










# Combating Prosthetic Infections: Synthesis, Characterization, and Evaluation of Magnesium-Doped Hydroxyapatite Nanofibers with Antibacterial Properties

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**Abstract.** Hydroxyapatite (HAp) is one of the most important in the family of calcium phosphates since it is an inorganic component of bone and it has been proven to be a biocompatible, osteoconductive, and osteoinductive material. However, it presents low antimicrobial activity making it susceptible to infections. This work aimed first to synthesize and characterize HAp nanofibers doped with Mg nanoparticles with a growth-oriented crystalline structure and second to evaluate their antimicrobial activity. For this, we synthesized HAp nanofibers by microwave-assisted hydrothermal method and carried out the structural characterization using the X-ray powder diffraction method. The morphological, topological, and microstructural characterization was performed with scanning electron microscopy. Given the results, synthetic Mg-HAp nanofibers exhibited a hexagonal morphology and high purity and crystallinity. In addition, the nanofunctionality show of Mg-HAp exhibits good antimicrobial activity.

**Keywords:** Nanobiomaterial · Hydroxyapatite · Antimicrobial Activity

## 1 Introduction

Bone is a connective tissue formed by a mineralized extracellular matrix, which provides support to the body and protection to some organs [1]; This matrix is made up of an organic phase and an inorganic phase, which is mainly made up of CaPs in the form of HAp crystals. Factors that affect bone tissue have a significant impact on general health; and it is that when bone defects are critical, they require the addition of biomaterials with characteristics like the chemical and structural properties of the tissue for their repair

since otherwise their regeneration would not be carried out completely [2, 3]. It was decided to work with HAp due to its chemical composition very similar to the inorganic component of natural bone tissue, which provides great biocompatibility and bioactivity with surrounding tissues, facilitating the union, proliferation, and differentiation of bone cells [4, 5]. This makes it a very attractive biomaterial to use in grafts. Although HAp has these qualities, studies reveal that it has a low antibacterial property, a necessity for its clinical application [4]. To increase its physical-chemical, mechanical, and biological properties, it was decided to dope the HAp with nanoparticles of Mg, which has been reported in previous studies that Mg is a biodegradable, non-cytotoxic material that improves all the properties of the HAp and has an antimicrobial effect directly on bacteria, affecting their cell membrane [6–8].

There is limited research discussing the exact mechanism by which Mg acts against bacteria. Nevertheless, there are a few proposals that offer some clarity to the ongoing discussion. According to Robinson et al., the addition of Mg alone cannot be attributed to the antibacterial inhibition; instead, it is associated with changes in pH. In their in vitro study, they observed diminished growth of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* when reaching levels in pH of  $>9$  [14]. This phenomenon finds support in the work of Nostro et al., who establish that an alkaline pH causes a lower adherence of bacteria due to bacterial surface hydrophobicity [15]. Additionally, a high alkalinity leads to an immoderate consumption of  $H^+$ , which reduces ATP synthesis [6].

Another proposed mechanism of Magnesium revolves around the generation of reactive oxygen species (ROS), which can damage cells by disrupting bacterial proteins and DNA. This leads to reduced bacterial adhesion, subsequently reducing biofilm formation [6]. In a study by Sawai et al. the antibacterial attributes of magnesium oxide were investigated, leading to the conclusion that the ROS generation was responsible for the antibacterial effects against *E. coli* and *S. aureus* [16].

The factors mentioned above could be acting alone or together. However, it is crucial to acknowledge that most evidence has been derived from in vitro studies. Limited in vivo evidence only suggests that the behavior of the material varies under physiological conditions, opening the door to further research.

For this reason, the objective of this work was to synthesize Mg-doped HAp nanofibers with a crystalline growth orientation through MAHM, characterize the material, and evaluate its antimicrobial activity.

## 2 Materials and Methods

### 2.1 Synthesis of HAp by MAHM

HAp synthesis was performed according to Alanís-Gómez et al. [9], in a Monowave 300 (Anton Paar). The reaction mixture's temperature was  $170\text{ }^\circ\text{C}$ , with a pressure of 6–11 kPa. At the end of the reaction, a white crystalline solid was obtained, which was filtered and washed using  $H_2O$ . Finally, the dust was desiccated to  $50\text{ }^\circ\text{C}$  inside a muffle.

## 2.2 Characterization of HAp Nanofibers

*X-Ray Analysis.* The analysis was made in a diffractometer D8 Advance [Bruker]. Sample preparation was not required. In the analysis, a Cu K radiation (to a wavelength of 1.54 Å) was used. The samples were scanned in a range of  $2\theta$  from  $10^\circ$  through  $90^\circ$  with a  $0.05^\circ$  step size.

*Electron Microscopy.* The morphological analyses of HAp were carried out using a JEO-JSM-6390-Scanning Electron Microscope (Jeol USA Inc). 20 kV was used in the acceleration voltage.

*X-Ray Fluorescence Analysis.* The X-Ray Fluorescence analysis (XRF) of the samples of HAp was made in X-ray fluorescence analyzer (AR Technologies, Inc.). All samples were distributed in 1 ml of ethanol as a dispersing agent.

## 2.3 Assessment of Antimicrobial Activity

Cultures of *Escherichia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 23235) and *Candida albicans* (ATCC 96901) were grown on Mueller-Hinton (MH) agar plates. Once the colonies had grown, sample was collected and suspended in a tube with nutrient broth. A sterilized cotton swab was immersed in the resulting suspension, and a lawn of bacteria was applied to the agar plates. Covered the sample with a sterile cotton bud, and a layer of bacteria was applied to an MH agar plate.

The Mg-HAp suspension was placed on filter paper discs and applied to the *E. coli* cultures. The side containing the particles faced downwards to ensure direct interaction with the agar and the bacteria, and the samples were incubated at  $37^\circ\text{C}$  for 48 h. The inhibition zones around the filter are scarred every 24 h.

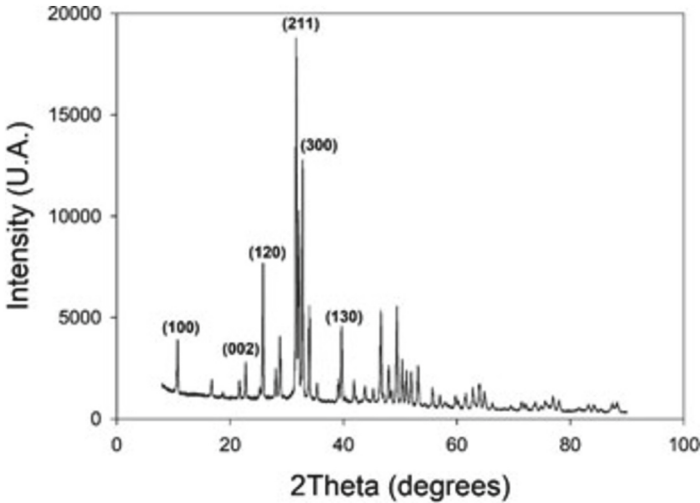
# 3 Results

## 3.1 X-ray Diffraction

For the analysis of the x-ray diffraction, our sample was compared to the powder diffraction file given by the International Center for Diffraction Data. The crystalline phases of our HAp-Mg sample corresponded to those of ICDD-PDF#86-1203. This indicates a consistency in the crystallography of the material and shows a successful synthesis method.

The X-ray diffraction image (Fig. 1) shows distinctive Bragg reflections corresponding to hydroxyapatite's crystallographic planes. We can also observe cleanliness in the pattern which demonstrates minimal noise interference.

The disposition of the Bragg reflexions are displayed as narrow bands with high intensities, which could signify high crystallinity and the presence of large crystals.



**Fig. 1.** X-ray analysis of Mg-HAp powder.

### 3.2 SEM

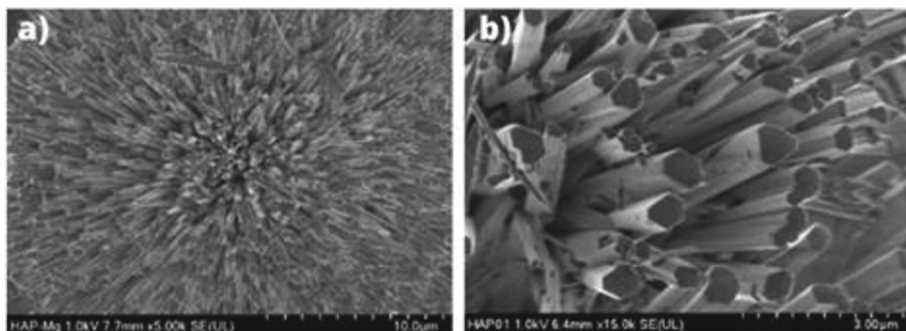
The SEM analysis captured a series of images at progressively closer magnifying intervals to examine the morphology of the fibers obtained. Figure 2a and 2b, taken at a moderate magnification, provide a general overview of the fibers' shape and arrangement. The images show a cluster of fibers that form a spherical structure.

Figure 2b, captured at a closer distance, reveals a clearer image of the fibers, providing more detail on their individual structure (15000x). However, it is until Fig. 2a with a magnification of 5000x that the hexagonal morphology of the fibers becomes evident, this being important since it improves its mechanical properties.

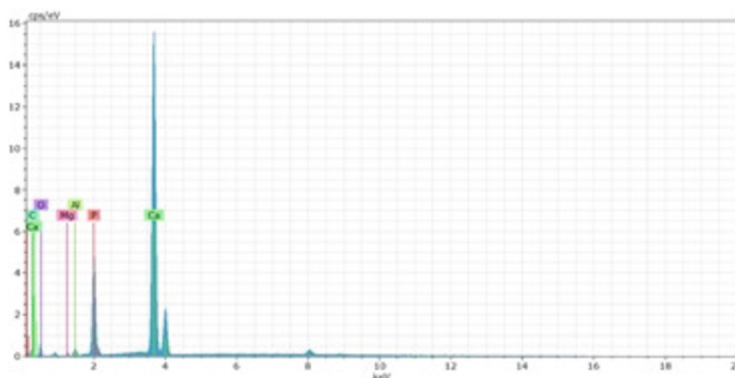
This image Fig. (2b) highlights their unique structural characteristics; attributed by the glutamic acid that adheres to phosphate groups and gives an acidic pH. This causes an intricate organized growth of the nanofibers in the c-direction, attributed by specific physical and chemical parameters used in the hydrothermal method: high temperatures, pressures and specific volume. These parameters are essential in providing an excellent medium for reaction speed and generation of smaller particles.

### 3.3 Energy Dispersive X-ray Spectroscopy

Figure 3 represents the elemental composition of our Mg-Hydroxyapatite sample, which is a crucial indicator to determine the relationship between phosphorus (P) and calcium (Ca). The most prominent peak in the Energy Dispersive X-ray Spectroscopy (EDS) spectrum corresponds to calcium (Ca) with an intensity of almost 16 (cps/eV), which indicates a significant amount of calcium within the sample. The spectrum also exhibits the presence of other elements such as phosphorus (P), magnesium (Mg), aluminum (Al), oxygen (O), and carbon (C).



**Fig. 2.** SEM micrographs of Mg-HAp showcasing the fibers morphology. a) 5000 magnification b) 15000 magnification.



**Fig. 3.** EDS analysis of Mg-HAp powder

The concentration of the different elements in our HAp sample is shown on Table 1, providing more detail on major and minor constituents. Major elements are indicated by % and minor elements by ppm.

**Table 1.** Element concentration analysis.

Compound	Concentration	Unit
MgO	1.179	%
P2O5	40.326	%
SO3	0.101	%
K2O	0.171	%
CaO	58.137	%
Fe2O3	417.9	Ppm

### 3.4 Kirby Bauer Disk Diffusion Susceptibility Test

The antimicrobial activity of magnesium doped hydroxyapatite was tested in an *in vitro* model with a HAp-Mg concentration of 100  $\mu\text{g/ml}$ /DMSO, against *Escherichia coli* (Fig. 4a), *Enterococcus faecalis*, *S. aureus* and *Candida albicans*. The diameters of inhibition of three independent experiments by each microorganism vs positive control (sensible antibiotic) are shown in Fig. 4b, in which moderate inhibition of bacterial growth can be observed (Fig. 4b).

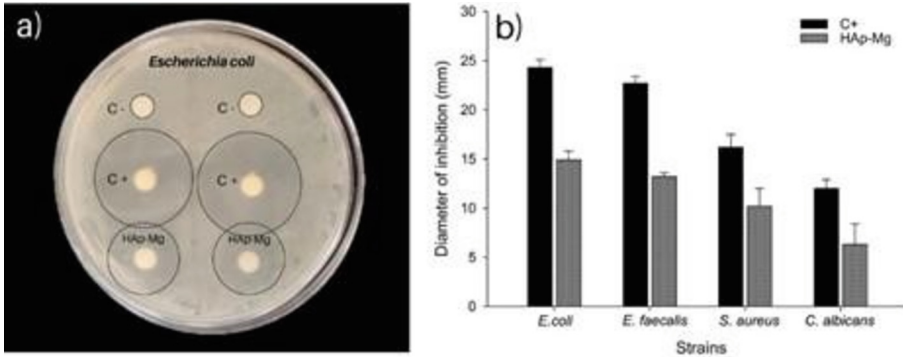


Fig. 4. HAp-Mg Antibiogram against *E. Coli* and inhibition diameters against different strains.

## 4 Analysis

Mg-HAp powder was synthesized through the microwave assisted hydrothermal method (MAHM), ensuring precision over parameters such as temperature and pressure. This method enabled us to produce nanofibers with superior crystallinity by initiating and guiding their growth. The high crystallinity in the resulting nanofibers Mg-HAp yielded a well-defined and organized structure, which led to the desired morphology [2, 11].

The powder obtained underwent X-ray analysis to provide more information about its composition and crystal structure. The crystallographic planes also allowed the identification of characteristic peaks and their corresponding Miller indices. The most intensive reflection occurred in the (211) crystallographic plane at  $2\theta = 34$  degrees, which is characteristic of hydroxyapatite. We can observe a well-defined spectrogram with narrow peaks. The presence of amino acids, as it has been mentioned in several studies, modifies the morphology and crystalline structure of HAp [12]. Therefore, the glutamic acid within the reaction functioned as a growth orientator, yielding the formation of nanofibers with strong definition and direction [2, 12].

Through SEM images we could deepen our understanding about the fibers' morphology and structure. We can highlight the formation of spherical structures made up of HAp nanofibers and intermixed with Mg particles. This structure occurs as a result of the deformations that take place on the crystal lattice when Mg ions are involved in the structure due to the substitution of  $\text{Ca}^{2+}$  ions by  $\text{Mg}^{2+}$  ions [13]. The examination of

the fibers at different scales also revealed their hexagonal nature, which suggests a well-defined crystallographic structure. An EDS was then performed to obtain an elemental analysis, which offers data to analyze the P/Ca relationship within the sample. However, it is important to notice the significantly higher peak of calcium, which means there is a stronger presence of these atoms in comparison to phosphorus atoms.

Given that the calcium peak is 15.5 cps/eV and the phosphorus peak approaches 7 cps/eV, it is possible to calculate Ca/P (15.5 cps/eV/7 cps/eV) which is approximately 2.21. The higher concentrations of elements within our HAp powder were diphosphorus pentoxide with a concentration of 40.326% and calcium oxide with a concentration of 58.137%. This Ca/P relationship results in 1.64, approaching the relationship in human bone of 1.67 [2]. The antimicrobial analysis was carried out by using HAp-Mg as the test sample, with a negative control consisting only of dimethyl sulfoxide (DMSO) and a positive control consisting of DMSO with an antibiotic, in this case ciprofloxacin 10 µg. To precisely measure the sensibility of a microorganism, the diameter of the inhibition zones was utilized as an indicator of the antimicrobial activity. The Mg-HAp presented an inhibition ring of 14 units with a concentration of 2%. The control positive also demonstrated intense antimicrobial activity while control negative didn't show any bacterial inhibition. This only indicates that our results are reliable.

## 5 Conclusion

This study focused on the synthesis and characterization of magnesium doped hydroxyapatite using controlled temperature and pressure using the hydrothermal microwave assisted method. The resulting particles showed distinctive crystallographic peaks in the X-ray diffraction analysis, demonstrating the preservation of the hydroxyapatite structure despite the incorporation of Mg.

SEM analysis revealed a hexagonal and unique morphology of Mg-HAp fibers attributed by the influence of glutamic acid, as a growth orientator in the c direction. EDS analysis reassured the elemental composition of our HAp, with notable concentrations of Ca/P, reaching similarities with human bone composition.

Through the Kirby Bauer Disk Diffusion Susceptibility Test, we observed an inhibition of bacterial growth when exposed to Mg-HAp at a 100 µg/ml/DMSO. These results point to promising advances on the exploration of Mg-HAp as an antimicrobial strategy in diverse medical and biomaterials applications.

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