

Research Article

Analysis of the Influence of Hydroxyapatite on the Morphology and Absorption Capacity of PHB Membranes

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Abstract

Biomaterials are substances, either natural or synthetic, capable of interacting with the biological system. The effectiveness of these biomaterials relies on their physicochemical, morphological, and mechanical properties, as well as their ability to adhere to tissue cells, promoting healing. These biomaterials can be incorporated into bioceramics such as HAp, promoting bone neof ormation. The objective was to obtain and characterize PHB and hydroxyapatite membranes with the prospect of their use as biomaterials for bone repair. This study outlines the process of preparing PHB/HAp composite membranes, including the chemical precipitation method used to obtain HAp powder and the membrane processing through solvent evaporation. The membrane morphology is analyzed using both optical and scanning electron microscopy (SEM), while the crystalline structure is evaluated through X-ray diffractometry. Water absorption tests are conducted following specific standards. Statistical analysis is carried out to interpret the results. In this study, PHB and PHB/HAp membranes were prepared using chloroform via solvent evaporation. The addition of HAp resulted in a rough and heterogeneous membrane surface. Membrane morphology was evaluated using optical microscopy, and PHB/HAp showed regular clusters, indicating the presence of HAp. X-ray diffraction analysis confirmed the crystallinity of HAp. The crystallinity of PHB membranes did not significantly change with the addition of HAp. The membranes exhibited absorption capacity in different mediums, being more absorbent in water, followed by artificial saliva and physiological solution. The PHB/HAp membrane lost more mass in physiological solution over time, while gaining more mass in water within 2 hours.

These features are crucial for biomedical applications. The results suggest that PHB/HAp membranes possess suitable morphological and structural characteristics for biomedical applications, with the potential for interactions with tissues and biological fluids.

Keywords: Biomaterial, Hydroxyapatite, Tissue Repair, Artificial Saliva.

Introduction

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Materials intended to interact with the biological system, biomaterials can be of natural or synthetic origin. These substances find applications in the fields of medicine, dentistry, tissue engineering, and pharmaceuticals due to their biocompatibility and ability to dissolve within the organism. The physicochemical, morphological, chemical, and mechanical properties of biomaterials influence their interaction with biological tissues. These properties can enhance the regeneration process¹⁻³. When applied to bone tissue, biomaterials have the capacity to replace and/or stimulate tissues that have lost their functions due to bone continuity loss. They facilitate the migration of osteoblasts or osteoclast precursor cells to the affected area, while also regulating factors that promote the recruitment of these cells to the bone. These cells are naturally present in hematopoietic tissues, such as bone marrow. Additionally, biomaterials must provide an appropriate structure that serves as a support for regenerative bone growth^{2,4-6}.

Biodegradable implants emerge as a tool capable of participating in the dynamic process of bone healing, contributing by reducing the weight of the support material, degradation of the material after a certain period, and eliminating the need for secondary surgeries for removal⁷.

In this study, polyhydroxybutyrate (PHB) was employed as the biomaterial, a natural polymer with biodegradable thermoplastic characteristics produced by bacteria from renewable carbon sources such as sugarcane. This polymer has been utilized in the manufacturing of sutures, bone prostheses, and capsules that gradually release drugs into the bloodstream due to its biocompatibility and easy absorption by the biological system. Moreover, this polymer exhibits crucial properties related to tissue healing and bone repair^{8,9}.

PHB can also be used for the production of biological membranes^{10,11} combined with Hydroxyapatite (HAp), which is one type of calcium phosphate¹². These are ceramic compounds formed by different Calcium (Ca)/Phosphate (P) ratios and are widely used as biomaterials for bone tissue replacement and regeneration applications. They are valued for their excellent

biocompatibility, bioactivity, osteoconduction, and for not eliciting toxic or allergic reactions^{3,7,13,14}.

HAp is present in the bones and teeth of all vertebrates, constituting 5% of the total weight of an adult individual. In its chemical structure, HAp is composed of three elements: Calcium (Ca), Phosphorus (P), and Oxygen (O)¹⁵ which is organized in a hexagonal crystalline structure ($a_1=a_2=a_3\neq c$ and $\alpha=\beta=90^\circ$ and $\gamma=120^\circ$)^{16,17}, with a unit cell composed of 10 calcium ions¹⁶.

The crystalline structure of HAp allows for both cationic and anionic substitutions, enabling the incorporation of a wide range of elements from the periodic table. The Ca^{2+} ions can be replaced by numerous monovalent (especially Na^+), divalent, and trivalent ions of rare earth elements (transition metals called lanthanides, including scandium and yttrium)^{17,18}. However, these possibilities seem to have a minor influence on apatites deposited in biological tissues due to the low quantities of these activating ions¹⁸.

All substitutions can lead to a structural disorganization of HAp, causing alterations in its physicochemical properties and inhibiting its normal functions. These variations can occur in crystallinity, lattice parameters, crystal dimensions, surface texture, material stability, and solubility (factors that alter degradation and in vivo behavior)¹⁸. However, in living organisms, cationic and anionic substitutions enable HAp to function as a reserve of calcium and phosphorus in a regulatory system for various ions in bodily fluids, through its release or storage. This demonstrates an increase in bone integration in orthopedic implants¹⁶⁻¹⁸.

HAp exhibits a strong affinity for proteins due to its capacity to adsorb proteins on its surface. In this context, synthetic HAp matrices have been extensively studied for their potential applications as biomaterials for implants¹⁹⁻²², additionally, synthetic HAp matrices have been explored as drug delivery systems²³. Furthermore, synthetic HAp matrices have found applications as radioactive sources for brachytherapy and tissue engineering^{24,25}. Moreover, synthetic HAp matrices serve as a support for the prolonged action of anticancer drugs in the treatment of bone tumors²⁶. Toothpaste is also added as an additional method to daily brushing in cases of early root caries lesions²⁷.

In order to optimize its biological properties, HAp can be used in the form of nanoparticles (nanoHAp)²⁸⁻³⁰, an injectable hydrogel based on polysaccharides comprising nanoHAp³¹ and 3D-printed scaffolds³²⁻³⁴. Additionally, HAp can be used to fill bone defects or voids, promoting bone regeneration and osseointegration when applied in orthopedic, dental, and maxillofacial applications³⁵.

The literature demonstrates that numerous experimental studies have been conducted to assess the response of HAp in bone tissue repair. Cullum et al.³⁶ used natural HAp in canine dental sockets and observed a significant increase in immature and highly cellular connective tissue, completely filling the defect one and two weeks after surgery. Additionally, at the margins of the original bone, there were initial activities of bone formation and resorption. No adverse reactions, such as inflammatory response or foreign body formation, were identified. Over time (four, eight, and 20 weeks), the connective tissue became denser and less cellular, promoting osteoid formation. After 30 weeks, all connective tissue was replaced by well-defined bone tissue, establishing a solid integration between the bone and HAp.

Franco³⁷, in an experiment treating tibial defects in dogs using pure synthetic HAp in combination with collagen and liposomes, observed significant bleeding during surgery in all animals that received HAp, except for those who received HAp associated with collagen. Ma et al.³⁰ developed HAp nanoparticles and incorporated them into polylactic acid (PLA) films using the solvent evaporation method. In vivo bone repair experiments conducted in a rat mandibular defect model proved that HAp/PLA films have broad applications as a biomaterial for bone repair due to their osteoinductive properties, biocompatibility, low cost, and mass production capability.

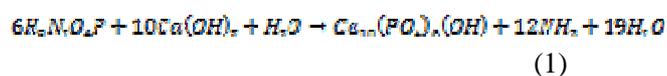
Given the presented information, the significance of the current study in obtaining and characterizing PHB membranes with HAp for potential use as a biomaterial for bone repair becomes evident. This research opens the door to numerous new possibilities in an area that holds great potential for further exploration. In this context, the objective of this study is to prepare and characterize PHB/HAp

membranes, as well as to assess their absorption capacity in different mediums, for potential applications in bone repair.

Experimental section

Membrane Preparation

The HAp powder was obtained through chemical precipitation using calcium hydroxide (Ca(OH)₂) and ammonium phosphate dibasic ((NH₄)₂HPO₄), as described in Equation 1. Initially, the reagents were dissolved in deionized water and mixed. The mixture was continuously stirred at room temperature. Subsequently, the precipitate was washed with distilled water. After this step, the suspension was dried in an oven at 100°C for 24 hours.



For the membrane processing, untreated PHB powder, supplied by PHB Industrial S/A (Serrana, SP – Brazil), was used. This polymer was derived from the fermentation of sugarcane sucrose by *Alcaligenes eutrophus* bacteria.

PHB/HAp membranes were prepared using the solvent evaporation method (solution casting). The compositions of the membranes with the PHB matrix and their respective concentrations are described in the Table.

Table 1 – Membrane Composition: PHB and HAp

Membrane	Composition (%)	
	PHB	HAp
P100	100	-
P50/H50	50	50

The preparation process involved initially immersing PHB in chloroform (CHCl₃) for 2 hours to expand its structure. Then, the mixture was heated to 55°C with constant stirring for the same period. For the P100 membrane, which contained only PHB, after returning to room temperature, the mixture was subjected to ultrasonication for 10 minutes and filtered using quantitative black stripe filter paper. The filtered liquid was then poured into

a Petri dish and left to rest under aluminum foil for 24 hours.

For the production of P50/H50 (PHB/HAp), the same preparation procedure as for P100 was followed, but with the addition of HAp before the ultrasonication step. This process resulted in the production of composite membranes, with potential applications in various fields due to the combination of PHB and HAp.

Morphological Characterization

The morphology of the membranes was analyzed using optical microscopy and scanning electron microscopy (SEM). For optical microscopy, a Hirox brand Optical Microscope was utilized, equipped with reflection and transmission capabilities, 2D accessories, and an amplification of up to 10X. This equipment was connected to an Image Analysis station, allowing detailed analysis of the membranes. Surface morphological analyses of PHB and membrane proportions were conducted through SEM using the SSX-550 Superscan SHIMADZU® microscope model. Before the analysis, the samples underwent a drying process in an oven at a temperature of 60°C for 24 hours and were then transferred to a desiccator for stabilization. To enhance sample conductivity, they were coated with a layer of gold using the SC-701 model metalizer from Sanyu Electron. The SEM equipment operated at a voltage of 15 kV, utilizing a tungsten filament. This process enabled a detailed analysis of the morphology and structure of the samples at high resolution.

Analysis of Crystalline Structure

The sample's diffractogram was obtained using a Shimadzu® XRD-6000 diffractometer, equipped with a PIXcel/3D radiation detector. For the analysis, a CuK α radiation source with a wavelength of 0.154 nm was used, operating at a voltage of 40 kV and a current of 30 mA. The scanning speed adopted was 0.014° per minute, covering 2 θ angle values ranging from 10° to 90°, with a scanning rate of 2° per minute. These settings allowed for obtaining a detailed diffractogram of the sample, which is crucial for the analysis of the crystalline structure and identification of phases present in the material.

Absorption Capacity Tests

The absorption capacity tests were conducted following the ISO 62:2008 standard and were referenced in previous studies conducted by Lima et al.³⁸, Bouchonneau et al.³⁹ and Almeida et al.⁴⁰. The objective of these tests is to measure the amount of water absorbed by polymeric matrices. The samples were prepared in 60x15mm Petri dishes and pre-dried in an oven at (50 \pm 2) °C for 24 hours, then cooled to room temperature in a desiccator. The samples were immersed in three types of solutions: deionized water, 0.9% physiological solution (Fresenius®), and artificial saliva with pH 5. Solution absorption was measured at predetermined time intervals, including 1 hour, 6 hours, 8 hours, 24 hours, 48 hours, 7 days, 14 days, 21 days, 28 days, and 35 days. After the initial immersion, the samples were placed in 300 mL of deionized water at room temperature (28 \pm 2) °C. At the specified time points, the membranes were removed from the water, excess surface water was removed with filter paper, and the sample masses were measured.

For each test, the mass absorption percentage was calculated using Equation 2:

$$A_{mass} = \frac{(m_2 - m_1)}{m_1} \times 100\% \quad (2)$$

Onde:

- m_1 it is the mass of the sample, in milligrams (mg), after drying and before immersion.
- m_2 it is the mass of the sample, in milligrams (mg), after immersion.
- A_{mass} it is the percentage of absorption for each sample.

This procedure allowed assessing the water absorption capacity of the samples under different conditions and over time.

Statistical Analysis

The results were statistically analyzed quantitatively and descriptively using Origin (Version 8.0), SPSS Statistics® (Version 20.0), and Microsoft Excel® (Version 2016), applying the Student's t-test. The tests were conducted at a

significance level of 5%, and the results were expressed as means and standard deviations.

Results and Discussion

Obtenção das Membranas

The PHB and PHB/HAp membranes were processed in analytical grade chloroform (Dinâmica®)⁴¹ due to the solubility of PHB in this solvent and its ease of evaporation at room temperature, membranes were formed using the solvent evaporation method.

To establish a comparative parameter, membranes of pure PHB (P100) and PHB/HAp (P50/H50) were

prepared. In Figure 1A, it can be observed that the pure PHB membrane has a homogeneous, non-porous surface with a slightly whitish coloration. Upon adding HAp (Figure 1B), the membrane's surface becomes heterogeneous, rough, and discontinuous, consistent with the findings of Sousa et al.⁴². Similar results were observed by Santos⁴³, who produced PHB and HAp membranes using the compression method. It was observed that the membranes had an irregular surface with the presence of unfused particles and pores distributed across the entire surface.

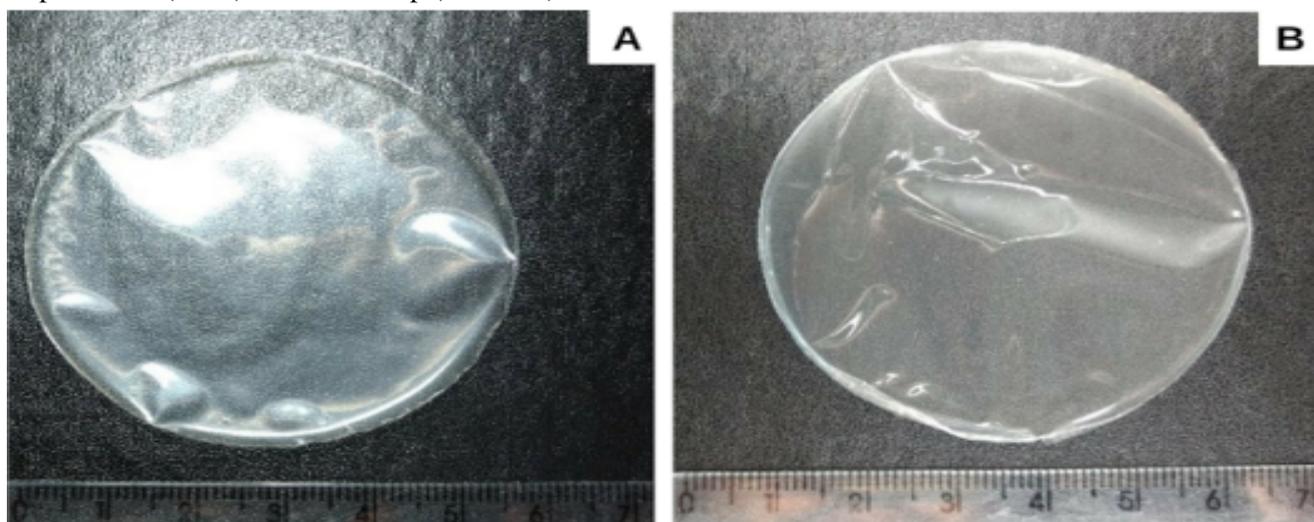


Figure 1 - PHB/HAp Membranes in Proportions: A) P100; B) P50/H50

Morphological Characterization

According to Wan et al.⁴⁴, the morphology of the biomaterial has an impact on its tissue adhesion, and the presence and size of pores can influence cell growth and subsequent proliferation. Figure 2 illustrates the optical micrographs of different

membrane proportions. In Figure 2A, a homogeneous and regular surface can be observed. In Figure 2B, clusters with more regular aspects can be visualized, possibly associated with the presence of HAp in the membrane composition.

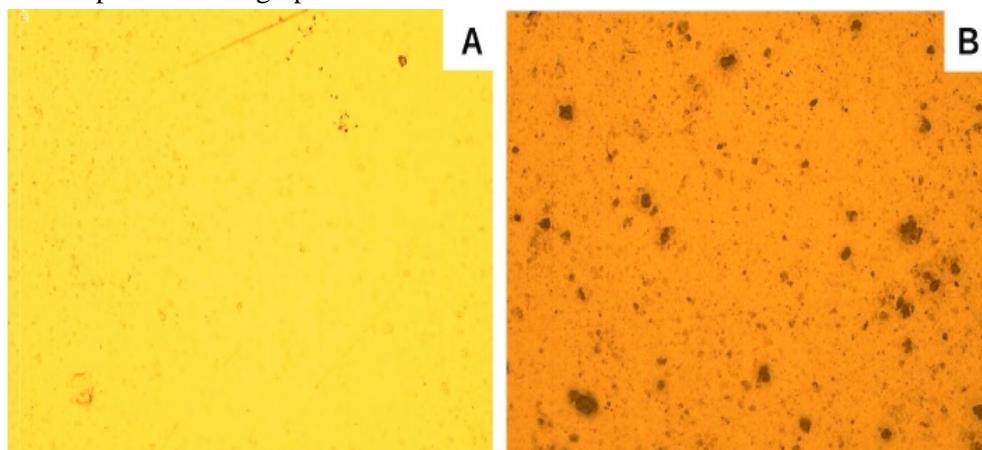


Figure 2 – Optical Microscopy at 10X Magnification of Membranes A) P100; B) P50/H50.

When analyzing the morphology of PHB powder using SEM, it is possible to observe a homogeneous and irregular structure that extends in all directions (Figure 3A). The image of pure HAp presents particles in laminar, irregular and homogeneous shapes (Figure 3B), corroborating the study by Oliveira et al.⁴⁵. The laminar structure of HAp

provides a surface area ready to interact with cells and biological fluids, which may be directly related to the biocompatibility of the biomaterial⁴⁶. It is crucial to emphasize that particle dimensions, their geometry, and surface texture are variables that influence cell adhesion and reproduction rates on the material^{47,48}.

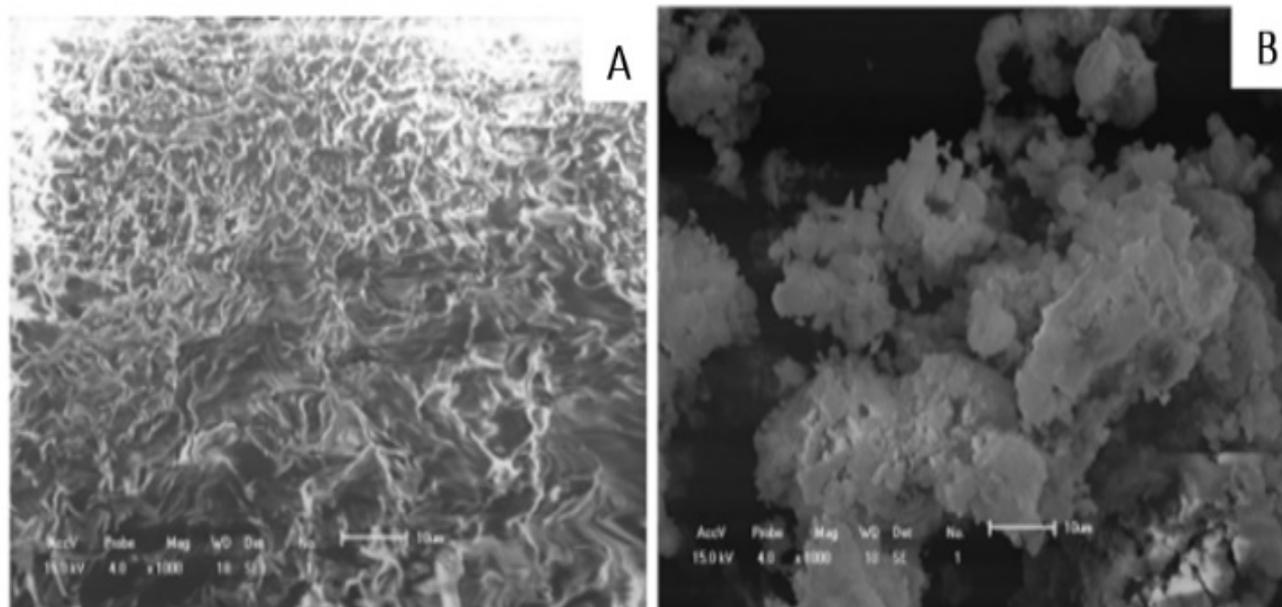


Figure 3 - Micrograph of samples: A) Pure PHB, B) HAp.

At a magnification of 2000X in the PHB/HAp membrane (Figure 4), it is possible to observe the presence of HAp clusters on the surface (indicated by arrows). The presence of these clusters on the membrane surface may enhance the adhesion, proliferation, and differentiation processes of cell types such as fibroblasts and osteoblasts, inducing cells to produce extracellular matrix components, thereby facilitating the healing process^{44,49}.

According to Anselme⁵⁰, these clusters can facilitate the presence of physicochemical bonds through ionic forces and Van der Waals forces, allowing the biomaterial to adhere to the tissue.

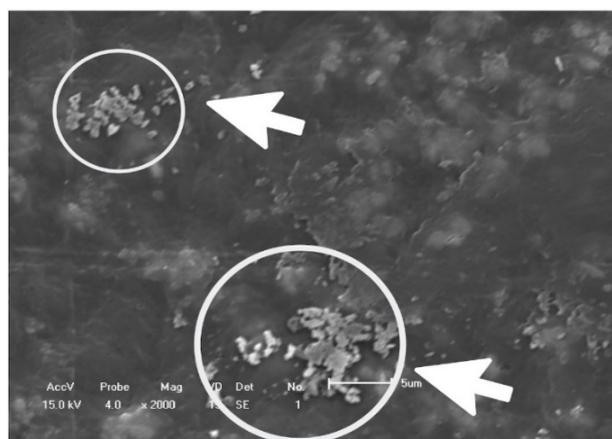


Figure 4 - Micrograph of the PHB/HAp membrane at a magnification of 2000x, highlighting the clusters of HAp on its surface.

Analysis of the crystalline structure

Crystallinity is a property that influences the dissolution rate of the biomaterial and is sensitive to the sintering temperature⁵¹. In Figure 5, the results of the HAp diffractogram are presented, showing characteristic peaks at 2θ angles of 30.25° , 33.74° , 37.40° , 39.81° , 46.67° , 49.30° , 51.47° , 54.97° ,

58.30°, 62.76°, and 76.19°. By comparing these crystallinity indices with those from the ICDD 00-001-1008 crystallographic database, it is evident that this material possesses a crystalline structure. This

finding aligns with the results obtained by Aquino⁵², whose study also identified similar crystalline peaks to those found in this research.

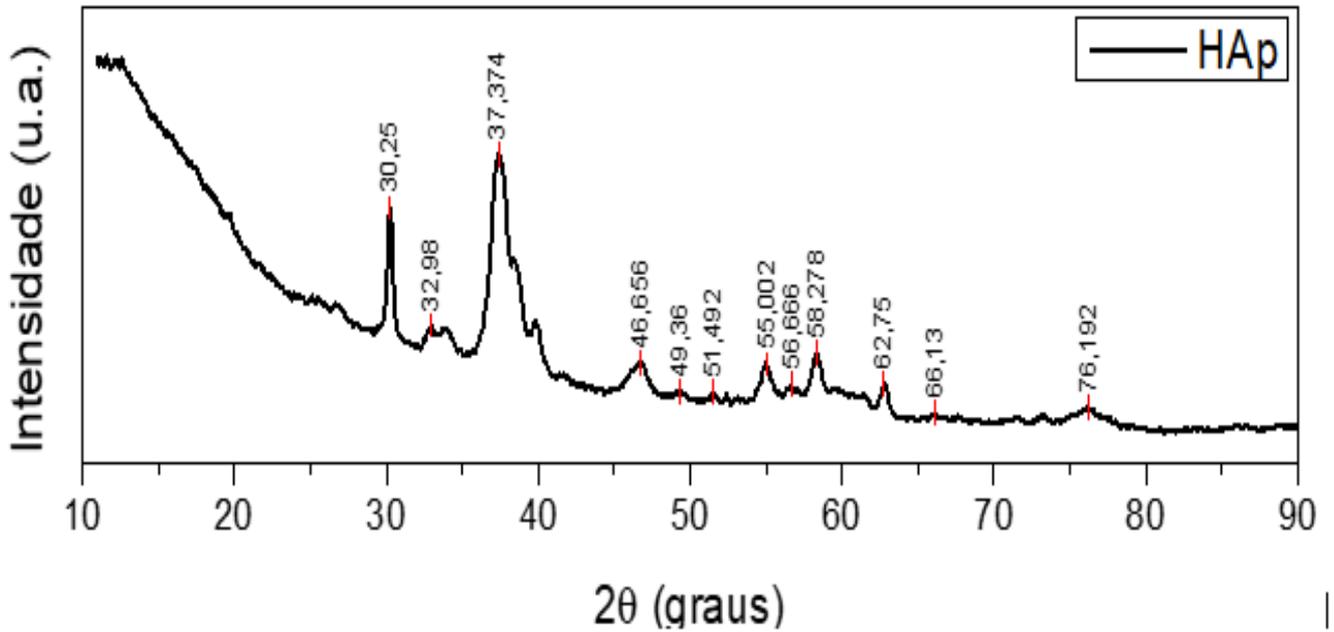


Figure 5.1 - X-ray Diffraction (XRD) Spectrum of HAp

In the analysis of crystallinity indices for the proportions of membranes represented in Figure 5, it was observed that the PHB membrane exhibited a low crystallinity index. Furthermore, upon

incorporating HAp into the membrane, the crystallinity did not undergo significant changes compared to the crystallinity of HAp in powder form.

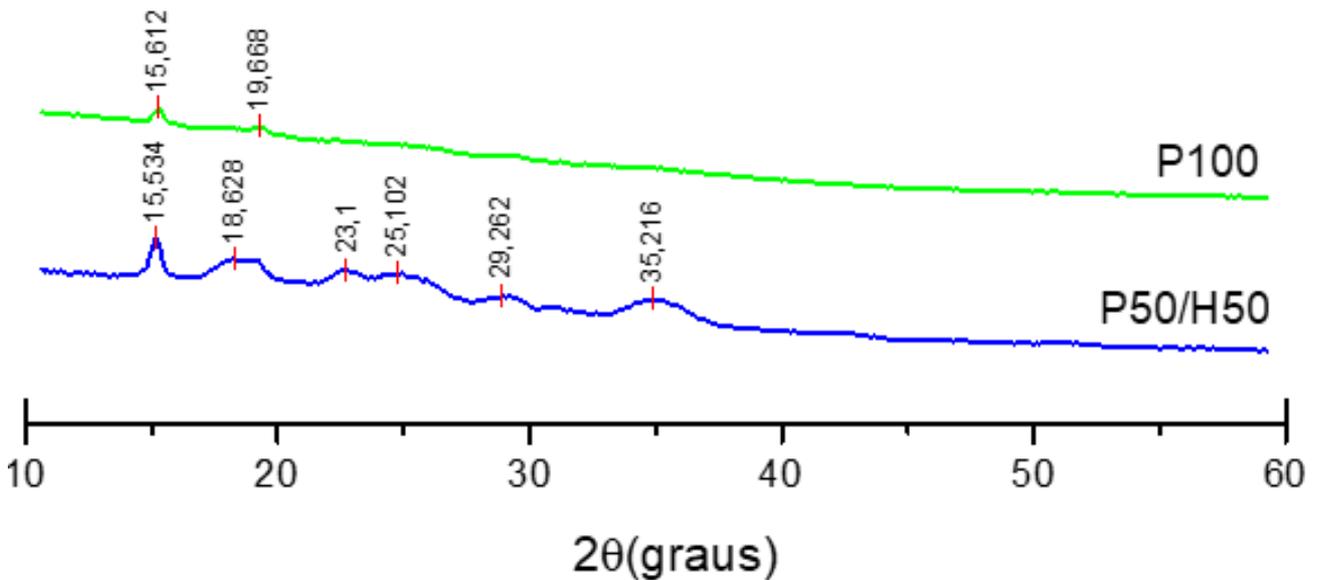


Figure 5.2 - X-ray Diffraction Spectra of PHB and PHB/HAp Membranes.

These findings are significant as the membrane was obtained at a temperature of approximately 50°C. As reported in the literature, changes in the synthesis temperature of these biomaterials also demonstrate a direct effect on their crystallinity and, consequently, on their bioabsorption⁵³. Sousa et al.⁵⁴, highlighted that factors such as restricted crystal growth, rapid cooling, and a shorter material conformation time likely influence the degree of crystallinity of the membranes.

Additionally, studies conducted by Duarte et al.⁵⁵ and Conz, Granjeiro e Soares⁵⁶ observed that crystallinity plays a fundamental role in the in vivo performance of biomaterials. Therefore, the lower the crystallinity index, the better the reabsorption and mechanical properties within the organism. It is important to note that increased crystallinity in materials can lead to modifications in their physical and chemical characteristics and tends to reduce their bioactivity⁵¹.

It is also noted that the peaks at P100 (29.28°) corroborate those found by De Sá et al.⁵⁷, who in their study identified a peak at 2θ equal to 29.57°, suggesting the incorporation of matrix constituents.

Characterization of Biological Properties - Absorption Capacity

The absorption capacity was evaluated in three different media: deionized water, 0.9% physiological saline solution (SF), and artificial saliva, in order to simulate the application conditions of the membranes. Deionized water and 0.9% SF were chosen because they are commonly used to hydrate membranes before in vivo application. Artificial saliva was selected to simulate a more complex environment, with salt concentration close to that found in the human body. This evaluation is crucial not only to replicate the original conditions of the cellular environment but also to adjust the mass transfer properties of the membranes⁵⁸.

The produced membranes demonstrated absorption capacity in different media, with higher absorption in water, followed by artificial saliva and physiological saline solution, as shown in Figure 6, respectively. The most significant mass loss occurred in PHB/HAp in physiological saline solution over time. For instance, after 30 days, the membrane lost 32.30% of its mass in this medium, which was the highest loss observed in all cases (p=0.000). The highest mass gain occurred in PHB/HAp in water over 2 hours, with an increase of 17.20% (p=0.000) in the membrane's mass.

It can be observed that there are some similarities and differences in the behaviors of the materials. Both PHB and PHB/HAp membranes demonstrated an overall mass loss in all media, but the rates and timing of this loss varied. The similarity lies in the trend of mass loss in all biological media.

The water absorption of a biomaterial intended for bone repair must be carefully considered and controlled, as this property can impact the performance and effectiveness of the biomaterial. According to Thein-Han et al.⁵⁹, this water absorption ability is a crucial factor for implantable materials, as it allows the absorption of bodily fluids and the transfer of nutrients and metabolites. A wettable surface is desirable in a biological environment as it can enhance the absorption of proteins on the surface⁶⁰. The hydrophilic nature of the surface also affects the dissolution of ion mass⁶¹.

The behavior of the membranes is subject to a margin of error due to adverse conditions in the environment where the test was conducted, such as air humidity. It is important to note that the behavior of the membranes in artificial saliva varied over time, including periods of mass loss and even a gain in mass at some point. Typically, artificial saliva seems to cause a gradual mass loss in the membranes, with fluctuations over time. After 30 days, the mass loss was 5.20%.

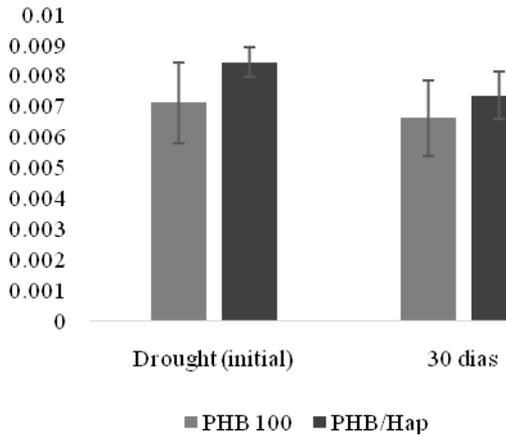


Fig.A

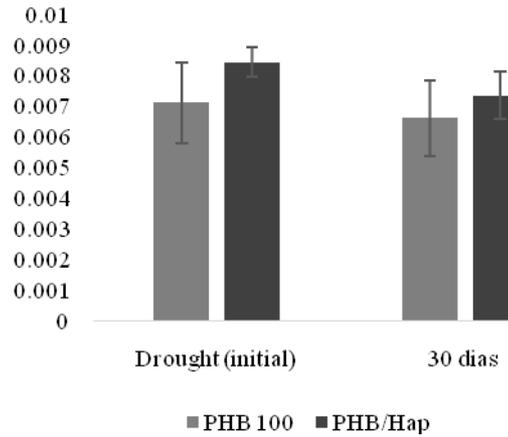


Fig.B

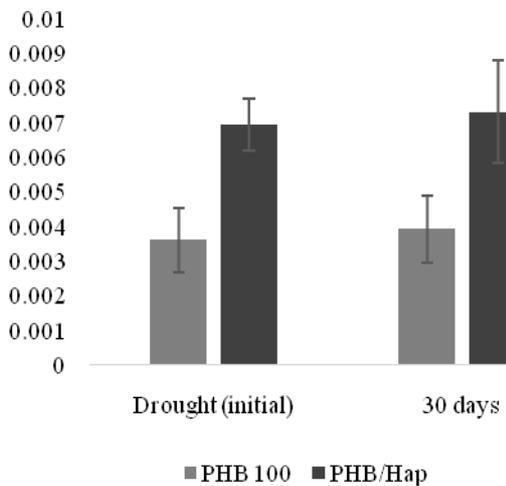


Fig.C

Figure 6 – Absorption Graphs of A) Water, B) Physiological Saline, and C) Artificial Saliva.

Conclusion

The results suggest that PHB/HAp membranes possess morphological and structural characteristics suitable for biomedical applications, with the potential for interactions with biological tissues and fluids. However, water absorption capacity varies depending on the environment, which should be taken into account when designing these membranes for specific applications. Nonetheless, the biological properties need to be determined to ensure their appropriate behavior in vivo.

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Conflict of interest: None to declare.

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