PRELIMINARY STRUCTURAL ULTRASONIC CHARACTERIZATION OF HYDROXYAPATITE

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Synthetic hydroxyapatite (HAp) is one of the most commonly used material for a large variety of biomedical applications. This paper presents structural, morphological and preliminary ultrasonic characterizations of the HAp nanopowder synthesized by an adapted co-precipitation method. To this end, the structural and morphological properties were evaluated by Scanning Electron Microscopy (SEM) and Energy Dispersive X-Ray Spectroscopy (EDS). Biological investigation were also performed in order to evidence the influence of HAp tablets on the development and growth of endothelial cells. The ultrasonic measurements obtained for the HAp dispersion allowed us to calculate the ultrasonic velocity and the attenuation. The study of ultrasonic waves propagation through dispersions could be used as future standard quality test, thus improving the quality of biomedical devices based on hydroxyapatite.

1. Introduction

In the last 50 years, a special attention has been given to ceramic materials used as biomaterials for a number of applications in the biomedical engineering field [1]. The need for creating new and enhanced materials for improving the quality of medical implants arose from the increasing number of the elderly population worldwide. Their susceptibility to accidents which often result in severe bone injuries has been an increasing concern among medics and researchers alike. In this context, the goal of many researches was to create more durable implants, thus improving the quality of life of patients suffering painful orthopedic surgeries [1]. Hydroxyapatite (HAp) is one of the most commonly used bioceramics in many medical applications. It is naturally found in the human biological system, being the main inorganic constituent of bones and teeth [2,3]. Due to its similarity with the natural mineral component of human hard tissue, synthetic HAp, having the chemical formula $Ca_{10}(PO_4)_6(OH)_2$, has been used in a series of biomedical applications as bone filler material [2,5-7], in bone tissue engineering scaffolds [2,8], as bioactive coating [2,5], or for soft tissue repairs [2,9-12]. Synthetic HAp has also been used for drug, proteins or gene loading and delivery systems [2,13-19] and in column chromatography for rapid fractionation of biomolecules [2,20-21]. The widespread applicability of synthetic hydroxyapatite

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is due to a series of remarkable properties, having an excellent biocompatibility with the human body and a very good osteoconductivity [2,8]. Given the fact that HAp is an important component of the modern medical field, it is important to also find new characterization methods which could provide vital information on the porosity and density of the material in order to assess its quality. In this way, only high quality samples would be used in clinical applications. Furthermore, considering that testing time is essential, fast and efficient characterization techniques are always preferable. In this context, a great interest has been directed to ultrasonic characterization of materials. This technique is already being used in medicine for a number of purposes such as ultrasonic imaging or ultrasonic evaluation of bones [22]. The great advantage of ultrasonic characterization is the potential to highlight material, mechanical and structural properties [22]. Moreover, another advantage of this type of technique is its nondestructive nature, the use of ionizing radiation being thus excluded [22].

The main goal of this study is to present a new method for characterizing hydroxyapatite dispersions through ultrasonic evaluation. On the other hand, in this paper we present for the first time the influence HAp nanopawders synthesized by co-precipitation method on the development and growth of endothelial cells.

2. Materials and methods

2.1 Samples

For the synthesis of the hydroxyapatite powder, ammonium dihydrogen phosphate [$(NH_4)_2HPO_4$] and calcium nitrate [$Ca(NO_3)_2 \cdot 4H_2O$] were used as reagents. They were purchased from Sigma-Aldrich and were used without further purification.

The $Ca_{10}(PO_4)_6(OH)_2$ ceramic powder was prepared using an adapted co-precipitation method, maintaining the molar ratio Ca:P=1:67 [23]. Two preliminary solutions were obtained by dissolving predetermined amounts of $Ca(NO_3)_2 \cdot 4H_2O$ and $(NH_4)_2HPO_4$ in deionized water. After continuous magnetic stirring for 2 h, the P containing solution was added drop by drop into the Ca containing solution and the new obtained mixture was magnetically stirred for 2 h at a temperature of 100^oC. During the reaction the pH was constantly adjusted and kept at 10. Subsequently, the deposited mixture was washed with deionized water several times. Finally, the obtained material (HAp) was dried in an electric oven for 72 h at a temperature of $100\ ^o$C.

2.2 Sample characterization
Scanning Electron Microscopy (SEM).

The morphology of the sample was evaluated by SEM, using a a HITACHI S2600N-type scanning electron microscope (SEM), operating at 25 kV in vacuum. The microscope was also equipped with an energy dispersive X-ray attachment (EDAX/2001 device). This secondary device was useful for the identification of the elemental composition of the sample.

Ultrasonic evaluation.

In order to evaluate the ultrasonic profile of the hydroxyapatite nanoparticles, specialized equipment was used. The ultrasonic transducer used for the measurements presented in this paper was a H5K model (produced by General-Electric, Krautkramer) with 5 MHz central frequency and a very short burst. For the acquisition of the ultrasonic signals, a Tektronix DPO 4014B oscilloscope was used (Figure. 1)
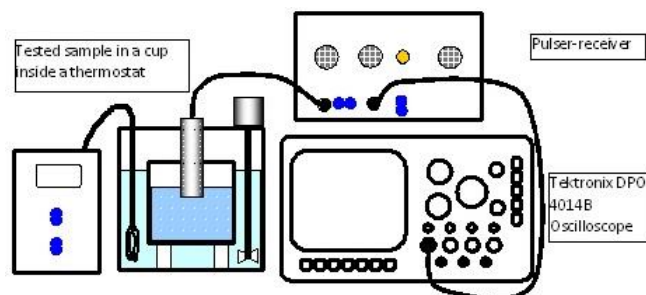
Fig. 1 The experimental setup

Water is frequently used as medium for nanoparticles dispersions. For ultrasonic pulse velocity measurements, all parameters affecting the velocity must be accurately determined. The water density is influenced by its temperature. It can be considered with good approximation, a linear velocity variation with temperature of 3 m/(s.K) around a temperature of 20°C. It can be thus expected that a 0.1°C error in temperature measurement leads to maximum 0.3 m/s error in pure water velocity, which is the reference fluid.

The calibration of the experimental setup was performed with double-distilled water at a temperature measured with an accuracy of 0.1°C. The fluid quantity was identical for the analyzed suspensions and the distilled water which is the reference. For a stabilized temperature valid for all the studied suspensions (23.3°C), the parameters of pure water are: the velocity of ultrasounds c=1492.07 m/s, the density of double-distilled water ρ=997.47 kg/m3, the adiabatic compressibility coefficient 4.5032e-010 Pa^{-1} and the attenuation coefficient 0.558845 neper/m. All measurements were performed at a central frequency f=5MHz.

The measured values of the densities: 999.7 kg/m^3 for double-distilled water and 11012.9 kg/m^3 for the hydroxyapatite dispersion were accurately determined with specialized equipment. The slight difference between the standard (997.47 kg/m^3) and the experimental (999.7 kg/m^3) values for water density was noticed. This small difference could be explained by small errors in temperature measurements and the temperature used for the standard value, the presence of dissolved air or less probable, the water purity.

2.3 Cell cultures

The biological material used in our experiments was comprised human endothelial cells, EAhy 926 cell line (Edgell C, 1983) grown in a culture medium DMEM with 4,5 ‰ glucose to which 10% fetal bovine serum and 2% antibiotic mixture (penicillin, neomycin and streptomycin) were added. The endothelial cells (0.85 x 105 cells/ml) were grown for 72 hours at 37°C and 5% CO_2 on tablets comprised of hydroxyapatite (HAp). After 3 days, the adhered cells were washed with phosphate buffered solution (PBS) and were processed for cell viability evaluation using LIVE/DEAD Viability/Cytotoxicity assay Kit (Lonza Walkersville, Inc). To another set of tablets comprised of HAp, assays to determine pH values were performed at 24 h and 48 h in DMEM culture medium with 4,5 ‰ glucose, without serum. Afterwards, these tablets were seeded with endothelial cells and the cell viability was determined after 6 days in the culture medium.

2.4 Cell viability

Living cells are distinguished by the presence of intracellular esterase activity, determined by enzymatic conversion of non-fluorescent AM calcein into highly fluorescent calcein. The polyanionic pigment – calcein is well retained in living cells, inducing an intense, green fluorescence, uniform in living cells (excitation/emission: 485 nm/530 nm). Dead cells were highlighted by staining them with ethidium homodimer (EthD-1). The pigment penetrates cells with destroyed cell membrane, leading to a 40-fold increase of the fluorescence after binding to nucleic acids, resulting in a red fluorescence of the dead cells (excitaion/emission: 530 nm/645 nm). The EthD-1 pigment is expelled by the plasma membrane of the living cells, which is intact. In order to be examined with a fluorescence microscope, the cells were incubated with a

fluorochromed mixture (1μM AM calcein, 2 μM EthD-1) for 45 min, fixed with 4% paraformaldehyde (PFA) in PBS 0.01M and examined with a fluorescence microscope. As reference, cells were grown in the absence of HAp tablet. Some of them were stained with AM calcein in order to mark the living cells, while others were treated with 70% ethanol for 30 min for membrane permeabilizaition and stained afterwards with EthD-1 in order to mark the dead cells.

3. Results and discussions

3.1 Morphology of the hydroxyapatite nano-powder

The morphology of the hydroxyapatite nano-powder was investigated by scanning electron microscopy. Fig. 2 presents the SEM image of hydroxyapatite. The obtained HAp nanoparticles have an acicular morphology and appear to agglomerate due to their much reduced dimensions.
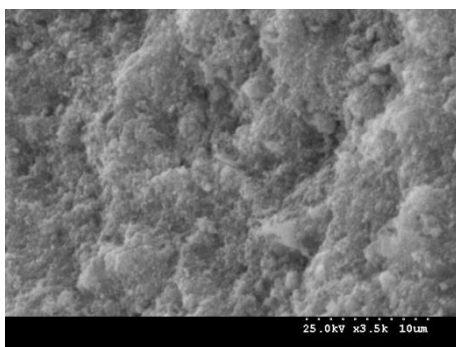

Fig. 2. SEM micrograph of hydroxyapatite (HAp) powder.

In Fig. 3 is illustrated the EDX spectrum of the HAp ceramic powder. It can be clearly observed that the main constituent elements of the studied sample are calcium (Ca), phosphorus (P) and oxygen (O). Furthermore, in Fig. 4 is presented the elemental mapping of the HAp sample. It can be observed that Ca and P are uniformly distributed throughout the entire sample. Also, the results suggest that the studied powder is homogeneous.
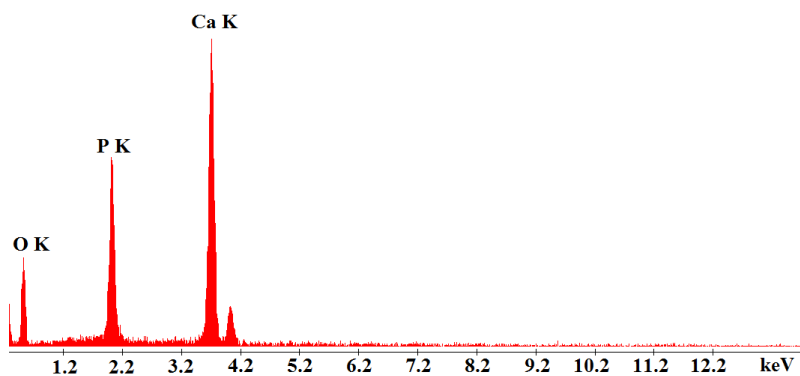

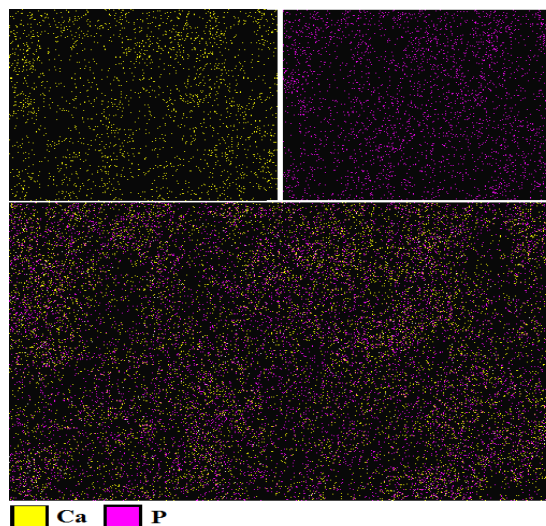*Fig. 3. EDX spectrum of the HAp nanopowder.*

Fig. 4. Elemental mapping of HAp sample.

Therefore, it can be ascertained that the hydroxyapatite characteristic components are found in the analyzed sample, and there were not present any other impurities.

3.2 Ultrasonic characterization of hydroxyapatite nanoparticles

Characterization of dispersions of nanoparticles by ultrasonic measurements has started to attract more and more interest from researchers worldwide. Although this is only the beginning of its development, the advantages of this type of technique are very appealing [24]. The time intervals between successive echoes in distilled water allow determining with good accuracy the distance between the transducer and the flat bottom of the aluminum cup used for all these measurements. The mentioned standard ultrasound velocity at the measured temperature was used for this purpose.

We have developed a specialized computer program for signals analysis, which can determine the time differences between echoes obtained in any fluid, with an accuracy of 1ns (Fig. 5). The small signals before the second and third echoes represent signals of multiple reflections on the flat bottom of the aluminum cup used to hold the tested fluids. These signals are ignored in the algorithm.

The algorithm is based on inter-correlation of the absolute values of the compared signals. After the analysis of signals obtained for double distilled water, the distance between the transducer face and the flat bottom of the measuring cup was computed: 47.91 mm. The water temperature during this measurement was 21°C, corresponding to a standard wave velocity of 1492.07 m/s.
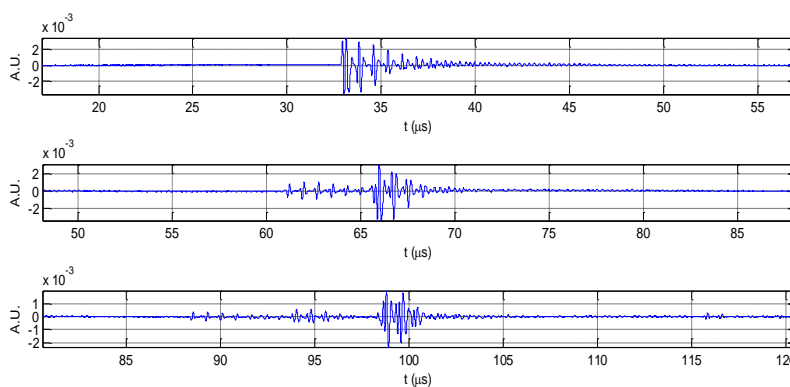

Fig. 5 The first three echoes acquired for double-distilled water

In Fig. 6 are presented the signals acquired for the hydroxyapatite dispersion with its first three echoes.

Taking all these parameters into account, the time differences between each of the three echoes obtained for double-distilled water and those of hydroxyapatite dispersion were obtained. Therefore, in the case of the first echoes of water and respectively hydroxyapatite dispersion, the time difference was 1.67 μs. For the second echoes, the time difference was 3.255 μs, corresponding to an absolute time difference of 1.628 μs. In a similar manner for the third echo, the time difference was 5.17 μs, thus establishing a total time difference of 1.723 μs.

From these time differences and the computed distance traveled by the signals, can be obtained the ultrasound velocity through the hydroxyapatite dispersion: 1454.17 +/- 1.06 m/s and an attenuation of 9.11055 Np/m, (1.04889 dB/m). The higher attenuation led to the disappearance of the weak signals produced by reflections in the cup bottom, which were detected for pure water (Fig. 5).
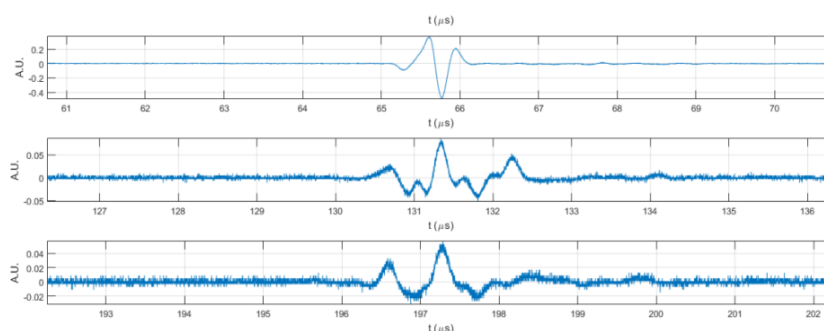

Fig. 6 The first three echoes acquired for hydroxyapatite
nanoparticles dispersed in double-distilled water

The preliminary results presented in this paper suggest that ultrasonic characterization of dispersions could be an essential tool in fast and accurate determination of physical material parameters. Considering that there are just a few experimental results presented in literature, it is important to continue the experimental study, for the practical application of this characterization technique.

3.3 Biological studies

For the first experiment (carried out on tablets unwashed with culture medium), after being cultured for three days, the viability of endothelial cells grown on tablets comprised of hydroxyapatite was analyzed. During the three days, it was observed that the tablets had a high cytotoxic effect on the culture cells compared to the reference. The culture medium was acidified even if it was changed two times (every 24 hours) during the three days of culture. The fluorescence microscopy images highlight the growth of a very small number of cells on tablets comprised of HAp, as well as the absolute death rate of the cells after they were stained with EthD-1 (Fig. 7). In addition, the HAp tablets exhibited a strong autofluorescence in the calcein field, inducing interferences in the observation of viable cells.
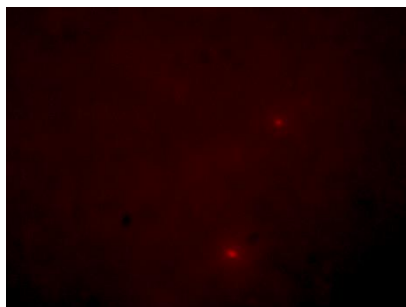
*Fig. 7. Image of endothelial cells grown on tablets comprised of HAp,
stained with EthD-1 (dead cells)*

By comparison, the endothelial cells grown as reference were at the stage of confluence, having a high viability highlighted by AM calcein staining (Fig. 8a). Staining cells treated with 70% ethanol with EthD-1 (for fixation and permeabilization of the membranes) evidences the total obliteration of the cells (Fig. 8b).
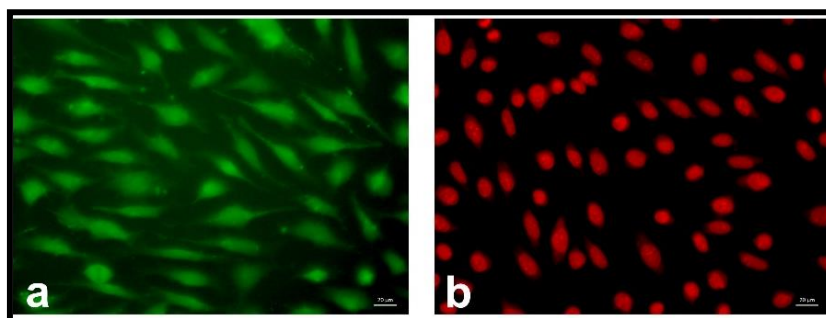

*Fig. 8. Image of endothelial cells grown as reference stained with AM calcein
for highlighting living cells (**a**) and with EthD-1 for highlighting dead cells (**b**)*

For a second set of tablets comprised of HAp, assays were performed to determine the pH values after 24 h and 48 h respectively in DMEM culture medium with 4.5 ‰ glucose without serum. The medium was harvested and replaced at 24 and 48 hours respectively. Afterwards, the same tablets were seeded with endothelial cells and cell viability was highlighted after being cultured for six days. Finally, after 8 days incubation in the culture medium of the tablets (and after the cells were cultured for six more days), the medium was harvested and the pH value was determined. It was observed a slight alkalinisation of the medium after 48 hours and 8 days respectively compared to the pH values acquired after 24 hours.

The cells were processed in order to determine the cell viability by marking the living cells (staining with EthD-1). After the cells were stained, they were mounted between two slides using a mounting medium containing DAPI (4',6-diamidino-2-phenylindole) and a specific pigment for nucleic acids which evidenced the nuclei of endothelial cells. These nuclei belong to both living and dead cells (arrow, Fig. 9). A slight growth of the number of endothelial cells that populate the HAp tablets could be observed.

*Fig. 9. Highlighting by DAPI staining the nuclei of endothelial cells
that populate the HAp and Zn:HAp tablets*

The fluorescence microscopy images showed a slight decrease in the number of dead cells (arrows, Fig. 10). Due to the strong autofluorescence, the living cells could not be distinguished. DAPI staining revealed the total number of cells (dead and alive) detected on the tablets seeded with cells. The qualitative difference between the DAPI staining (revealing all the cells) and the EthD-1 staining (revealing dead cells) highlighted a relatively small number of viable cells present on the HAp tablets.
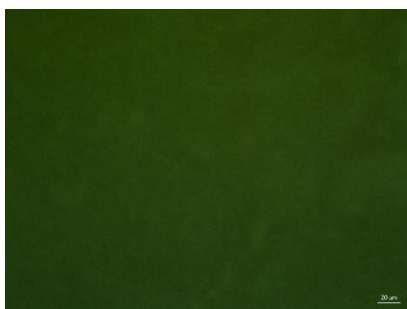

Fig. 10. Dead endothelial cells on HAp tablets highlighted by AM and EthD-1 staining

The viability results obtained for the HAp tablets were compared with the results obtained for the cells grown as reference. After 8 days in the culture medium, the endothelial cells reached the preconfluence stage, the number of cells being adequate to cover almost the entire surface of the slide (Fig. 11). By staining with AM calcein and EthD-1 we could observe living (green) and dead (red) cells (Fig. 11a) as well as dead cells previously treated with 70% ethanol (Fig. 11b). These results show the low density of cells on the HAp tablets compared with the density of cells grown as reference.
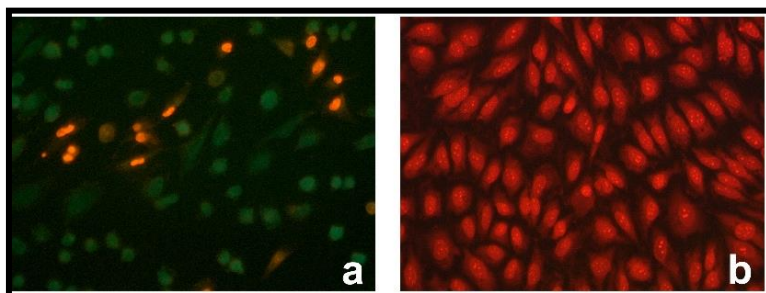

*Fig. 11. Endothelial cells grown on glass slides (a) – living cells (green) and dead cells
(red) stained with AM calcein and EthD-1; (b) dead cells – previously treated with 70%
ethanol and stained with AM calcein and EthD-1.*

4. Conclusions

Structural, morphological and ultrasonic characterizations of hydroxyapatite powder synthesized by an adapted co-precipitation method were presented in this paper. The SEM study revealed that the HAp sample is composed of acicular nanoparticles which tend to agglomerate due to their reduced size. On the other hand, the EDX and elemental mapping analysis highlighted the main constituent elements (P, O and Ca) and their uniform distribution throughout the sample.

During the biological experiments, the viability studies showed (by fluorescence microscopy) that the HAp tablets had a cytotoxic effect on the endothelial cell growth. Maintaining the tablets seeded with endothelial cells for three days in the culture medium caused a strong cytotoxic effect, resulting in a low cell population and a high death rate. A strong acidification of the medium was also observed. Later on, maintaining the tablets in the culture medium for 48 hours and then populating them with cells resulted in a moderate increase of the pH values, which led to a slight increase of the endothelial cell population and an increase of the number of living cells. However, the cell density detected remained low compared with the density of cells grown as reference.

The novelty of our study consists of preliminary ultrasonic measurements conducted on HAp nanoparticles uniformly dispersed in distilled water. By means of a specialized computer program for signal analysis, we were able to determine and analyze time differences between echoes measured in distilled water and in the HAp dispersion. Therefore, we obtained valuable information on the velocity and attenuation of ultrasonic waves propagating through the dispersion. The ultrasound velocity through the dispersed nanoparticles hydroxyapatite can be used as standard test for the quality. Other parameters such as size and concentration in water are supposed to be controlled and identical.

In conclusion, our results provide a non-destructive, fast and accurate characterization technique for nanoparticles dispersions.

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