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Properties of Microcrystalline Chitosan-Calcium Phosphate Complex Composite

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ABSTRACT

Nature itself uses materials like, cellulose to provide the structure of plants, chitin as the exoskeleton of several insects and molluscs, collagen for mechanical support in connective tissues and so on. At present, the socioeconomic situation of the modern world has raised the interest in renewable materials being used in regenerative medicine. The composition of MCCh/ß-TCP complex in sponge shape is derived from the junction of two or more different materials, containing organic and inorganic materials, including bioactivity and biodegradability as a characteristic. The chemical characteristics of MCCh/ß-TCP complex composites showed that both of the components organic and inorganic exist in the material. All sponge preparations, with MCCh/ß-TCP have a well-shaped 3-dimentional structure, a highly porous and interconnected and homogenous pore structure to ensure a biological environment conducive to cell attachment and proliferation as well as tissue growth, providing the passage of nutrient flow. These materials can be used in future for medical applications as a base for scaffolds production and as implants in regenerative medicine.

Keywords: Microcrystalline Chitosan; Calcium Phosphate; Functional Biopolymer; Medical Applications

1. Introduction

Different types of materials have been developed from chitosan and calcium phosphates that are biocompatible, osteoconductive, resorbable and osteoinductive. The large wide range of materials available for hard tissue regeneration reflects methods of production and application to reconstruct part of skeleton.

All methods may achieve restoration and/or replacement. They all possess inherent limitations, such as use site, donor-site morbidity, an obligatory graft resorption phase, contour irregularities, insufficient autogenous resources, disease transmission, major histocompatibility, structural failure, stress shielding, infection and so on [1,2].

Calcium phosphates, polysaccharides and composite materials, are developed during last 20 years to be used for bone problems. Natural polymers have low mechanical strength compared to bone, and on the other hand

have good biocompatibility and bioactivity. Ceramics are brittle and have low fracture toughness. With all these properties of polymers, ceramics are together producing composite materials having a reasonable approach [2].

Bone is of composite nature and consists of 69 wt% calcium phosphate, 21% collagen (polymer), 9% water and 1% other constituents building up a very complex microstructure which is very difficult to imitate and gives most of the superior mechanical properties to bone [2-4].

Biomaterials studies in this research are classified into surface-active materials such as hydroxyapatite (HAp), ß-tricalcium phosphate (ß-TCP), and chitosan and its derivatives such as MCCh [5-7].

Surface-active materials bind to bone through an apatite layer formed on the material surface after implantation. It is assumed that the velocity of layer formation and new cell correlates with bioactivity. However, how bioactivity affects the formation of the apatite layer is still understudying. It has not been made clear whether the apatite layer can be formed only under the influence

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of the bone tissue [8-10].

Chitosan exhibits a variety of physicochemical and biological properties, such as low toxicity and allergenicity. Its biocompatibility, biodegradability and bioactivity make it a very attractive substance for diverse applications as a biomaterial in pharmaceutical and medical fields [11-14].

Polyaminosaccharides, such as chitosan (poly(B-(1,4)-2-amino-2-amino-2-deoxy-D-glucopiranose)) and its derivatives, are characterized by excellent biostimulation properties which facilitate reconstruction and vascularization of damage tissues, and also compensate the shortcomings of cells components [13,15]. This cationic property is the basis of many of the potential applications of chitosan and its derivatives that can be considered as a linear polyelectrolyte with a high charge density which can interact with negative charged surfaces, like proteins and anionic polysaccharides [13,15].

Microcrystalline chitosan is characterized by special properties of initial chitosan such as biocompatibility, bioactivity, non-toxic, hydrophility with same creation of molecular and super-molecular structure during its manufacture, in addition, a inner surface formation and reducing the size of crystals in the polymer. It's suitable for medical application, especially for wound dressings and drug delivery [15-17].

The calcium phosphate family constitutes mainly of inorganic materials currently used in dental and orthopedic reconstructive medicine. Specifically, hydroxyapatite (HAp) and ß-tricalcium phosphate (ß-TCP) were developed as bioceramics in the early 1980s and nowadays are the most common calcium phosphates used in medical applications [8,18]. Despite their relative importance, both ceramics show a number of drawbacks that reduce their clinical performance. The biodegradation of HAp in physiological environments is too low to achieve the optimal formation of bone tissue [8,15,17,18].

On the other hand, ß-TCP shows fast release of Ca^{2+} and PO_4^{3-} ions when exposed to physiological fluids and could be considered as bioactive [10]. Selecting the appropriate blend of both calcium phosphates, the mixture gradually dissolves in the physiological environment, releasing Ca^{2+} and PO_4^{3-} ions and inducing the bioactive behavior suggesting mainly mechanical bonding. The material that remains during dissolution acts as a template for the newly formed bone [9-11,17,19]. Biphasic alcium phosphates composites with ß-tricalcium phosphate (ß-TCP), $Ca_3(PO_4)_2$, and hydroxyapatite (HAp), $Ca_{10}(PO_4)_6(OH)_2$, are compounds with a high potential for bio applications, which combine the excellent bioactivity of HAp with the good bioresorbability of ß-TCP [10-12,17,19]. To improve the suitability of chitosan and its derivatives for bone tissue engineering, the biocom-

posites of MCCh, ß-TCP and hydroxyapatite could be applied.

2. Materials and Methods

2.1. Materials

Initial chitosan from Primex Co trade name Chito Clear FG90 was used, characterized by: average molecular weight (M_v) = 342 kD, deacetylation degree (DD) = 82%, ash content = 1.7%. Microcrystalline chitosan (MCCh paste) characterized by: average molecular weight (M_w) = 330 kD, deacetylation degree (DD) = 82%, ash content = 0.7%, water retention value (WRV) = 598%, dry polymer content = 2.79%, pH = 7.38. Microcrystalline chitosan/ß-TCP paste characterized by: water retention value (WRV) = 560%, dry complex content = 3.6%, (Content of MCCh: 80% and ß-TCP: 20%), pH = 7.40. Tri-calcium phosphate (ß-TCP), ($Ca_3(Po_4)_2$)—Sigma Aldrich Lab., Germany. Hydroxyapatite ($Ca_{10}(Po_4)_6(OH)_2$)—Sigma Aldrich Lab., Germany. Plasticizer-Glycerol ($C_3H_8O_3$) 99%, pure p.a., Sigma-Aldrich, Germany. Hydrochloric ac. 37.8% p.a., manufactured by Fluka.

2.2. Methods

2.2.1. Analytical Methods for MCCh/ß-TCP Characterization

1) The water retention value of complex or MCCh (WRV) is determined by submerging 0.5 g ± 0.0001 g of MCCh or complex in 50 cm^3 of distilled water. Next it is centrifuged for 10 min at 4000 rpm. The weight of the sample is determined after centrifuging and after drying to constant weight after 20 hours at 105°C ± 1°C.

2) Determination of complex or MCCh polymer content - approx. 5 g ± 0.0001 g of complex sample is placed in a laboratory dryer and dried at the temperature of 105°C ± 1°C to constant weight.

3) Deacetylation degree of chitosan or MCCh is determined by the potentiometric titration method using the first derivative UV-spectrophotometry (DUVS). The concentration of chitosan solution is up to 100 cm^3 with anhydrous acetic acid 0.0100 M. The presence of D-glucosamine was corrected for by a reference curve for N-acetyl-D-glucosamine.

4) Ash content in chitosan or MCCh—the quartz crucible is heated at 800°C for 3 h to constant weight. After cooling to ambient temperature in the desiccators and weighed on the analytical balance.

2.2.2. Preparation of Microcrystalline Chitosan/ ß-TCP Complex

Microcrystalline chitosan/ß-TCP complex was prepared according to a method elaborated in the Institute of Biopolymers and Chemical Fibers, Polish patent application P 393758 (27/01/2011).

2.2.3. Elaboration of the Quantitative and Qualitative Composite MCCh/ß-TCP Complex and HAp

The main objective of this study was to elaborate qualitative and quantitative composition of MCCh/ ß-TCP complex and HAp. The study used a microcrystalline complex form. The quantitative and qualitative compositions of composite are given in **Table 1**.

2.2.4. Preparation of Composite Sponge

The preparations were carefully homogenized and next freeze-dried method was applied by dryer ALFA 1 - 4 made by Christ Co. The temperature range from (−25) to 10°C and vacuum from 0.1 to 0.53 mbar during 20 to 24 hours depending upon size of the charge. Drying accomplished that way resulted in the preparation of sponges with a smooth surface without defects.

2.2.5. Preparation of Microcrystalline Chitosan Powder

The microcrystalline chitosan powder was prepared by freeze-drying method using ALFA 1 - 4 made by Christ Co. The freeze drying proceeded in the temperature range of (−20) to 10°C and vacuum 0.1 - 0.7 mbar. The drying time was 20 - 24 hours to obtain sponge and after the powder.

2.2.6. SEM Study

The particle size of commercial ß-TCP and HAp powder and distribution in the polymer matrix in a sponge form, was observed using a scanning electron microscopy (SEM)—FEI Quanta 200, USA.

2.2.7. Infrared Spectroscopy

Infrared spectroscopy Fourier transform (FTIR) is a non-destructive technique that was used to identify the functional groups through their chemical bonds, which generate a spectrum of infrared bands characteristic of each connection type. The infrared analysis was in the

Table 1. The formulations characteristic of composites in sponge form.

Symbol of sample	Weight proportion of components (wt%)			
	MCCh/ß-TCP complex suspension	MCCh (powder)	HAp	Glycerol
Sample 1	1[*]	0	0	0.5
Sample 2	1[*]	0	0.5	0.5
Sample 3	1[*]	0	1	0.5
Sample 4	1[*]	0	2	0.5
Sample 5	1[**]	0	2	0.5
Sample 6	1[*]	1	2	0.5

[*]Dry complex content 2.0%; [**]Dry complex content 2.95%.

range of 500 to 4000 cm^{-1}, resolution 4.0 cm^{-1} with a Spectrum Genesis Series. Samples were prepared for analysis with KBr, in the form of tablets. To verify the presence of functional characteristic groups of ß-TCP, HAp and MCCh in the composite material.

2.2.8. Determination of Particles Size in HAp and ß-TCP Powders

The technique used was by laser particles sizer by Sympatec Hellos H1330, type BF (sympatec GmbH, Clausthal, Germany).

2.2.9. X-Ray Diffraction

Two calcium-phosphates in form of powder have been investigated by means of WAXS-diffraction. Both powders HAp and ß-TCP were simply placed between two polypropylene films for running the WAXS-scan in transmission mode. The following parameters were adjusted. X-ray radiation—CuKα-doublet, non-monochromatic B-portion suppressed by Ni-filter. Mode of operation—Transmission, anode Voltage—40 kV, tube current—40 mA, step width—0.025°, scan rate—5 sec/step, aperture slit—1 mm, anti-diffusion slit—1 mm, detector slit—1 mm.

2.2.10. Determination of Ca and P in HAp and ß-TCP Powders and Composites

The aim of this study is to determine and compare the quantity of Ca and P that remains after sponges preparation of the composites, by ICP-OES, Germany, by method of microwave digestion according to SOP2.5L126, edition 3-quantification of Calcium and Phosphorus (DIN EN ISO 11885).

3. Results and Discussion

3.1. FTIR Study

The main peaks of energy vibration identified in the composite the ß-TCP, HAp and MCCh (**Figures 1** and **2**). The characteristic functional groups of orthophosphate (PO_4^{-3}), hydroxyl (OH)$^-$, phosphate (HPO_4^{-2}) the latter one in trace amount, shown characteristic peaks of calcium phosphates material. The presence of carbonate was observed, in trace, in the commercial ß-TCP and HAp material, that suggest in some commercial ß-TCP and HAp the CaO and Ca(OH)$_2$ are used to get an ideal stoichiometric relation between Ca/P in the material and also related with the preparation process of the samples that were prepare in atmosphere conditions with presence of carbon dioxide and air.

It is known that synthetic ß-TCP is the material more stable and more soluble in human body, than HAp, among the calcium phosphates [9,18,20]. These features can increase the regeneration of bone tissue in the presence of an implant.
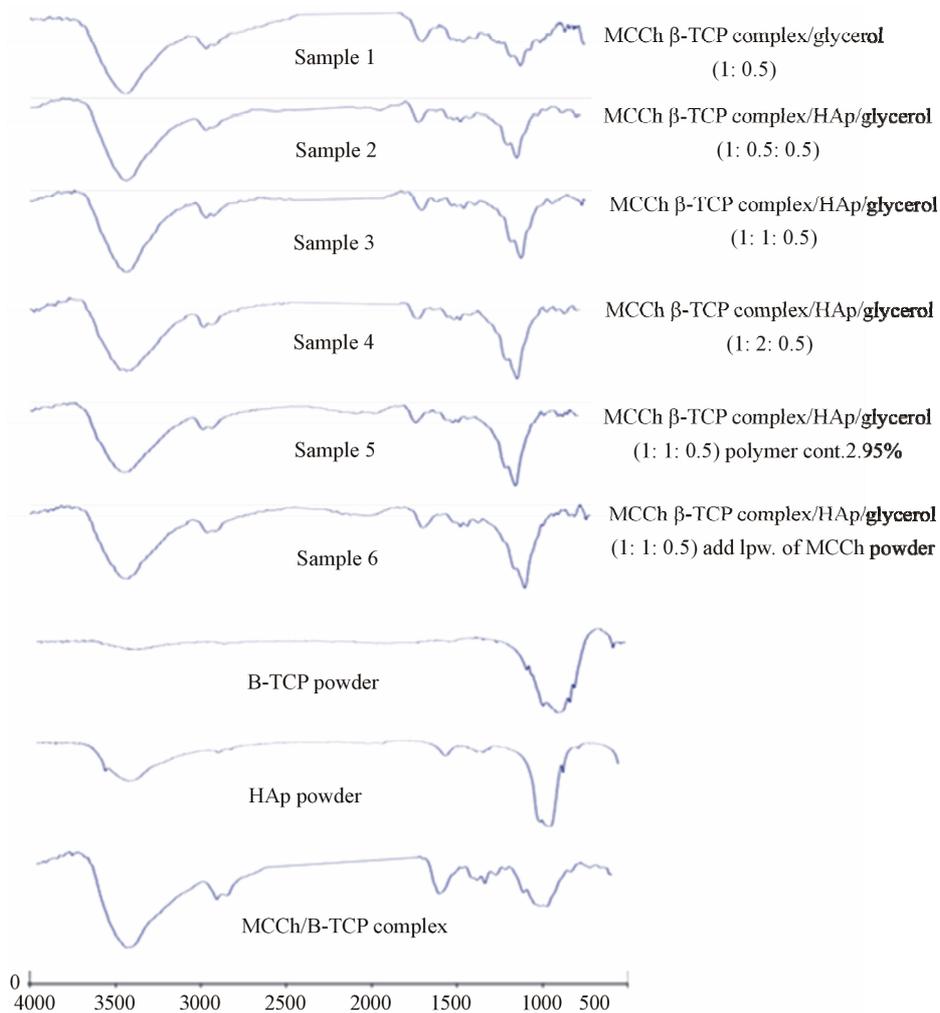
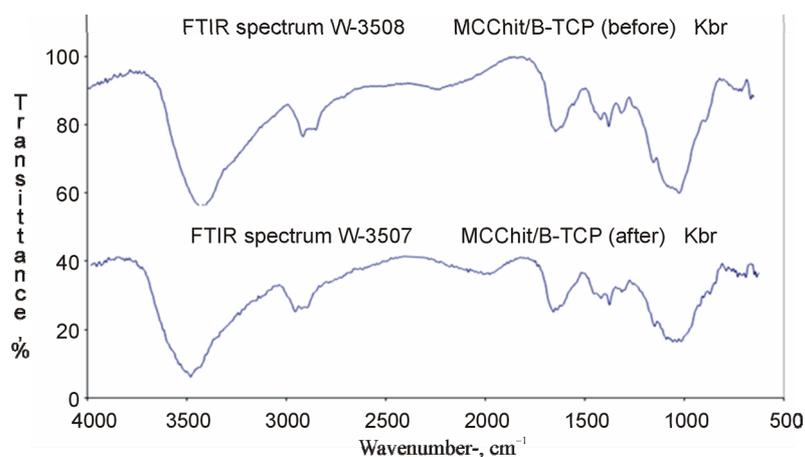**Figure 1. FTIR spectrum of the MCCh/ß-TCP and HAp composites.**

Figure 2. FTIR spectrum of the MCCh/ß-TCP before and after purification process.

In the composite, the absence of bands at 460 and 740 (cm^{-1}) and an isolated band, 600 cm^{-1}; characteristic of a-TCP, indicating that the starting material is only composed of ß-TCP, also shows a small amount of CO_3^{-2} in 1428 cm^{-1}. This calcium phosphate is easily identified by presenting a broad band in 900 - 1200 cm^{-1}; observed the presence of the peak at 724 cm^{-1}, characteristic of the symmetric mode (P-O-P) assigned to distortion of P-O.

Since the peak at 1211 cm^{-1} is characteristic of a non-degenerate deformation of hydrogen groups -OPO$_3$, -H, O-PO$_3$, common ions HPO$_4^{-2}$, the presence of this group may be a consequence of the interaction of water molecules in the structure [18,20-22].

Also in the composite samples showed in 3338 cm^{-1} and 1650 cm^{-1} the O-H, H-O-H group peak, referring to the adsorbed water. The peak observed at 968 cm^{-1} of low intensity, corresponds to non-degenerate symmetric stretching of P-O bonds of phosphate groups. The bands 1041, 1081 and 1098 (cm^{-1}) represent the asymmetric stretch modes, respectively, the P-O bonds of phosphate groups [8,18,21].

In the composite is confirmed also the presence of the functional groups NH$_2$ (1539 cm^{-1}), Amide I (1648 cm^{-1}), amide II (1557 cm^{-1}) from MCCh material. The main peaks of energy (1026, 1086 cm^{-1}) identified the C-O skeletal vibrations stretch of saccharide structure.

The peak observed at 1154 cm^{-1}, of high intensity, corresponds to anti-symmetric stretching of C-O-C bond groups. The bands 1026 and 1086 (cm^{-1}) represent the symmetric stretch modes, respectively, the C-O bonds characteristics of the saccharide structure groups [16,21,23]. The **Figure 2** shows the FTIR spectra of complex before and after purification process. The spectrum exhibits peaks of the same characteristic such as: a strong and broad peak between 1154 cm^{-1} and 1026 cm^{-1} and 1086 cm^{-1} that refer to the skeletal vibrations stretch of saccharide structure and a peak at 1636 cm^{-1} attributed to the free primary amino group (NH$_2$) in MCCh [16,21,23]. The main peaks of energy vibration identified in the ß-TCP are characteristic functional groups of orthophosphate (PO$_4^{-3}$) at 1100 cm^{-1}, hydroxyl (OH)$^-$ at 3430 cm^{-1},

phosphate (HPO$_4^{-2}$) at 1000 cm^{-1}.

A broad peak appears from 3338 cm^{-1} corresponding to the -OH stretching absorption band in the samples. The chemical interactions between the inorganic and organic constituents in the composite, probably take place via the ionic and electrostatic bonding between Ca^{2+}, phosphate groups and the amino group of chitosan, that confirm the positive charge of the MCCh and the negative charge of the ß-TCP [21,23].

The FTIR spectrum before and after purification process show very similar peaks that suggest the same product, validating the method of preparation of MCCh/ß-TCP.

3.2. SEM Study of the Sponges

The objective of the investigation was to estimate the ß-TCP and HAP, suitability in the sponge preparations form and distribution in the polymer matrix, showed in the **Figures 3-5** and according with **Table 1**.

The preparations of composites sponges formed a well-shaped 3-dimensional structure and interconnected porous. This was also supported by the high porosity which can be used in future as a base for scaffolds production. The crystals of HAp and ß-TCP and clusters formation from both calcium phosphates aggregate well in the polymer matrix. Addition of MCCh in powder form in the suspension doesn't affect the uniformity and distribution of the HAp and ß-TCP in the polymer matrix showed in **Figure 6**. The SEM analysis showed great ability of calcium phosphates to agglomerate, the small particles tend to agglomerate due to the increased intensity of the attraction forces compare to repulsive forces,
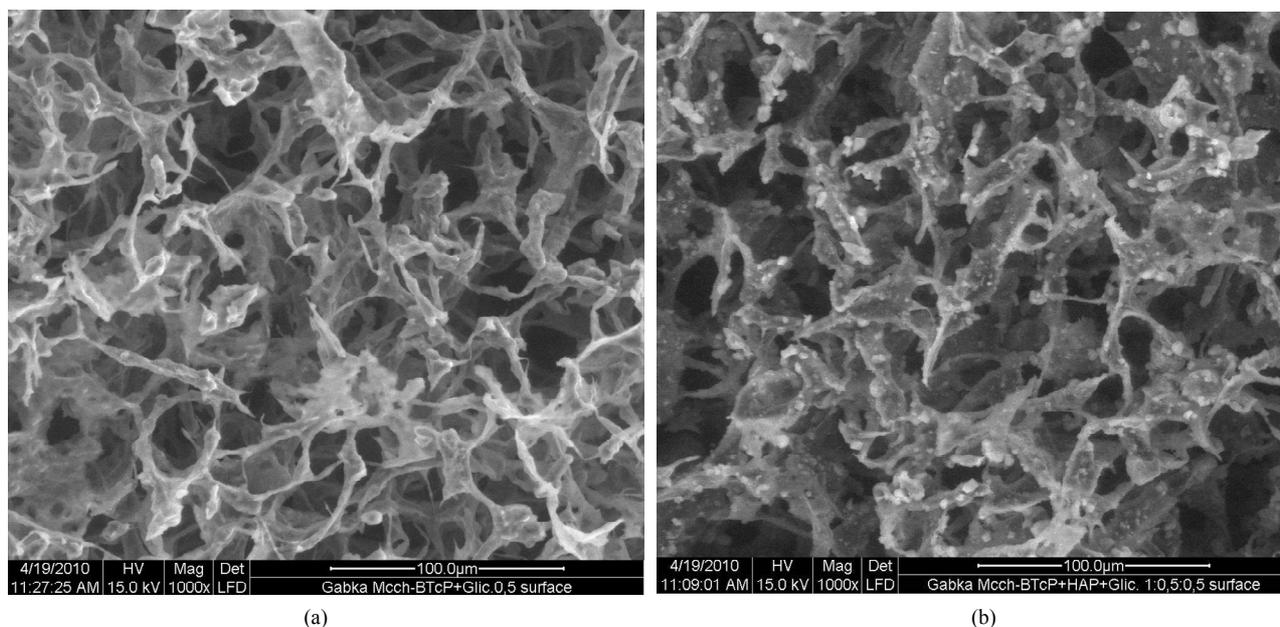

(a)   (b)

Figure 3. SEM microphotos cross-section 1000× (a), sample 1 (b) sample 2.
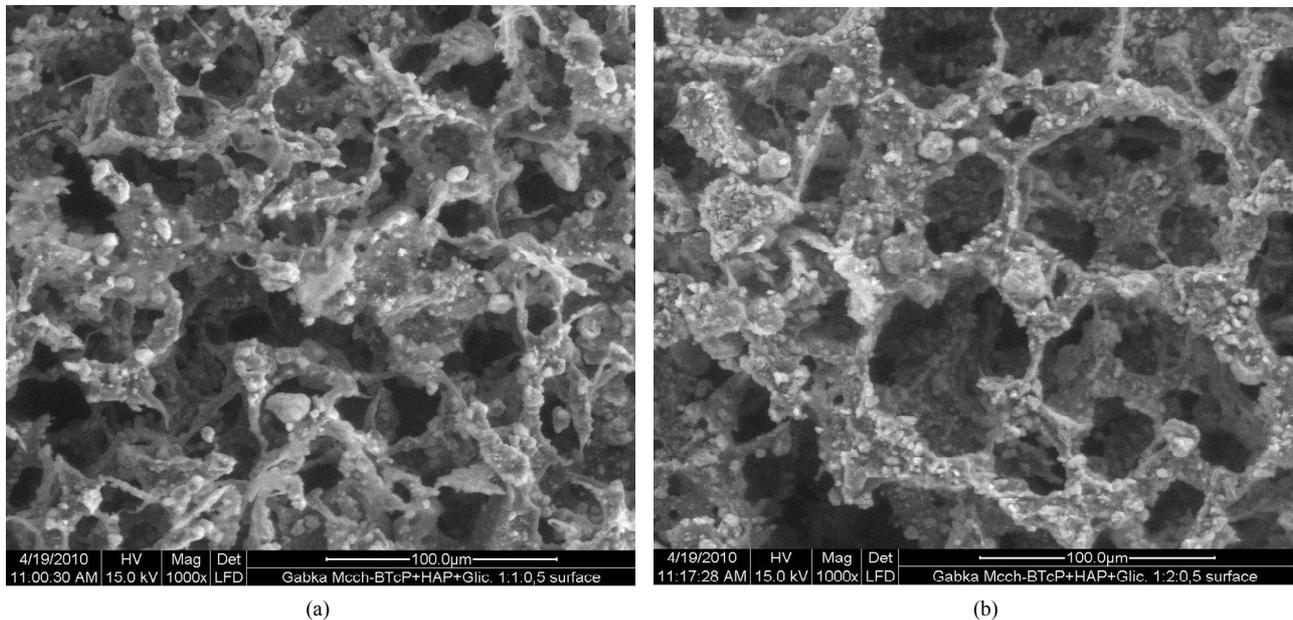
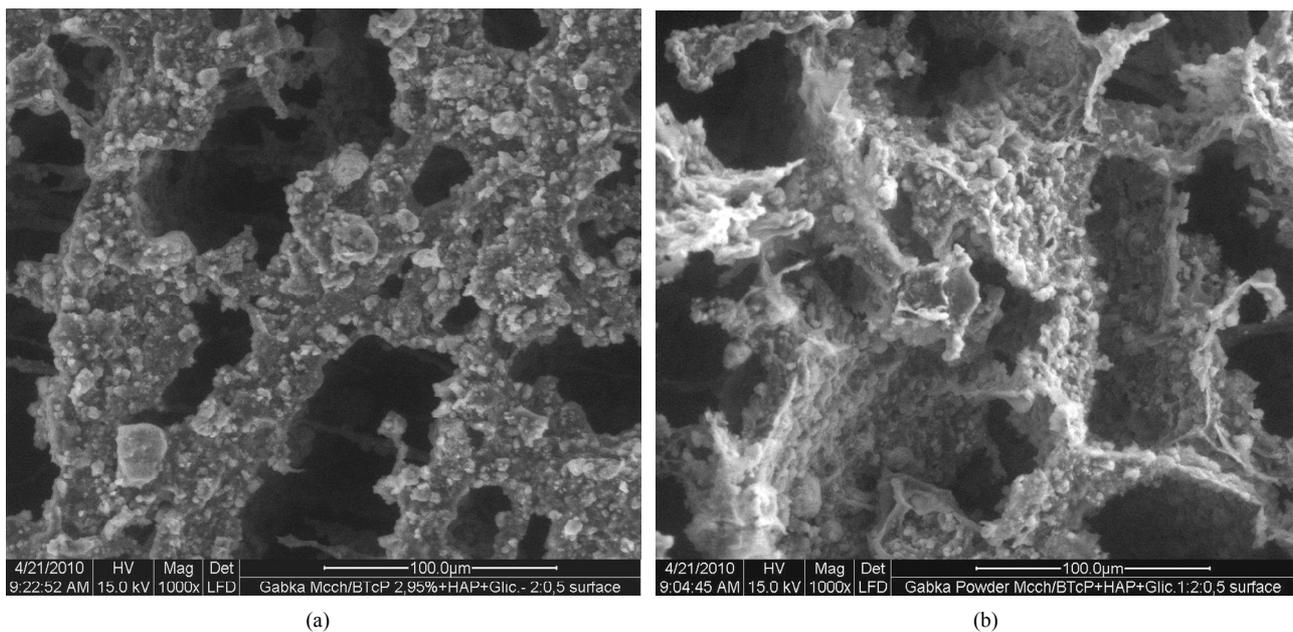**Figure 4. SEM microphotos cross-section 1000× (a), sample 3 (b) sample 4.**

Figure 5. SEM microphotos cross-section 1000× (a), sample 5 (b) sample 6.

because the charge distribution on the surfaces and the water adsorption. The addition of hydroxyapatite gradually increased the rough surface.

3.3. X-Ray Diffraction

The aim of this study is to confirm the crystalline and amorphous phases of the HAp, ß-TCP powders showed in **Figure 6**.

The crystalline phase of powders is directed related with calcium phosphate dissolution and less time for new tissue stimulation and formation [20].

However the intensity of peak at 32.5° and the intensities of peaks at 26° and 40° is also a characteristic peaks of HAp. Regarding that the peaks values were observed, the structure showed is hydroxyapatite and no different new crystal phase was observed in the **Figure 6**. The increased sharpness and intensities and decreased broadness of mentioned peaks can be explained by the increase in crystal sizes according with particles size analyses showed in Section 3.4, relatively small crystals of the HAp and ß-TCP which were in powders form and with particle size range less than 10 microns.

The X-ray diffraction of the calcium phosphate powders showed a considerable amorphous halo in the HAp powder between $10° < 2\theta < 20°$, the ß-TCP was found to be completely amorphous.

In material applications for engineering tissue regeneration, the relatively high solubility of amorphous calcium phosphate in aqueous environments make suitable or a good choice as a mineralizing agent. When compounded with appropriate polymeric material, amorphous calcium phosphate bioactivity may be particularly useful in enhancing the performance of composites in physiological environmental [19].

3.4. Determination of Particles Size in HAp and ß-TCP Powders

The aim of this study was to determine the particle size and morphology of the commercial HAp and ß-TCP in form of powder used in this research. The **Figures 7(a)** and **(b)** show the ß-TCP and HAp respectively.

Notice the morphologies of the powder differ in shape, the ß-TCP powder is shown in the **Figure 6(a)**, is more spherical that HAp powder. Also the powders showed great ability to agglomeration and cluster formation, explained by a not homogenous charge distribution on the surfaces and high ability for water adsorption. Typically the very small particles tend to agglomerate due to the increased intensity of the attractive forces over the repulsive forces, due to charge distribution on the surfaces.

The analysis of particles size shown a standard deviation cannot be given so easy. The particle size depends firstly on particle shape (aspect relation). Most calculation models refer to ball-shaped bodies. The more the particles deviate from this model the more the deviation rises. Secondly, the broadness (width) of particle size

distribution has an influence on the standard deviation using this method. In a case where this distribution covers only a narrow part, for instance 2 to 5 μm, then the deviation is lower than 10%. However, the measurement itself is very precise. The measurement of the powders used in this research had procedure of three-times for each sample and receives deviations of results lower than 2%, the samples of both powders had a range from 0.5 to 50 μm. The analysis showed a ß-TCP grain size fits in the range 3.0 - 9.0 μm and grain size of HAp fits mainly in the range 2.0 - 7.0 μm, and with fair agreement with the literature [9].

The particles size distribution graphics of the commercial calcium phosphate powders HAp and ß-TCP used in this research are shown in the **Figures 8** and **9** respectively.

The measurements of particles size by laser particles size gave a specified surface area covered by each powder HAp is $3.41\ e + 04\ cm^2/g$ and ß-TCP $2.53\ e + 04$
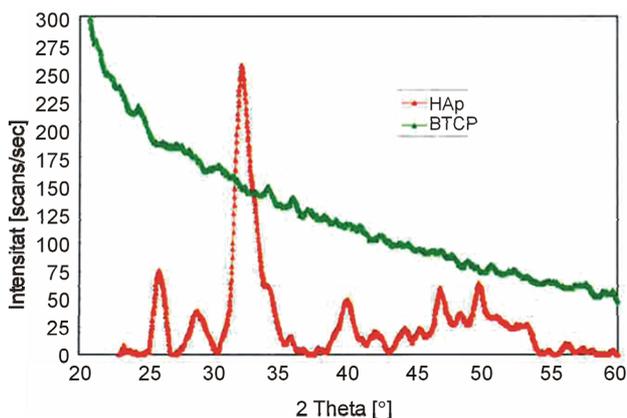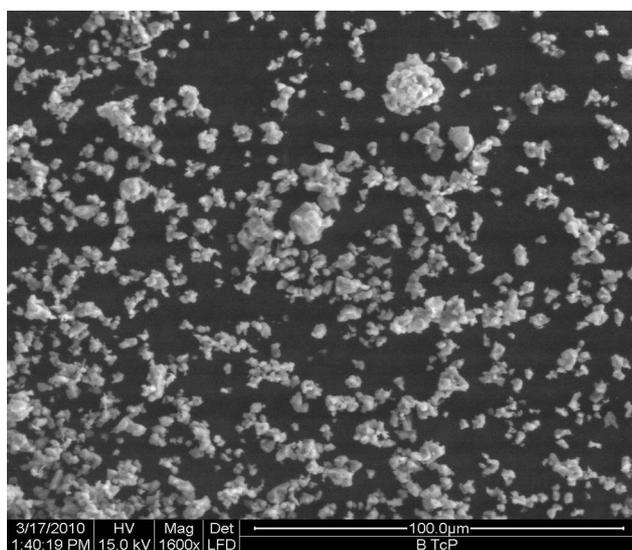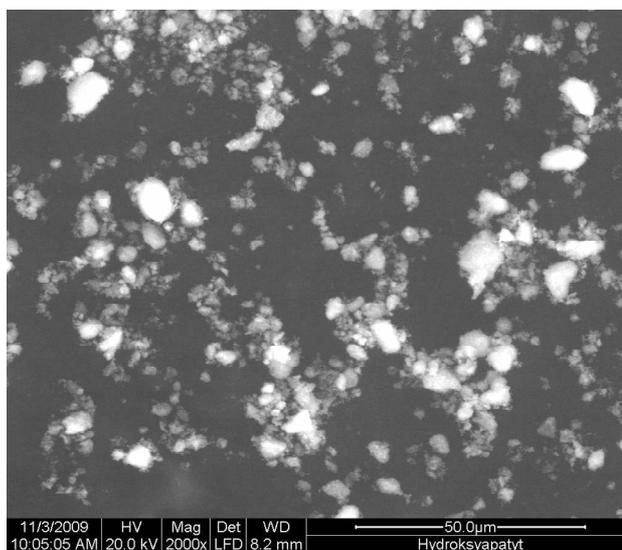

Figure 6. X-Ray spectrum of the ß-TCP and HAp powder.

<div align="center">(a)   (b)</div>

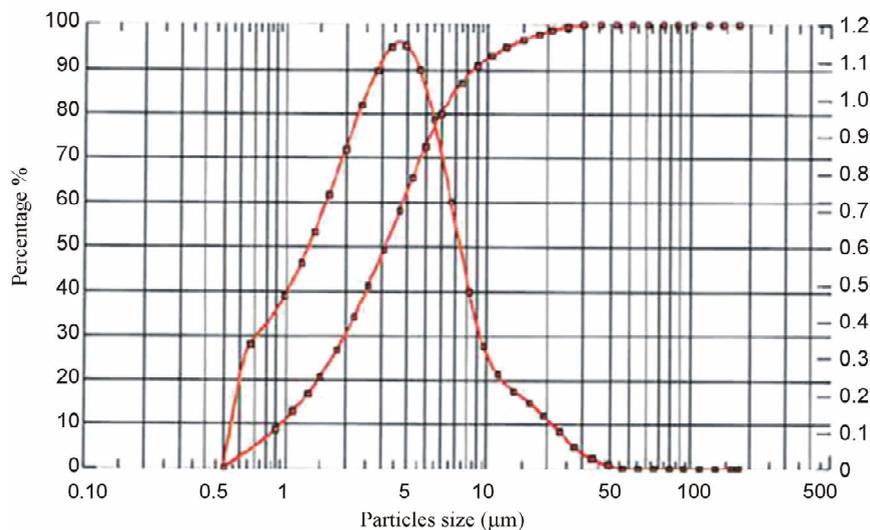**Figure 7. Morphology of particles of the commercial ß-TCP and HAp respectively, powder form.**

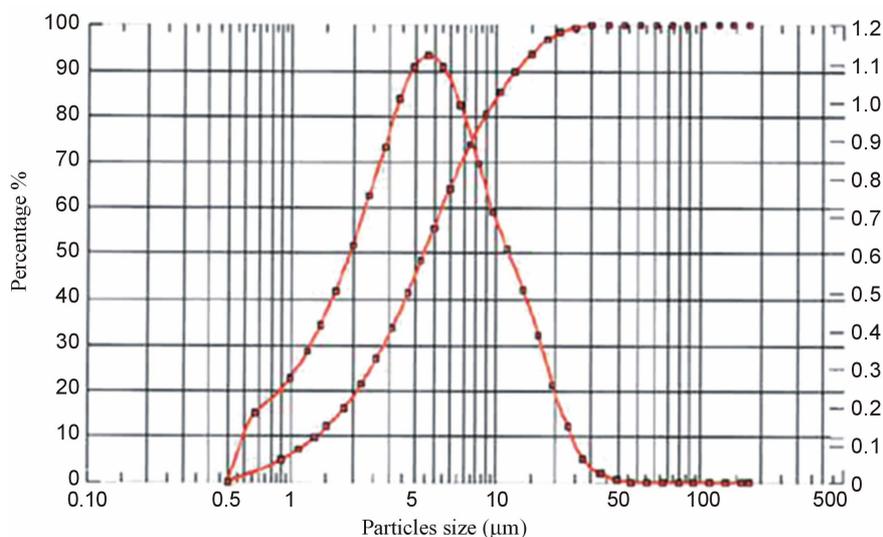**Figure 8. Grain size and distribution of synthetic HAp powder.**

Figure 9. Grain size and distribution of synthetic ß-TCP powder.

cm^2/g (**Table 2**). Also it was found around 10% of the calcium phosphate particles used in this research (HAp and ß-TCP) was nano size that also indicate a faster and easier release of calcium and phosphate ions in physiological environmental following the literature [9,19]. There are many factors related with the performance of the biocomposite for hard tissue regeneration that reflect in the preparation of suspension, mechanical properties and calcium and phosphate ions in the physiological environmental as a particles size, shape, particles distribution and also ratio of inorganic part in the polymer matrix.

3.5. Determination of Ca and P Content in Composites

The aim of this analyze is to determine the content of Ca

and P that remains after sponges preparation. This relation is also mentioned in the literature that minerals in study are the most important elements for hard tissue regeneration activity and are direct related with calcium phosphate dissolution and less time for new tissue stimulation [9,19]. **Table 3** shows the proportion of the Ca and P in composites using complex as a base for preparations.

The presence of Ca and P in the samples describes a good relation between those materials involved, suggesting a good bone tissue regeneration activity. The cationic nature of the HAp and microcrystalline chitosan and anionic nature of the ß-TCP also responsible for high electrostatic bounding reflect in the structure, a ceramic composite formation in all preparations that also is related with solubility and viscosity of the composite in the

Table 2. Particles size of commercial HAp and ß-TCP.

POWDERS	HAp	ß-TCP
Particles size (90%), (µm)	8.81	12.78
Particles size (50%), (µm)	3.16	4.48
Particles size (10%), (µm)	0.97	1.35
Superficial area (cm^2/g)	3.41 e + 04	2.53 e + 04

Table 3. Weight proportion of Ca and P composites.

Symbol of sample (film form)	Composites	Ca (ppm)	P (ppm)
HAp powder	Initial	447,200	186,500
ß-TCP powder	Initial	425,400	199,000
MCCh	Initial	108	186
MCCh/ß-TCP complex	Initial	53600	28,200
(Sample 1)	MCCh/ß-TCP complex/glycerol (1:0.5 wt%)	42100	23,500
(Sample 2)	MCCh/ß-TCP complex/ HAp/glycerol (1:0.5:0.5 wt%)	115,000	51,700
(Sample 3)	MCCh/ß-TCP complex /HAp/glycerol (1:1:0.5 wt%)	148,000	66,400
(Sample 4)	MCCh/ß-TCP complex /HAp/glycerol (1:2:0.5 wt%)	183,000	80,800
(sample 5)	MCCh/ß-TCP complex /HAp/glycerol (1:2:0.5 wt%)	185,000	82,100
(Sample 6)	MCCh/ß-TCP complex /HAp/glycerol (1:2:0.5 wt%)	158,000	70,000

physiological environmental. The results showed a relation between Ca and P approximately 2/1 (wt%) respectively, that show a high ppm content of Ca and P in all samples.

4. Conclusions

Based on the study it should be noted:

1) The FTIR analysis of the composites showed a characteristic peak of MCCh, ß-TCP and HAp in the samples, validating also the method to obtain the complex.

2) The X-ray diffraction of the calcium phosphate powders showed a considerable amorphous halo in the commercial HAp between $10° < 2\theta < 20°$, and the ß-TCP was found to be completely amorphous.

3) The SEM analysis showed a ß-TCP and HAP grain size mainly in the range of 10 µm, average particle size of 12.78 µm and 8.81 µm. The composite sponges showed a homogenous distribution of the calcium phosphate blend in the polymer matrix with homogeneous and interconnected porous microstructure.

4) The high content of Ca and P in the composites showed a relation approximately 2/1 (wt%) respectively, and very high content of calcium and phosphate ions.

5) The study showed the feasibility of freeze drying method which resulted in three dimensional interconnected micro-porous network structure, which could be used in the future as a scaffold material.

5. Acknowledgements

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