermogravimetry allows you to determine the thermal stability and the speed of decomposition of different materials in study. Is the required amount of sample mass is placed on the scale, and the temperature rises from 25 to 600°C with a heating rate of 20°C/min in an atmosphere of nitrogen. The equipment used is STA 625 Polymer Laboratories.

2.4 Preparation of composites

For the preparation of the prosthesis a solution 4% w/v of Chitosan in acetic acid 1% v/vis used as solvent, homogenized once proceeds to filter the solution to remove impurities, then take an aliquot of 10.0 ± 0.1 mL and HAP as additive is added to 3% w/v keeping stirring overnight. Once homogenized solution, is neutralized with 0.2 N NaOH until a 5.5 pH approx., completed this procedure the solution is deposited on 7.5 plastic pipe cm long and 1 cm in diameter then be frozen for 24 hours at - 62°C, approximately. Frozen samples are freeze-dried for three days to obtain desired sponges.

Once the samples are prepared solutions of: 0.5; 1.0; 1.5 and 2.0% w/v of polylactic acid dissolved in chloroform, the sponges are made through a conditioning system solution of polylactic acid. This component granted sponges better physico-chemical, mechanical stability and a lower solubility. Optimized once the technique to obtain the desired sponges proceeds to prepare the sponges inside theclean area in order to have them free of contamination.

Following the same methodology, but first we proceed to sterilize everything autoclaving for 30 minutes at 121°C, materials that cannot be autoclaving are sterilized in the UV Chamber within the area of controlled contamination, this mode is ensures the total sterility of materials to be used, to ensure if the material is free of contaminants this is sent to for analysis.Thirty five samples (seven with each formulation composites) were prepared.

2.5 Characterization of the prosthesis based on Chitosan, hydroxyapatite and polylactic acid.

Biomaterials prepared were characterized both chemical as morphologically using techniques such as FTIR and TGA (described above) in addition to morphology by microscopy (AAS), UV-visible spectroscopy, atomic absorption spectroscopy Electronics (SEM), solubility test, analysis of mechanical properties.

2.6 Calcium determination by Atomic Absorption.

Possible interference caused by the presence of phosphate are reduced or eliminated byadding to the 0.5 %v/v lanthanum and KCl 0.1% v/v. Working wavelength is 422,7 nm. This procedure consists in place of sample treatment weighing a portion of the prosthesis to the 50 mg previously dried and ground, which totaled porcelain crucibles. They were taken to the furnace, where the

temperature gradually raised up to 500°C and left for 5 hours. Crucibles were removed and the samples were placed in 50 mL flasks. These were treated with 1 ml of hydrochloric acid 4 N to be filtered and carried by nanopore water to a final volume of 50mL.

Once the samples are prepared, a calibration curve using a standard of 1000 ppm calcium solution, then a 20 times dilution is performed to obtain a solution of 50 mg/L. which will help us to make the curve in the range ideal for work. 50 mg/L solution are the standards of 1, 2, 3, 4 and 5 mg/L for the preparation of the calibration curve, once measured this we proceed to measure the samples.

2.7 Morphological characterization of Composites by Scanning electron microscopy (SEM).

The evolution of changes in the morphology of the prosthesis of Chitosan that occurred after the addition of HAP and PLA in different concentrations, was analyzed by scanning electron microscopy. Electron microscopy was used to check the size and quality of the generated pores, also to observe the final morphology of samples. For that, the samples were frozen in liquid nitrogen for 5 minutes approximately to fracture them easily and observe the surface of fracture, once in the workplace these were metallized gold in order to ensure its conductivity. As in other studies, used a scanning electron microscope ETEC Corporation Autoscan29.

2.8 Solubility for prostheses prepared test.

Saline serum was used for the analysis of solubility (0.9% NaCl), in the studies to make samples were taken from swabs of 30 to 100 ±0. 1 mg, all of them are left for 24 hours with 10.0 ± 1mL of ethanol 70% v/v to be sterilized them and then washed with nanopure water. Subsequently, the samples are treated with 20.0 ± 1mL of normal saline 0.9%, each sample is left for a period of 24 hours. After the time has elapsed, all samples are washed with nanopure water to then be dried for 6 hours in a desiccator at room temperature, then these are weighed until constant weight and mass difference show us the solubilized percentage of the samples.

2.9 Mechanical properties

The use of a particular biocompatible composite in the design of a biomedical device forces to assess their ability to withstand mechanical loads that will be submitted during the fulfillment of their specific function. The mechanics of deformable solids studies the behavior of deformable solid bodies to different types of situations such as applying loads or thermal effects. These behaviors, more complex than the rigid solids, are studied in solid mechanics deformable to introducing the concepts of strain and tension. A typical application of the mechanics of deformable solids is determined from a certain original solid geometry and applied forces on it, if the body meets certain requirements of strength and rigidity. Machine for the testing ofcompression consists of a fixed arm and one mobile, both equipped with a few dishes and JAWS to ensure samples in the place of measurement. The samples are analyzed in universal testing machine, model CMT 2502, SANS using a size of 16 mm and 8 mm diameter samples.

3. RESULTS

3.1 Parameters of chitosan

The nitrogen content in the studied Chitosan is: 7.3 ± 0.8%. For potentiometric titration was weighed: 0.1060 ±0.0001g, the result is 85% of deacetylation. The MW of chitosan was 250.000 g/mol (viscosity method).

3.2 Infrared spectroscopy of Chitosan (FTIR)

FTIR spectrum of Chitosan is presented below, where features of this biopolymer gangs manifested allowing ratifying the chemical identity of Chitosan. Here you can appreciate the bands corresponding to OH and NH (3415 cm^{-1}), characteristic of alcohols, amines and amides present in the structure of the deacetylated Chitosan. They are also bands of C=O and NH, characteristics of amides, fundamentally 1659 - 1588 cm^{-1}.

Approximately between 1660 -1555 cm^{-1} are the characteristic amide I vibration bands ($\tilde{\nu}$ C-O,1659 cm^{-1}) and amide III (δN-H,1380 cm^{-1}), respectively.

3.3 Infrared spectrum of polylactic acid additive (PLA).

A summary of the absorption bands of the PLA with their respective type of vibration and the range in which they appear. The most characteristic bands are: $\tilde{\nu}$C-H at 2996 cm^{-1},$\tilde{\nu}$C=O at 1752 cm^{-1}, δCH_3; C-H at 1454 cm^{-1}, $\tilde{\nu}$C-O at 1180-1046cm^{-1}, δC-O; C-C at 1263-1376cm^{-1}.

3.4 Infrared spectrum of the additive hydroxyapatite (HAP)

Hydroxyapatite, as well as other infrared spectrum shows the characteristic bands of the material. The spectrum presents the characteristic bands of hydroxyapatite, one of which correspond to OH and found at 3433 cm^{-1} which correspond to stretching vibrations, other bands corresponding to the vibration ν_3PO4^{3-} appearing at 1028 cm^{-1} and in the part of the fingerprint vibration bands of ν_4PO4^{3-}at 603 cm^{-1} and the 565 cm^{-1} these correspond to vibrations of deformation.

3.5 Chitosan Composites preparation

Samples were carried out successfully, a total of 30 biomaterials were prepared outside the area free of contaminants and 35 within it, some of them are shown in the following photographs: the following image shows the difference between (a) the biomaterial of Chitosan without additives and (b) prepared and reinforced with PLA and HAP, biomaterial to the naked eye can see that material prepared with Chitosan and without additives is fragile and is not homogeneous.

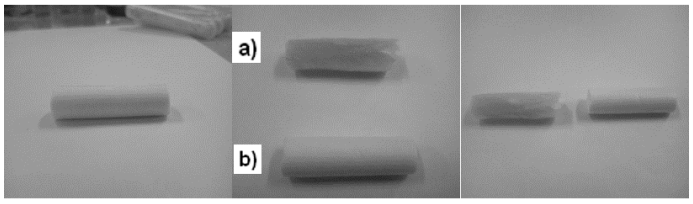

Figure 4. Comparison of biomaterials without (a) and (b) coated with PLA.

Figure 5. Sterile composites prepared in clean area.

3.6 Comparative infrared spectrum of composites.

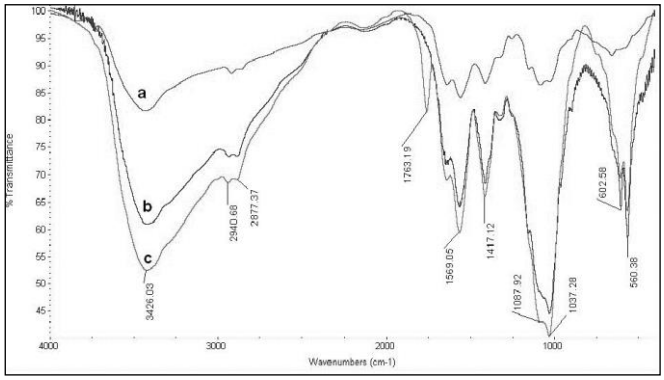

Figure 6. Infrared spectrum of composites:**a)** CH 4% ;**b)** CH 4 % + HAP 3% y **c)** CH 4% + HAP 3% + 1,5% PLA.

Figure 6 compares three composites one of 4 % Chitosan, with HAP and Chitosan with HAP and PLA. Also showing the characteristic bands that appear on the previous analysis. As these infrared were carried out in quantitative terms the increase in intensity of the bands can be observed in adding a new additive as shown in the bands (a), (b), and (c). New bands of the first material not possessed it is shown in the spectrum (c), very characteristic bands of the added additive which are for example the 1763 cm^{-1} which corresponds to the C =O or the carbonyl ester and are bands of polylactic acid.

Also, for the HAP appear to 602 cm^{-1} and at 560 cm^{-1} which correspond to vibrations of the $PO4^{3-}$ This suggests we actually realized an interaction between Chitosan and added additives.

3.7 Thermal study of the Chitosan composites (TGA).

Chitosan shows that at 137 °C there is a loss of 9.2% of the initial mass due to loss of moisture from the material. The significant loss of mass starting at 234°C refers to the processes of depolymerization and decomposition of different acetyl amino groups of chitosan being 304°C the decomposition temperature. In figure 7 there are two decomposition temperatures al 316 and 368°C. On the other hand, in figure 8 there are two decomposition temperatures al 310 and 375°C.

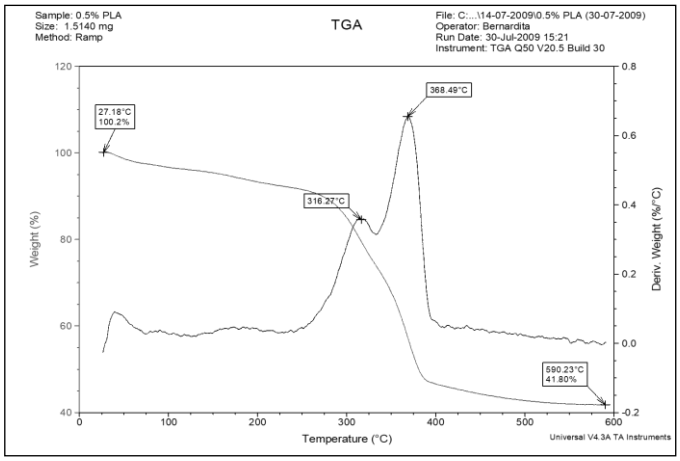

Figure 7. Thermogram of composite: 4% CH + 3%HAP + 0,5 % de PLA.

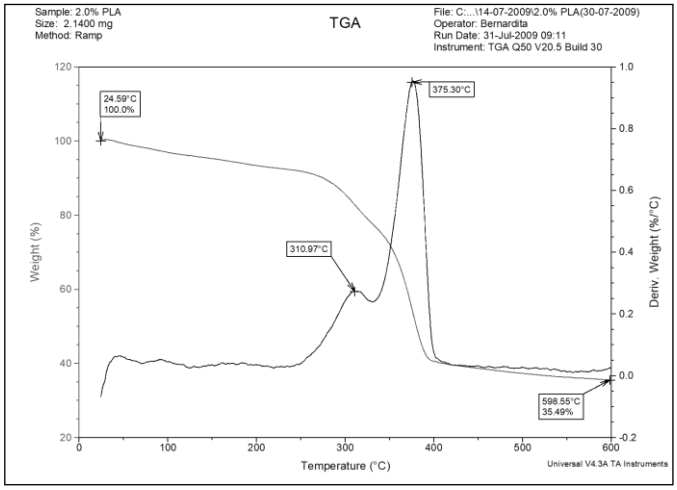

Figure 8. Thermogram of composite: 4% CH + 3%HAP + 2,0%PLA.

Table 1 shows decomposition temperatures in compositesin the several processes of decomposition. In this table is observed from 40 up to 100 °C a loss of water containing samples they come up to 130 °C approximately corresponding to occluded waters. The composites decomposition temperatures with PLAaddition from 0.5 to 2.0% changes from 310 to 316°C being pure PLA 351 and pure chitosan 304°C. Around 360-370 °C are the latest decompositions of the material, which correspond to the interaction of HAP with chitosan and PLA, demonstrating the presence of the composites.

Table 1. Decomposition temperatures of different composites.

Samples	Peak 1 (ºC)	Weight loss (%)	Peak 2 (ºC)	Weight loss (%)
CH4%	234	2.8	304	58
HAP	590	8	---	---
PLA	---	---	351	39
CH4% + HAP3%	151	12	304	42
CH4% + HAP3% + PLA 0,5	316	20	368	44
CH4% + HAP3% + PLA 1,0	315	20	363	36
CH4% + HAP3% + PLA 1,5	316	20	374	49
CH4% + HAP3% + PLA 2,0	310	18	375	47

3.8 Scanning electron microscopy (SEM)

Below is shown the SEM analysis carried out, showing the average pore size obtained in the composites with new additives, also shows some of the corresponding electron micrograph of the composites.

Table 2. Pore size for the Chitosan 4% + HAP 3% and polylactic acid at different concentrations.

Composites at different concentrations of PLA	Average porous size (μm)
Without PLA	162
0,5 PLA	160
1,0 PLA	147
1,5 PLA	145
2,0 PLA	146

The SEM analysis shows a strong tendency to the decrease of the average pores increasing the percentage of polylactic acid, of this unusual trend is observed in 1.5% percentage PLA, this does not meet the trend that arises, as to the increasing the percentage of polylactic acid dissolution viscosity also increases, making more difficult the entry of the dissolution to the pores of biomaterials, this can be seen best in biomaterials with PLA 2%.

Next micrographs show the fracture surfaces of the three-dimensional structures of biomaterials with and without additives. Where you can see porosity on the surface, likening them to a real, this is important in bones for the mobility of nutrients and fluids to the biomaterial for bone regeneration.

Shown that the pore size of the composites decreases by adding more levels of additives. This is due to that increasingly the add any additive other than space between charges becomes shorter in size.

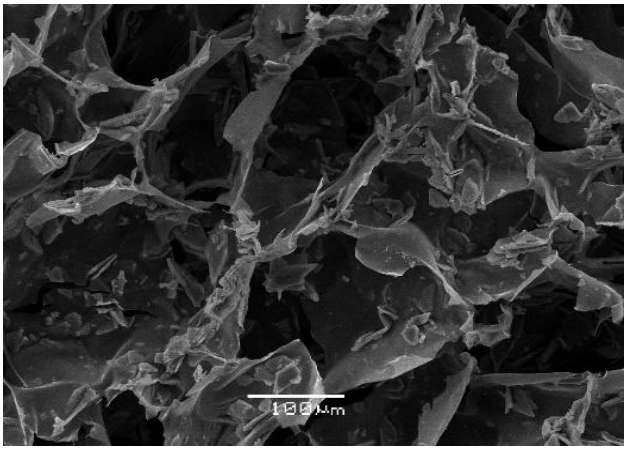

Figure 9. CH4% + HAP3% + 0,5% PLA.

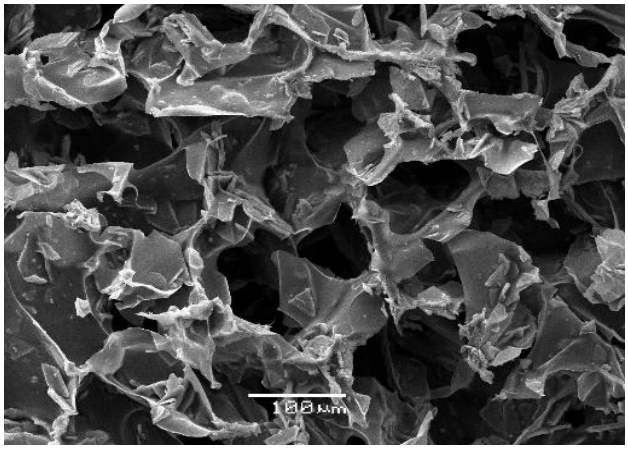

Figure 10. CH4% + HAP3% + 1,0% PLA

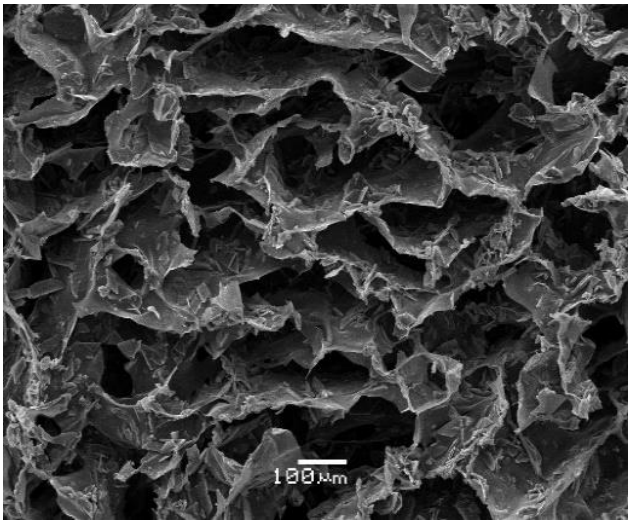

Figure 11. CH4% + HAP3% + 1,5% PLA.

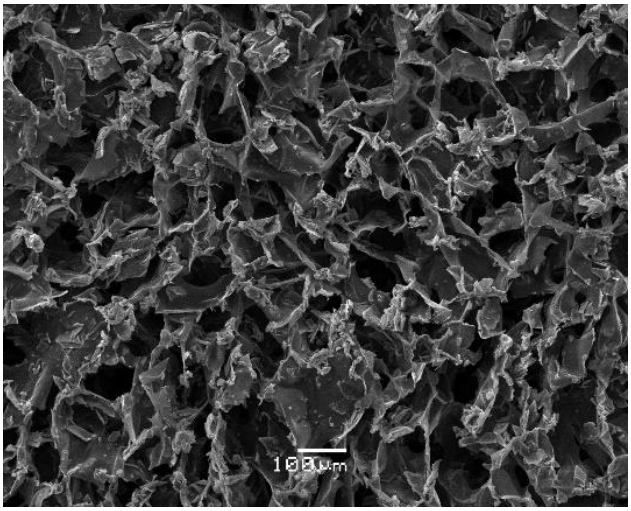

Figure 12. CH4% + HAP3% + 2,0% PLA

3.9 Test of solubility for chitosan composites.

This test helps us to know how soluble it is the sample prepared with new additives, in a physiological environment simulated, while less soluble is the sample, it will be much better to be used in implants that are to be achieved, the low solubility will extend the life of the biomaterial without loss of its three-dimensional structure.

Table 3. Test of solubility for prosthesis at different concentrations of PLA.

Sample	Initial Mass (mg)	Final Mass (mg)	Solubilization (%)
CH4% + HAP3%	53.1	45.8	10.72
CH4% + HAP3% + PLA 0,5	56.5	53.2	5.89
CH4% + HAP3% + PLA 1,0	80.6	76.5	5.08
CH4% + HAP3% + PLA 1,5	52.1	50.4	3.86
CH4% + HAP3% + PLA 2,0	49.2	47.3	3.26

Table 3, shows the solubility of composites, there is a clear tendency to decrease the solubility when you add a higher concentration of PLA, although this difference in solubility is small, is repeated in all the prepared samples, remember that the sample for this and other studies is n = 10. The same is shown in the earlier analyses such as FTIR which were prepared with the same concentrations.

Analysis of mechanical properties of biomaterials.

The results of the mechanical properties of the prepared biomaterials are presented below**.**

Table 4. Data for analysis of mechanical properties

Samples	Strength force (kN)	Compression resistance (MPa)	Elasticity Young modulus (MPa)
CH4% + HAP3%	0.02	0.31	5.97
CH4% + HAP3% + PLA 0,5	0.02	0.33	6.71
CH4% + HAP3% + PLA 1,0	0.02	0.46	8.27
CH4% + HAP3% + PLA 1,5	0.03	0.41	9.59
CH4% + HAP3% + PLA 2,0	0.02	0.62	12.32

The study of the mechanical properties indicates a sense marked increase of compressive strength, increasing the percentage of PLA also increases resistance to deformation leading to an increase in the force required to achieve deformation of the prosthesis. Following are the graphical of composites to different percentages of PLA.

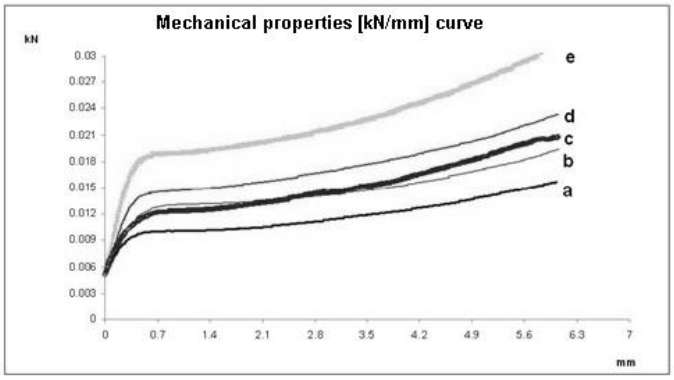

Figure 13. Graph of mechanical properties of Chitosan composites with different concentrations of PLA.

Figure 11 shows an increase in the slope, it increases the concentration of polylactic acid in the sample analyzed, in this chart all these trend lines contain CH 4%, 3% HAP that is modified is the percentage of polylactic acid. As you can be seen in the trend line (a) which corresponds only to CH 4% + 3% HAP, presents lower slope, which is expected since it is not very resistant to compression, other lines correspond to (b) 0.5% PLA; (c) 1,0% PLA; (d) 2.0% PLA and (e) 1.5%. PLA. Trend marked again until reaching the 1.5% PLA, which presents one resistance greater than material coated with 2.0% PLA, this behavior is attributed to which this concentration 2% of PLA, the dissolution do not enter inside the pores and covers partially the composites.

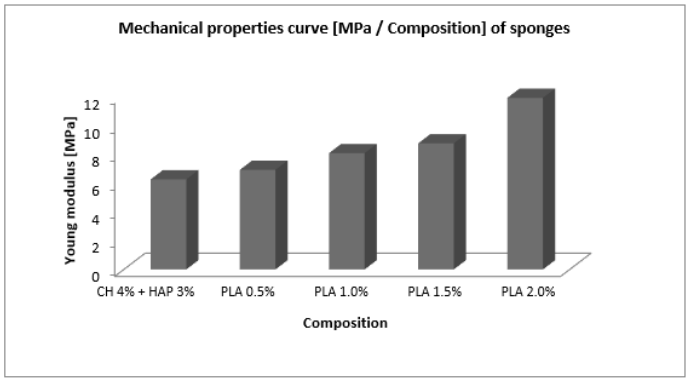

Figure 14. Mechanical properties of biomaterials by increasing the percentage of polylactic acid (PLA)

This graph showed the increase of the Young modulus along with the increases of percentage of polylactic acid, as shown in the concentration 2.0%PLA being the highest.

CONCLUSIONS

The dissolutions of polylactic acid concentrations 0.5; were carried out successfully 1.0; 1.5 and 2.0% to give greater to the composites stability and is achieved by coating with different percentages of PLA.

Biomaterials were evaluated by all the methodologies described, concluding that within all biomaterials prepared which presents best properties to be used as scaffold of bone growth, is containing Chitosan up to 4%, hydroxyapatite 3% and 1.5% polylactic acid.

The solubility study shows gets get one much lower solubility, to cover biomaterials with PLA, quality Pro to obtain a correct bone regeneration.

On the characterization of the prosthesis by means of analyses of calcium and phosphorus, we find there is a 1.6 Ca/P ratio which tells us that indeed the biomaterial is present of calcium phosphate in the form of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) showing that is no change in its chemical structure.

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