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Hydroxyapatites Obtained from Different Routes and their Antimicrobial Properties

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Abstract: Among applications of ceramics in technological context, hydroxyapatite (HAp) stands out in the scientific community due to chemical biocompatibility and molecular similarity with the structures of bone and dental tissues. Such features are in addition to its antimicrobial properties. This work aimed firstly to synthesize hydroxyapatite by two different routes: hydrothermal (HD HAp) and co-precipitation (CP HAp), and secondly to verify the antimicrobial properties of these materials through direct contact tests against *Staphylococcus aureus* (SA10) and *Escherichia coli* (EC7) bacteria. These materials were characterized by XRD, Raman, and TEM. Antimicrobial tests showed inhibitory efficacy of 97.0% and 9.5% of CP HAp for SA10 and EC7, respectively. The HD HAp showed inhibitory effect of 95.0% and 0.0% for SA10 and EC7, respectively. The inhibitory effect of the tested materials against *Staphylococcus aureus* may be related to the HAp hydrophilicity.

Introduction

The concern for the well-being and quality of life of humans is of utmost importance. Advances in medicine have provided an increase in life expectancy, which has driven research aimed at the development of new biomaterials used in the repair of bone defects caused by disease or trauma [1]. Thus, the use of biomaterials in the repair of damaged parts of the bone tissue has revolutionized medicine and dentistry.

The usage of chemicals capable of facilitating repair of bone tissue has been a constant target of research, and interest in the group of calcium phosphate based biomaterials is increasing every day [2]. In this context, hydroxyapatite (HAp) has been the subject of various studies because it is biocompatible, constituted of the principal mineral phase found in bone and teeth. In addition, HAp has other important properties such as bioactivity and osteoconduction [3-6].

Many studies have searched for functional biomaterials with antimicrobial properties, which this material should possess. In addition to its functionality, it may become more effective when there is no bacteria growth. Thus, it is necessary to determine the antimicrobial properties of identical materials obtained through different procedures and routes, since most of biological studies track a single route [8]. In the antimicrobial studies, the most analyzed bacteria are *Escherichia coli* and *Staphylococcus aureus* because they belong to different classifications and are also among the most common bacteria.

This study aimed to obtain hydroxyapatite by hydrothermal and co-precipitation methods, and subsequently evaluate the hydroxyapatites antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*.

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Materials and Methods

Chemicals

Reagents without previous purification were used in all experiments: calcium hydroxide (ISOFAR) - $Ca(OH)_2$ and ammonium phosphate dibasic (VETEC) - $(NH_4)_2HPO_4$. Deionized water Milli-Q® (Millipore Corporate) was used as the solvent. All reagents were used without any previous purification or treatments to begin the synthesis.

Obtaining hydroxyapatite

To obtain HAp, an aqueous solution containing calcium hydroxide (1.6950g), ammonium phosphate (1.8126g) and MilliQ® water (40 mL) was prepared, and then it was subjected to the following synthesis protocol: hydrothermal and co-precipitation methods. In the hydrothermal method this aqueous solution was placed in a magnetic reactor of hydrothermal Teflon and was stirred for 30 minutes in a magnetic stirrer. Then, the solution was kept under heat for 2 hours. After this period, the resultant solid material was washed with MilliQ® water three times, centrifuged at 3000 rpm for 5 minutes, and dried at 80°C. Finally, the dried material resultant was crushed (HAp/HD).

For the co-precipitation method, the reactants were added to a beaker which was maintained under constant magnetic stirring at 25 °C for 24 hours. After this period, the resultant solid material was washed and dried as described above and was called HAp/CP. Materials were characterized by XRD, TEM, and Raman.

The comparison between the crystalline planes found in the obtained material (HAp) and the crystallographic data of the pure material assures the material's phase formation. Vibrations from the ionic groups that compose the material confirm which elements are present, securing the material's synthesis. The images show the morphological features of the material, such as porosity and compaction.

The X-ray's diffraction of a determined material shows peculiar and singular characteristics of a material concerning atoms' organization, so XDR is an efficient technique to identify materials. Since each ionic groups' vibrations are unique features, Raman shows vibrations on the bonds of the material's constituents, causing each specific vibration to identify the present clusters. The images of TEM show the morphological features of the material, such as porosity and compaction.

Evaluation of the Antibacterial Activity

Antibacterial activity of the materials was evaluated by direct contact in solid medium according to Zheng and Zhu (2003) [7], as described below. A bacterial suspension (10^8 cfu/mL) was prepared in saline solution (NaCl 0.85%). Then, 2000 µL of this suspension were transferred to an Eppendorf tube containing 2000 µg of the material. This mixture was stirred, and then 100 µL were seeded by the spread plate method onto plates containing nutrient agar. The plates were incubated at 37°C for 24 hours. After this period, the number of bacterial colonies on each plate was counted. As a positive control, the materials were replaced by saline solution (2000 µL). All assays were performed in triplicate, and the results were normalized by the calculation of the arithmetic average. The inhibitory effect produced by each test material was calculated according to the equation:

$$\eta = \frac{N_1 - N_2}{N_1} x\ 100\%$$

where η stands for the inhibitory effect, N_1 is the arithmetic average of the colony forming units grown on control plates, and N_2 is the arithmetic average of the colony forming units grown on test plates. Strains of *Escherichia coli* and *Staphylococcus aureus* were used in this research.

Results and Discussion

Figure 1 presents diffractograms of HAp obtained by hydrothermal (a) and co-precipitation (b) methods. The XRD patterns for the materials were indexed using the Crystallographic Data Sheet 00-001-1008, whose main peak was found at 32.05°. In both materials this plan was found at 32.13° which corresponds to the plan (211) regarding the hydroxyapatite phase [11, 12]. By comparing the XRD diffraction patterns, it is possible to verify that there were no changes in the peak's profile shown by the phases formed, which indicate that HAp was formed on two routes of synthesis.

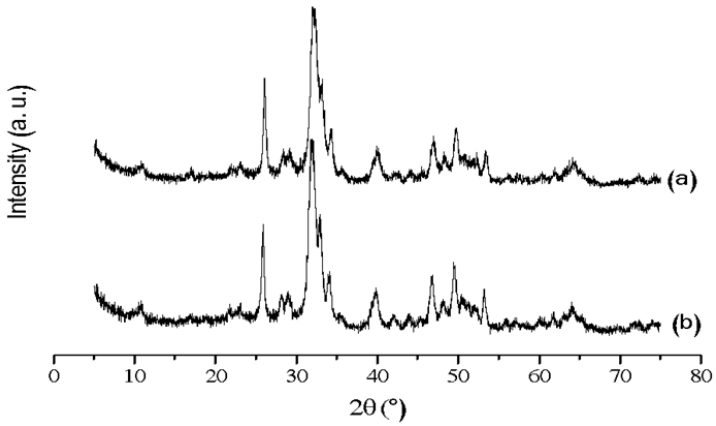

Fig. 1. XRD of HAp/HD (a) and HAp/CP (b)

The vibrational properties of the pure materials obtained by co-precipitation and hydrothermal methods were analyzed by Raman spectroscopy (Figure 2). In both spectra, it is possible to identify bands at 963 cm^{-1} related to the symmetric stretching phosphate (PO_4^-) and two bands at 592 and 429 cm^{-1} on the deformation of these phosphate groups present in the HAp [13].

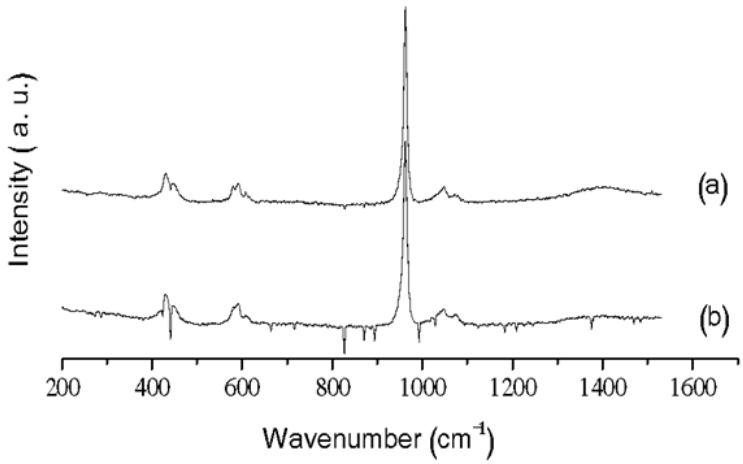

Fig. 2. Raman spectrum: HAp/HD (a) and HAp/CP (b)

Figure 3 presents the transmission electronic microscopy (TEM) of pure materials obtained by co-precipitation (Figure 3a and 3b) and hydrothermal (Figure 3c and 3d) routes.

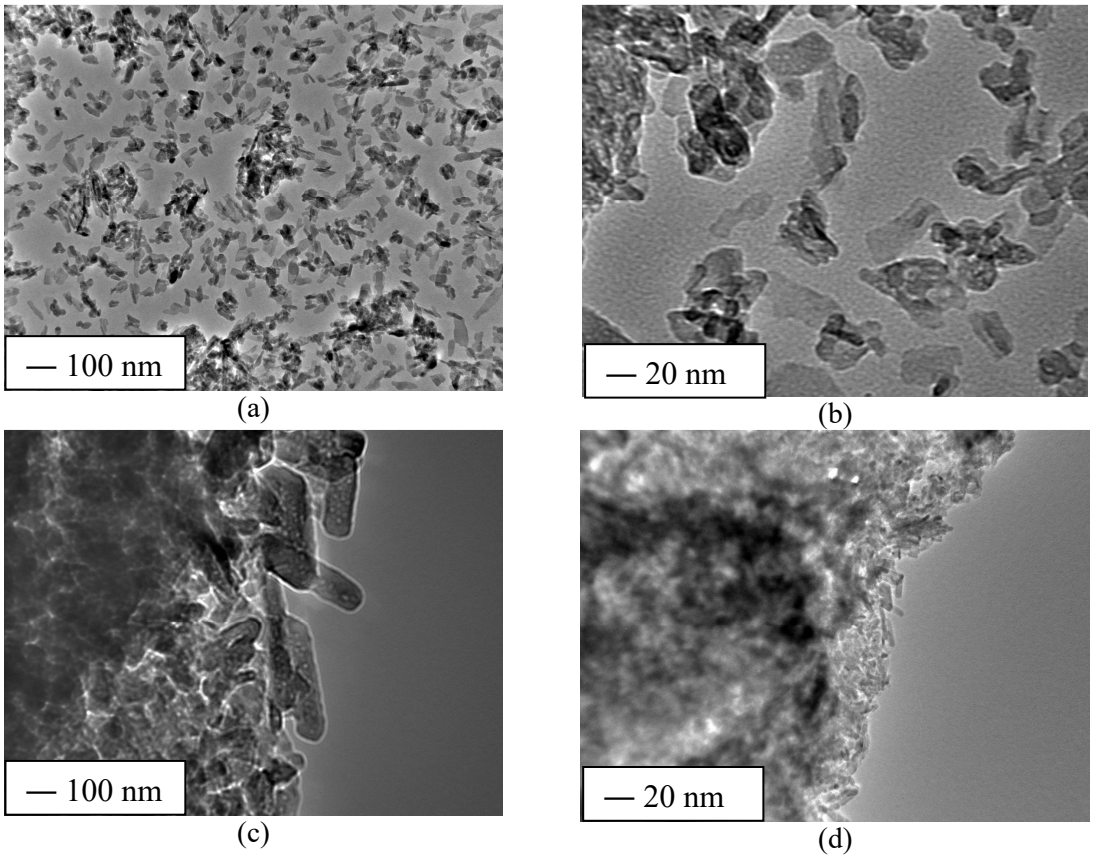

Fig. 3. TEM of materials: HAp/HD (a), HAp/HD (b), HAp/CP (c), HAp/CP (d).

Both materials have particles with varying morphology in the nanometer range. However, the material obtained by the hydrothermal route showed a greater uniformity of particles. On the other hand, the material obtained by co-precipitation presented a greater dispersion of particles, while in the hydrothermal material, the particles have agglomerated more.

Both HAp/CP and HAp/HD were able to inhibit the *S. aureus* (SA10) growth (Figure 3). For *S. aureus* strain, HAp/CP showed an inhibitory effect of 97.0%, while HAp/HD was able to inhibit 95.0% of the bacterial cells. The inhibitory effect against *S. aureus* showed by HAp/CP was greater than the inhibitory effect presented by HAp/HD, which can be related to a better dispersion of the HAp/CP particles (Figure 3).

Gram-positive bacteria as *S. aureus* have a cell wall composed by a thick layer of peptidoglycan, a polymer of carbohydrates and charged amino acids, which makes it highly hydrophilic. The antibacterial activity observed for hydroxyapatite against *S. aureus* may be due to the hydrophilic nature of this material, which is related to the presence of hydroxyl groups, which may favor the interaction with the cell wall of Gram-positive bacteria.

On the other hand, HAp/CP showed a weak inhibitory effect against *E. coli* EC7 (9.5%), and HAp/HD did not show antibacterial activity (0.0%) against this strain. Gram-negative bacteria as *E. coli* has a relatively hydrophobic outer membrane composed by amphipathic molecules as lipopolyssacarides and phospolips, which acts as a very effective barrier for spontaneous diffusion of lipophilic compounds [14]. The lack of antibacterial activity against *E. coli* strain could be related to the presence of the hydrophobic outer membrane, which could impair the interaction with hydroxyapatite [15].

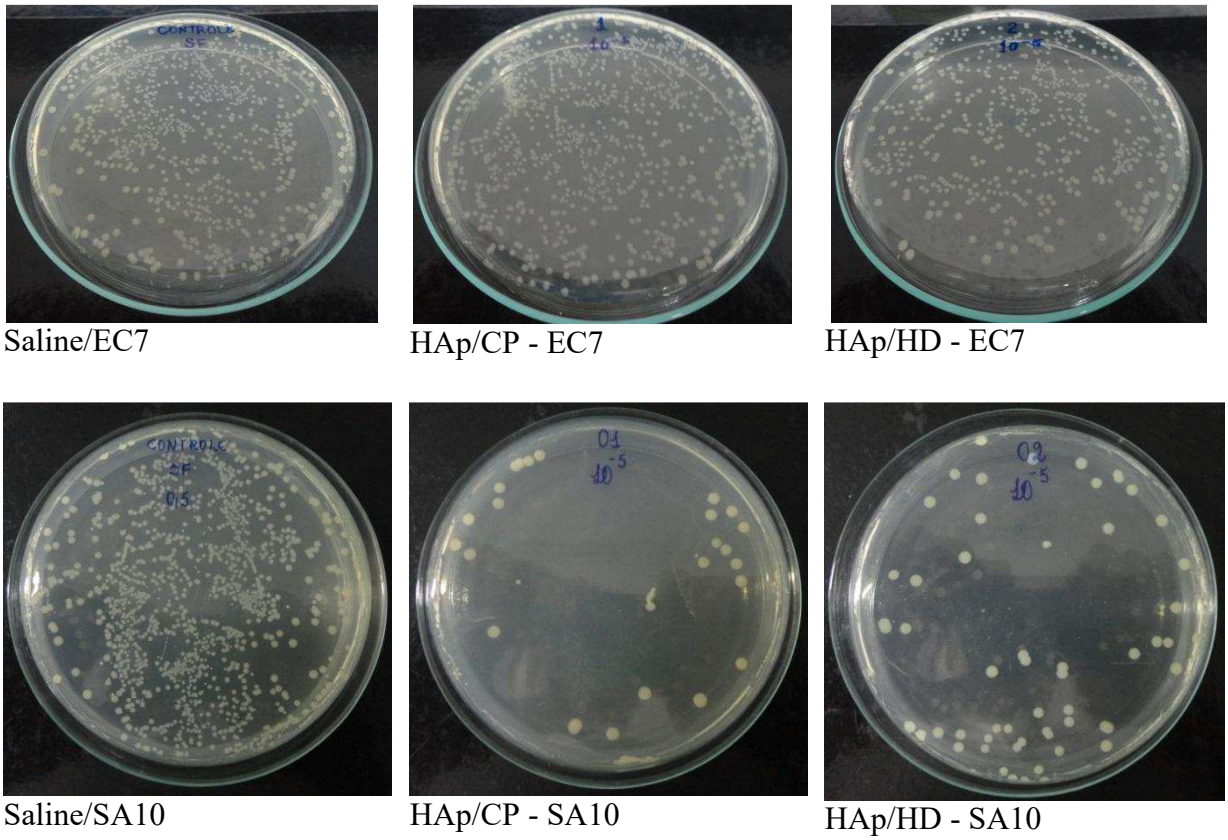

Fig. 4. Inhibitory effect of hydroxyapatites tested against EC7 and SA10 strains.

Conclusion

Both routes of synthesis used in this study were effective in the formation of hydroxyapatite. The obtained material's diffractogram showed the existence of crystalline plans presented on the crystallographic data of HAp. However, HAp/CP and HAp/HD showed differences in morphology and particle aggregation. Both HAp/CP and HAp/HD presented good activity against *S. aureus*, but did not have a good inhibitory effect against *E. coli*. Hydroxyapatite formed by co-precipitation showed better antibacterial activity, which shows promise for production of biocomposites adsorbed with antiseptic substances aimed at preventing infections associated with orthopedic and dental implants.

Acknowledgements

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