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Synthesis of antimicrobial powders for tissue engineering

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ABSTRACT- This work focuses on the synthesis and physicochemical characterization of materials based on calcium phosphates for use as biomaterials, either for bone filling or recovery of bone prostheses. The synthesis of the powder materials was performed by precipitation in aqueous solution (Synthesis wet) at ambient temperature. Bioactive molecules were incorporated into apatite crystal structure, such as fluoride ions and antibacterial agents (Cu^{2+}, Zn^{2+} ions) that accelerate the bone healing and preventing local infection around material. Many technical methods for physicochemical analysis such as X-ray diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR) and Thermal Gravimetric Analysis/Differential Thermal Analysis (TGA/DTA) are used for the characterization of materials. The results show that the final product may consist of a pure phase (Apatite of formula $(Ca, M)_{10}(PO_4)_6(OH,F)_2$, with M=$Cu^{2+}$, Zn^{2+})) or a mixture of various components in controlled proportions. The evaluation of antimicrobial effects conducted on powders is performed against pathogenic bacterial strains of Escherichia coli and Staphylococcus aureus. The results are very promising and demonstrate the improvement of antimicrobial properties of prepared material.

Keywords: Biomaterials, Fluorohydroxyapatite (FHA), infection, antimicrobial.

I.Introduction

The hard tissue, bone and teeth, are organo-ceramic composites that have a very complex microstructure. The main mineral component of bone calcium phosphate is hydroxyapatite (HA), which is part of the family of calcium orthophosphate. It is the most widely used ceramic material for the development of artificial bone or recovery of bone prostheses in relation to its good properties of biocompatibility. To get closer to the mineral part of the bone that contains many ionic substitutes, many studies have been conducted on the influence of various ions such as fluoride, strontium or groups such as carbonates or silicates.

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The aim of this work is characterized by different techniques XRD, FTIR, TGA/DTA fluorohydroxyapatite powders doped with antibacterial agents, then assess their bactericidal effect against both bacterial strains; Escherichia coli and Staphylococcus aureus.

II. Experimental procedures

II.1. Synthesis of doped powders

The phosphate apatite powders were prepared by the precipitation method in aqueous solution (wet synthesis), the solution used is a mixture of two solutions containing 0.042M $CaCl_2.2H_2O$ 0,025M and $(NH_4)2HPO_4$, dissolved in double distilled water, and then we add to the solution 0.012M NH_4F.

For doping with antimicrobials agents, the amounts of $CuCl_2.2H_2O$, and $ZnCl_2$ are dissolved in the electrolyte with the other reactants.

The concentrations of the various reagents are chosen so as to obtain a ratio (Ca+M)/P of about 1.67, M=Cu, Zn [1].

In our case (M+Ca)=0.042M [1], with [M]=100ppm [2, 3].

The gel formed is dried in an oven at 80°C and calcined at 400°C for 1hour.

II.2. Antibacterial activity's evaluation of the powders

II.2.1. Strains tested

The antimicrobial activity of the samples (powders) was tested towards two bacterial strains, Escherichia coli (E. coli, CIP 7025) at negative Gram, and Staphylococcus aureus (S. aureus CIP 7025) at positive Gram, the choice of these two strains allow to compare their reactions to synthetic powders.

II.2.2 Preparation of the bacterial suspension

From a young cultured on agar medium, a dense bacterial suspension (equivalent to 10^6UFC/ml) [2, 3] is prepared for each strain (E. coli, S. aureus) by separating 3-5 colonies in 5ml of sterile physiological saline, the suspension density is then measured using a spectrophotometer (Schimadzu UV mini 1240).

II.2.3. Antibacterial activity evaluation

The antimicrobial activity of doped powders Fluorohydroxyapatite is evaluated using the diffusion technique in Mueller Hinton agar, as recommended by the antibiogram committee of the French Society of Microbiology [2, 4].

The agar was poured into Petri dishes to form layers (about 4 mm thick). The seeding of the inoculums of microorganisms on the medium is done by swabbing technique. A swab sterile is moistened in the bacterial suspension then passed across the medium surface (3 passages at 60° offset orientation of the box and to the swab). Let dry for 10 min [1].

The powder form of pellets (about 16mm diameter and mass 0.12g) [2], are deposited on the surface of the medium of Figure-1 (4 pastilles on a petri dish) at an important distance between to avoid overlapping inhibition zones [1].

The dishes were incubated at 37 ° C for 24 hours.

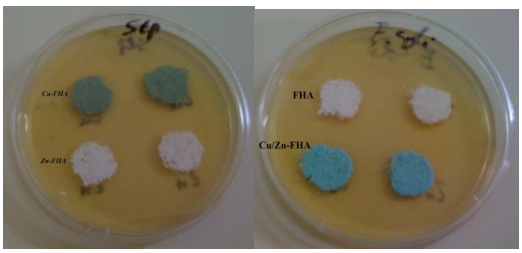

Fig 1. Deposit pellets.

The reading of the results was performed by measuring the average diameters of inhibition zones (mm) around the pellets [1,5]. Two tests were performed for each powder and take the average.

II.2.4. Determination of minimum inhibitory concentrations (MIC) of the doped powders

The Minimum Inhibitory Concentration (MIC) of synthetic powders is determined using the method of agar diffusion (Mueller Hinton). Mueller Hinton agar was supplemented with increasing concentration of a stock solution of each powder having the following end concentrations: 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128 mg/ml. A series of Petri dishes is cast for each concentration, homogenized and dried. From a bacterial suspension of 10^6 CFU/ml, 10 µl are deposited at spot on Mueller Hinton medium. The dishes were incubated at 37°C for 24h.

The minimum inhibitory concentration is the lowest concentration of apatite powder capable of inhibiting any visible growth to the naked eye.

All materials used were sterilized by autoclaving at 120°C for 20 minutes before the experiments.

III. Characterization of synthesized powders

III.1. Characterization by X-ray diffraction (XRD)

The X-ray diffraction patterns of synthetic powder are shown in Figure-2. In all fluorohydroxyapatite powders doped, the majority phase is the fluorohydroxyapatite which is well crystallized.

We have also identified the presence of other phases, Calcium Phosphate Fluoride Chloride ($Ca_5(PO_4)_3F_{.94}Cl_{.1}$) as shown in Figure-2.

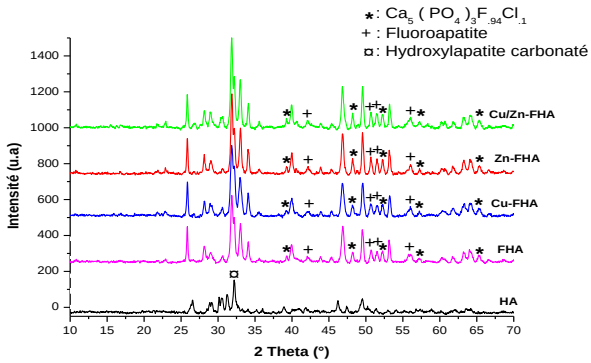

Fig 2. X-ray diffraction spectra of synthetic powders: HA, FHA, Cu-FHA, Zn-FHA, Cu/Zn-FHA.

The Cl^- ions are incorporated into the apatite; their presence develops an acidic environment on the surface of bone, which active osteoclasts cells in bone reduction. These environments lead alkaline salts of bone mineral to be solubilized. In a second time, their presence activates the action of enzymes secreted by osteoclasts that digest the organic matrix. However the greater the chlorine content, the more bones cause problems of toxicity [6].

The XRD spectrum of the HA powder, shows the appearance of the main peaks of carbonated hydroxyapatite substituted in site B ($Ca_{10}(PO_4)_3(CO_3)_3OH_2$). The carbonate ions ($CO_3^{2-}$) permit two types of substitutions within the apatite structure. They are called type B when the phosphate ions (PO_4^{3-}) are substituted and type A when it is the hydroxide ion (OH^-). Substitution at both sites generates mixed carbonate apatite type AB. The structure and chemical composition of the carbonated apatite tends to those of biological apatite [7]. Several in vitro studies have shown that the carbonates increased solubility and bioactivity of the hydroxyapatite and accelerate the formation of the apatite layer [7].

III.2. Infrared analysis (FTIR)

The infrared absorption spectra of hydroxyapatite powders and those doped with various elements, calcined at the temperature of 400°C are shown in Figure-3. These spectra's show almost the same absorption bands. The main lines are allocated to the hydroxide and phosphate groups of hydroxyapatite. The intense bands at 1100, 1025cm^{-1} corresponding to the asymmetric stretching vibration ($\nu 3\ PO_4^{3-}$) of the phosphate group , those at 950 cm^{-1} symmetric stretching mode ($\nu 1\ PO_4^{3-}$) while the strips between 560-600 cm^{-1} are attributed to the anti-symmetric deformation mode ($\nu 4\ PO_4^{3-}$). The weak lines at 472 cm^{-1} are those of the symmetric deformation mode ($\nu 2\ PO_4^{3-}$) [2, 8].

The absence of characteristic bands of OH^- ions (630 and 3560 cm^{-1}) is owing to the broad band between 3200 and 3700 cm^{-1} which corresponds to the water adsorbed on the surface of the powders as well as centered at 1635 cm^{-1}. These bands are similar to those reported by S. Kanna et al (2002) [9] and X. F. Xiao et al (2006) [10].

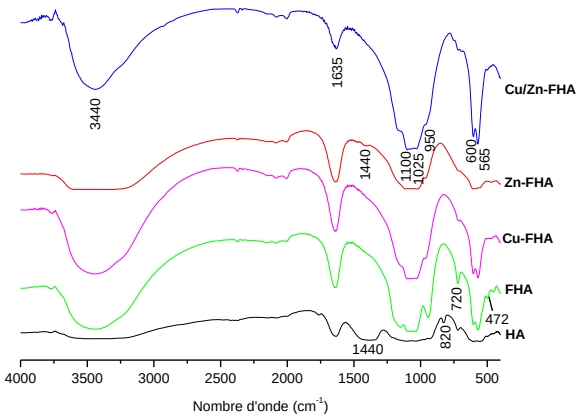

Fig 3. FT-IR spectra of different synthetic powders

Weak bands at 1440 cm^{-1} and 880 cm^{-1} are also observed on HA and Zn-FHA powders. They correspond to apatite carbonate groups [11]. MH Fathi et al (2009) [8] showed that the weak band at 720 cm^{-1} is attributed to the OH^- group, and the intensity of this band increases with the introduction of F^- ions in the apatite leading to a predominant configuration of OH-F group.

If we compare the spectra of fluorohydroxyapatite and doped flourohydroxy-apatite powders we notice that the bands of the phosphate groups in vibration mode ν1, ν2 and ν4 decrease in intensity with the incorporation of zinc ions, copper ions or the mixture (copper/zinc), corresponding the reduction of the crystallinity of fluorohydroxyapatite powder.

On the other hand, we also observe the disappearance of the characteristic bands of carbonates with the incorporation of zinc, copper or mixture (copper/zinc). The disappearance of the carbonate ion can be explained by ion-exchange between calcium ions, zinc ions and copper ions causing a change in the mesh size [12].

For all types of synthesized powders we found wider bands characteristic of poorly crystallized phases [11, 13].

III.3. Thermogravimetric analysis (TGA) and differential thermal analysis (DTA)

The evolution of mass loss (TGA) and the heat flow function of powders developed within different temperatures in an inert medium are shown in Figure-4.

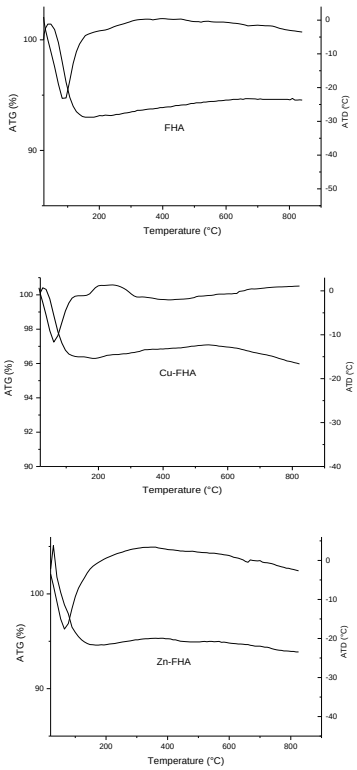

Fig 4. TGA and DTA curves of synthetic powders : (a) FHA, (b) Cu-FHA, (c) Zn-FHA.

The differential thermal analysis curves are almost identical. The profile of the curves (a, b and c) shows endothermic peaks at about 93°C for the FHA powder and at 70°C for the Cu-FHA and Zn-FHA powders that are bound to the elimination of the physiosorbed water. These are accompanied by significant loss of mass and which are in order of 8%, 4% and 12% respectively for FHA, Cu-FHA and Zn-FHA powders.

No other phenomena were observed, despite another slight decrease in mass (1%) of the Cu-FHA sample probably due to the partial volatilization of crystalline water, as already observed by F. Hanna et al (2003) [14].

IV. Effects of the antimicrobial activity

The results of antimicrobial diffusion tests on disk are shown in Table 1. and Figure-5.

Table 1. Diameter of inhibition zones of powder testeD

Strains	FHA	Cu-FHA	Zn-FHA	Cu/Zn-FHA
S. aureus	16mm/ 16mm	23mm/ 16mm	23mm/ 16mm	28mm/ 16mm
E. coli	16mm/ 16mm	19mm/ 16mm	18mm/ 16mm	20mm/ 16mm

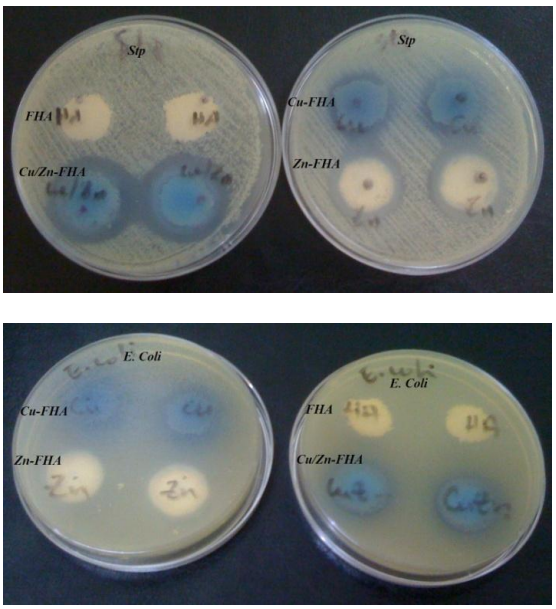

Fig. 5. Test results of the inhibition zones: (a) Towards S. aureus, (b) Towards E. coli.

The results showed that only the doped antimicrobial powders affects both strains S. aureus and E. coli, there is no inhibition zone for powder FHA. The inhibition zones were averaged 18mm/16mm and 25mm/16mm for E. coli to Gram-negative and S. aureus to Gram-positive, respectively (Table 1). These results show that the Gram positive bacteria are more sensitive with respect to Gram negative bacteria. Resistance of E. coli is due to the structure of its wall and the composition of the outer membrane [2].

Mr. Jelinek et al (2009) [15], found in their work on "Antibacterial properties of Ag-doped hydroxyapatite layers Prepared by PLD method" that the mechanism of influence of these ions on the stem depends on many factors: the speed at which ions are released, the smaller particles and nanoparticles have better results and another factor may be the crystallinity of the powder which slows the process.

The Cu^{2+} and Zn^{2+} ions are incorporated on the surface of fluorohydroxyapatite crystals, which facilitates the contact between them and the cell membrane of micro-organisms and contribute to the overall reduction in the number of viable cells in agar medium [2].

IV.1. Determination of minimum inhibitory concentrations (MIC)

The determination of the Minimum Inhibitory Concentration (MIC) is commonly used to determine the sensitivity of a strain to antibiotics [16]. The application of this technique for synthetic powders, allows to determine the threshold of bacteria resistance to antibacterial agents incorporated into the apatite powders.

The MIC of an antibiotic is defined as the lowest concentration able to prevent visible growth of the bacteria under standardized conditions [16].

In this test, the MIC will be determined in Mueller Hinton agar. The results obtained (Table 2) show variability between strains and between their responses to different powders tested.

Table 2. Results of tests mic performed on powder

Strains	Concentrations Samples	0,25	0,5	1	2	4	8	16	32	64	128
S. aureus	Cu/Zn-FHA	+	+	+	+	+	+	-	-	-	-
	Cu-FHA	+	+	+	+	+	+	+	+	-	-
	Zn-FHA	+	+	+	+	-	-	-	-	-	-
	FHA	+	+	+	+	+	+	+	+	+	+
E. coli	Cu/Zn-FHA	+	+	+	+	+	+	+	+	-	-
	Cu-FHA	+	+	+	+	+	+	+	+	+	-
	Zn-FHA	+	+	+	+	+	+	+	+	-	-
	FHA	+	+	+	+	+	+	+	+	+	+

+ : growth,- : no growth

The MIC values ranged from 0.25 mg/ml to more than 128 mg/ml (Table 3).

Table 3. Minimum inhibitory concentrations of samples (mg/ml) vis-a-vis stem «Staphylococcus Aureus» and «Escherichia Coli»

Strains	Samples	CMI (mg/ml)
S. aureus	Cu/Zn-FHA	16
	Cu-FHA	64
	Zn-FHA	4
	FHA	> 128
E. coli	Cu/Zn-FHA	64
	Cu-FHA	128
	Zn-FHA	64
	FHA	> 128

MIC FHA powder could not be determined. However, it is greater than 128 mg/ml in both strains (S. aureus and E. coli), because growth of the strain was observed at this concentration. Strains of E. coli seem to be more resistant with MIC ≥ 64 mg/ml towards the three doped powder. Unlike S. aureus seems very sensitive to the Zn-FHA powder with a MIC=4 mg/ml. Both strains are very sensitive to zinc relative to copper.

V. Conclusion

The results allowed to demonstrate that chemical modification of hydroxyapatite powders by bio-elements was possible to induce a significant decrease in bacterial adherence which depends on various parameters, among them the nature of strain; Gram negative and Gram positive.

Extensive research, directed toward a better understanding of the interaction between apatite particles doped with metal ions, and membrane proteins of micro-organisms should shed light on the mode of action of these apatite to be used as an antimicrobial material.

References

[1] Q. L. FENG, F. Z. CUI. Ag-substituted hydroxyapatite coatings with both antimicrobial effects and biocompatibility. Journal of materials science letters. 18 (1999) 559-561.

[2] V. Stanic, S. Dimitrijevic, J. Antic-Stankovic, M. Mitric, B. Jokic, I.B. Plecas, S. Raicevic. Synthesis, characterization and antimicrobial activity of copper and zinc-doped hydroxyapatite nanopowders. Applied Surface Science 256 (2010) 6083–6089.

[3] Q. Ling Feng, T. N. Kim, J. Wu, E. S. Park, J. O. Kim, D. Y. Lim, F. Z. Cui. Antibacterial effects of Ag-HAp thin films on alumina substrates. Thin Solid Films. 335 (1998) 214-219.

[4] R. Moreda. Antibiogramme par la méthode de diffusion en milieu gélosé. D'après l'édition

janvier 2007 du comité de l'antibiogramme de la société française de microbiologie. Lycée Docteur Lacroix – Narbonne. (2009).

[5] L. Yingguang. Y. Zhuoru. C.Jiang. Prepamtion, Chaiactelization and Antibacterial Property of Cerium Substituted Hydmxyapatite Nanopatticles. Journal of Rare Earths 25 (2007) 452–456.

[6] M. PALARD. Synthèse et frittage d'hydroxyapatites phosphocalciques silicatées. Ph.D. Univ Limoges, 2007.

[7] E. DELVAT. Contribution au développement de nouveaux alliages biocompatibles à base de titane. Ph.D. Univ Rennes 2006.

[8] M.H. Fathi, E. Mohammadi Zahrani. Mechanical alloying synthesis and bioactivity evaluation of nanocrystalline fluoridated hydroxyapatite. Journal of Crystal Growth 311 (2009) 1392–1403.

[9] S. Kannan, A. Balamurugan. S. Rajeswari. Development of calcium phosphate coatings on type 316L SS and ther in vitro response. Trends Biomater. Atrif. Organs. 16 (2002) 8-11.

[10] X.F. Xiao, R.F. Liu, Y.Z. Zheng. Characterization of hydroxyapatite/titania composite coatings codeposited by a hydrothermal–electrochemical method on titanium. Surface & Coatings Technology. 200 (2006) 4406–4413.

[11] R-N. BORŞA. Elaboration de poudres et de dépôts de phosphates de calcium silicatés à usage de biomatériaux. Ph.D. Univ Toulouse 2008.

[12] Jidong Li, Yubao Li, Li Zhang, Yi Zuo. Composition of calcium deficient Na-containing carbonate hydroxyapatite modified with Cu(II) and Zn(II) ions. Applied Surface Science. 254 (2008) 2844–2850.

[13] E. BOUYER. Étude de la préparation de poudres et de dépôts à partir de suspension par plasma inductif. Le cas de l'hydroxyapatlte phosphocalcique. Ph.D. Univ. Sherbrooke, CANADA 1997.

[14] F. Hanna. Z.A. Hamid. Electrodeposition of biomedical hydroxyapatite coatings on titanium alloy substarte. Pigment & Resin Technology. 32 (2003) 319-325.

[15] M. Jelínek, T. Kocourek, K. Jurek. Antibacterial properties of Ag-doped hydroxyapatite layers prepared by PLD method. Materials Science & Processing. 10 (2009) 00339-010-5911.

[16] G. UNTEREINER. Etude de l'impact de bactéries environnementales sur la spéciation de l'uranium en vue de processus de bioremediation. Ph.D. Institut des Sciences et Industries du Vivant et de l'Environnement (Agro Paris Tech) 2008.