JOURNAL OF OPTOELECTRONICS AND ADVANCED MATERIALS Vol. 9, No. 12, December 2007, p. 3827 - 3831

Characteristics of hydroxyapatite thin films

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Thin films of hydroxyapatite (HAp) ($Ca_{10}(PO_4)_6(OH)_2$) were vacuum deposited on different substrates (quartz and silver) in connection with biomedical applications. Characteristics of thin films were putted into evidence by (SEM) and (XPS) analysis. The FT-IR spectroscopy was performed on hydroxyapatite powder and thin films deposited on quartz. The deposition of HAp thin films presents good surface quality as a smooth and an adherent layer. The biological tests confirm the characteristics of these thin films as bioactive materials. The hydroxyapatite thin films supports have a micro cell configuration that allows them to be used for obtaining medical biocompatible supports.

(Received November 29, 2007; accepted December 4, 2007)

Keywords: Hydroxyapatite, Vacuum deposited technique, XPS, Osteoblasts culture (DMEM), MTT test

1. Introduction

Nanotechnology has strongly affected biomaterials science and engineering, once reduced condensed matter domains may deeply alter biomaterials' electronic/thermodynamic properties and thus the response of living tissue to them [1]. The biomaterials are those substances that do not present pharmaceutical properties when included in a biological environment and have the power to substitute the working of different bodies and tissues [2]. These biomaterials play an important part due to the interesting property of chemical reactivity with the biological fluid by making a link to the living bone and its soft connection tissue that facilitates the bone regeneration. The commercial coatings are obtained for the most part of these biomaterials by RF sputtering deposition technique in spite of some disadvantages as poor adhesion on the substrates or inhomogeneous composition. In the last years, the pulsed laser deposition (PLD) technique is viewed as a promising technique for high quality biomaterials thin films deposited on different substrates.

Among these biomaterials, the hydroxyapatite (HAp) $Ca_{10}(PO_4)_6(OH)_2$ is the model structure for the apatites forming the inorganic components (96%) of bones and teeth. To achieve a better understanding of process of biomineralization and to enable design of artificial biomaterials a thorough understanding of the structure and of chemistry of HAp and other calcium orthophosphates is fundamental [3]. Calcium phosphates and specially hydroxyapatite are some of the most important bioceramics for bone reconstruction, and in the last years a great effort has been made to improve HAp mechanical properties and bioactivity. Regarding the structure of HAp, in the dental enamel the structural unit of HAp is formed by many prisms in the size range of tenths of microns, which run from the enamel dentin junction to the enamel surface. These prisms are formed by many elongated crystals inside an organic matrix. In the organic phase, the fibers of collagen serve as a matrix for the precipitation of HAp, determining the structure of the crystals. The collagen gives to bone its elastic resistance. Bone exhibits natural HAp crystals with needle-like or rod-like shapes, well arranged within the polymeric matrix of collagen type I. These natural nanoparticles formed in physiological environment have a more dynamic response when compared to synthetic material with larger particle size. The excellent biocompatibility and bioactivity is sustained by many *in vivo* experiments that have confirmed the advantages of a bioceramic with porous structure for tissue ingrowth [4]. The application of the porous HAp ceramics is limited as the strength of the porous ceramics decreases exponentially with the pore volume ratio [4].

This paper presents some results regarding the properties of HAp coatings deposited on quarts and silver film using the technique of thermal evaporation in medium vacuum. The comparison between evaporation technique and commercial coatings by RF sputtering is related to the use of low energy atoms, high vacuum path, larger grain size and in general poor adhesion.

2. Experimental methods

2.1 Sample preparation

Powder of artificial hydroxyapatite was supplied by Fluka at 90% purity. The powder was pressed and then, was used a specific quantity for vacuum deposition. The HAp thin films were deposited by thermal evaporation using a HOCH VAKUUM Dresden system. The thin films were deposited on quartz (S 1) and quartz/silver(S 2). For evaporation in medium vacuum ($p \sim 8 \times 10^{-6}$ torr) it was used a wolfram boat, and the intensity of the maximum current through boat was I^{max}= 80 A in a time interval t > 60 sec. The thickness of the thin films were d(S 1) ~ 140 nm and d(S 2)~ 150 nm on a silver film of d_{Ag}~ 470 nm. The as obtained HAp thin films were characterized by different techniques namely: XPS, FTIR, SEM, EDS and biological tests.

2.2 FT-IR analysis

In order to characterize the HAp coatings on quartz we present a study regarding the IR active groups by the help of FT-IR spectrometer type Spectrum BX (7800-350 cm^{-1}) in transmission mode with the resolution: 8 cm^{-1}.

2.3 SEM and EDS analysis

The surface morphology and growth mode of the deposited HAp thin films were investigated by scanning electron microscopy (SEM) in a XL-30-ESEM TMP system For the elemental analysis the electron microscope was equipped with an energy dispersive X-ray (EDS) attachment.

2.4 XPS analysis

Soft X –ray Photoelectron Spectroscopy (XPS) is one of the most important technique for the study of the elemental ratios in the surface region of a thin film deposited layer. The surface sensitivity of XPS which is typically 40-100 Å makes the technique ideal for the measurements of oxidation states, oxide layer thickness on biomaterials powders and thin films. The ratio Ca/P in atomic percentage is obtained in details in a VG ESCA 3 MK II XPS installation ($E_{K\alpha}$ = 1486.7 eV). The vacuum in the analyzer chamber was p~ 3×10^{-8} torr. The X-rays are emitted by an anti-cathode of Al, U=12.5 kV, filament emission current I=20 mA, flood gun: 2 V, electron current I=0.3 mA, voltage on electron multiplier U=2.8 kV. The XPS recorded spectrum involved an energy window w= 20 eV with the resolution R=50 eV, and with 256 recording channels. The XPS recorded spectra were processed using Spectral Data Processor v 2.3 (SDP) software.

2.5 Biological tests

2.5.1 Cell culture

Osteoblasts were grown in Dulbecco Modified Eagle's Medium (DMEM) supplied with 10% fetal bovine serum, DMEM sodium pyruvate, 2% glutamine and antibiotic mix. Medium compounds were purchased from Gibco (UK). The cells were incubated at 37°C, 5% CO_2 and the split was performed using tripsin-EDTA solution 1x (Sigma-Aldrich) and phosphate-buffered saline (PBS) from Gibco.

Osteoblasts used to determine the cell proliferation, viability and citotoxicity interaction with S1 and S2 thin films has been obtained from the upper part of the patient's femur. These patients undergo the surgery intervention in arthritis disease when the haunch articulation is removing.

Primary osteoblast culture from bone explants was designed according to Gallagher et al (1996) protocol [10-12]. The pieces from bone tissue are transferred into a sterile recipient with PBS. Obtained tissue is detached from soft conjunctive tissue of the external bone area. The tissue is rinsed in sterile PBS and removed in Petri dishes which contain a small volume of sterile PBS proportionally to the size of the pieces.

Next step was to place the explant fragments in DMEM with antibiotics supply, washing successively with antibody solutions, cultivate in DMEM medium supplied with 15% Bovine Serum Albumin (BSA), 2% glutamine and buffered with natrium bicarbonate.

The first osteoblasts from explants arise after 7-10 days of incubation (5% CO_2 atmosphere, T=37°C) and were suitable for split after 4-6 weeks; after the second passage, the culture contains strictly fibroblasts. Subsequent splits were performed at confluence (2×10^6 cells/plate) in about 10 days, with a 1:3 ratio. Confluent cultures have been treated with trypsin for 2-3 min and then centrifuged at 1.500 rpm for 10 min. Cells were re-suspended in minimal DMEM volume, counted with Burker-Turk chamber and evenly distributed on sterile supports, previously treated with polylisine.

2.5.2 Cell viability

Biocompatibility test of the S1 and S2 thin films has been done using primary osteoblast cell line. After osteoblast culture achievement, the cells were treated with trypsin 0, 05% and splitted in 35/35 mm Petri dish.

Cells were seeded at a density of 10^5 cells/ml in Petri dish and incubated on H2 and H4 thin films for 48 hours. The cell viability was determined by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) reduction test. The cells were incubated (5% CO_2 atmosphere, T=37°C) for 4h with MTT (0, 1 mg/ml).

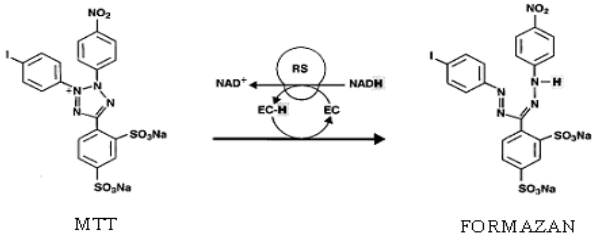


Fig. 1. MTT is reduced to formazan in mitochondria of the cells.

The viability cell number is directly proportional to the production of formazan as in the scheme presented in Fig. 1. The isopropanol was added to dissolve the insoluble purple formazan product into a coloured solution. The absorbance was quantified by measuring the wavelength at 595 nm by TECAN spectrophotometer.

3. Results and discussion

3.1 FTIR analysis

The infrared spectra of the sample HAp and S1 (Fig. 1) show the vibration modes of powder hydroxyapatite (HAp) and sample 1 (S1). In these spectra, the characteristic bands of HAp are observed [5-7]. The bands at 550-600 cm^{-1} (sample 1) can be attributed to the PO_4^{3-} ions (v4) [8]. Bending bands of HAp at 600 cm^{-1} and 594 cm^{-1} attributed to the PO_4^{3-} ions (ν_4) merge to form a strong band at 597 cm^{-1}. The most prominent HAp band is the band at 1040 cm^{-1} (v3) can be attributed to the PO_4^{3-} ions [9]. The most prominent sample 1 band at 1100 cm^{-1} are assigned to the PO_4^{3-} ions, ν_3 vibrations mode (asymmetric stretching). For CO_3^{-2} groups, a small peak at 875 cm^{-1} corresponding to ν_2 vibration (asymmetric stretching) in the sample 1 were observed. The band at 1400 cm^{-1} arises from vibrations of CO_3^{-2} ions for ν_3 vibrations mode (asymmetric stretching) in HAp. Vibrations associated to the vibrations of hydrogen-bonded water molecules adsorbed on the surface are detected by the band 1640 cm^{-1}.

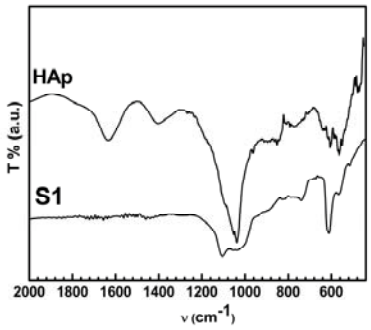


Fig. 2. FTIR spectra of the powder hydroxyapatite (HAp) and sample 1(S1).

3.2 EDS and SEM analysis

We note that the EDS spectra (Fig. 2) can be used in this case only for a qualitative analysis. Indeed, a quantitative analysis is not possible, as the oxygen $K\alpha_{1,2}$ lines includes contributions both from the thin films and from the SiO_2 substrate. Hydrogen is missing since as known, elements lighter than boron can not be detected by EDS.

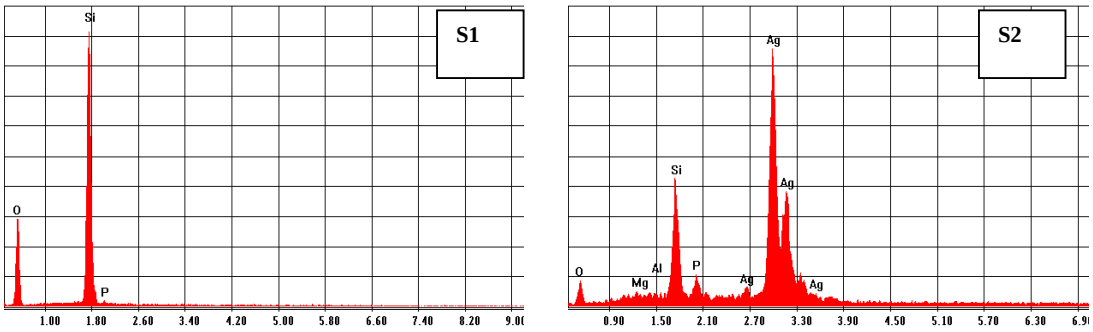


Fig. 3. EDS spectra of thin films: sample 1 (S1) and sample 2 (S2).

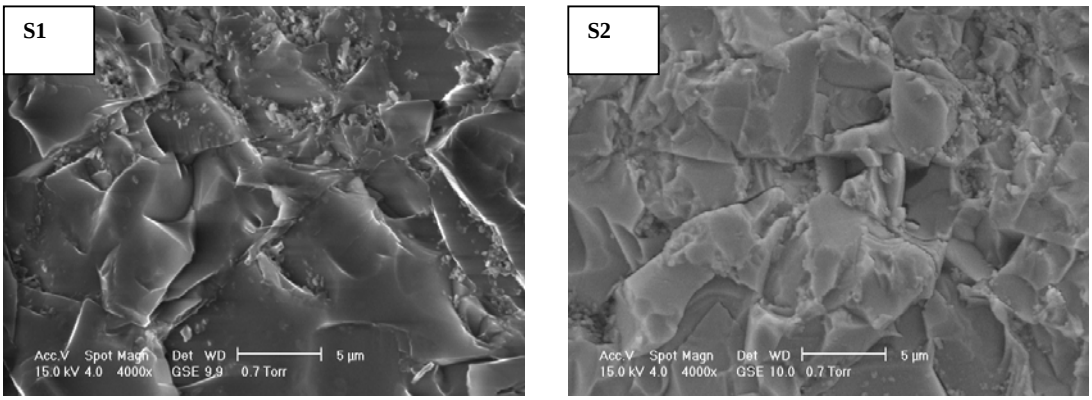


Fig. 4. Typical SEM micrographs of thin films: HAp (S1) and HAp (S2).

In order to analyze the morphology and crystallite size, a SEM analysis has been conducted. The samples 1 and 2 show the well defined platelet morphology with almost the same aspect for sample S1 and S2 prepared in similar conditions but on different substrates.

3.3 XPS analysis

The well defined signal of elemental surface composition is raised from the XPS analysis. The calibration line is based on C (285 eV), and besides the signals of O and C the most interesting characteristic elements are Ca and P. For Ca: A ($2p_{1/2}$), B ($2p_{3/2}$) 347eV is the biding energy, it is present a low chemical shifts in calcium composites especially in phosphates; 2.31 eV is the half-width at the peak position.

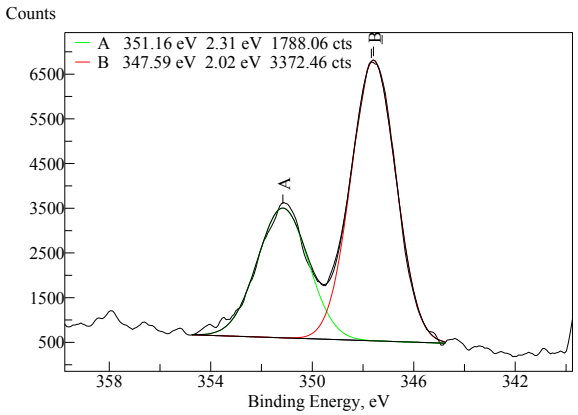


Fig. 5. XPS spectrum for Ca in the frame of sample 2(S2).

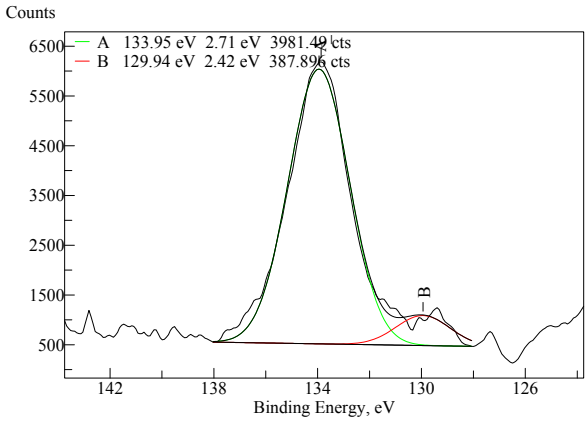


Fig. 6. XPS spectrum for P in the frame of sample 2 (S2).

For Phosphorus the deconvolution lines are: (A $2p_{1/2}$) and B ($2p_{3/2}$); where an A-regarding phosphate is a 2.71 eV half-width at peak position and B is a possible a satellite line. Regarding the atomic ratios percentage Ca/P the situation is:

(S2)	P	Ca		
	76.7	23.2	% atomic	Ca/P (~0.302)
(S1)	50	50.2		Ca/P (~1)

The possible comment: P a volatile element migrates to the surface in the case of (S2) probably due to a greater current intensity through the wolfram boat I_{max}~ 90 A t> 75 sec and in the thin film the atomic percentage goes worse. The sample S1 (HAp deposited on quartz) at a lower intensity, and at a reduced deposition time is a better quality film in the view of atomic percentage as regards the chemical stoichiometry. The standard deviation in atomic percentages is strongly related to matrix effects as presented in standard ISO 18 118/2004, affected in a range (0.3-3).

3.4 Biological tests

MTT assay is a laboratory test and a standard colorimetric assay (an assay which measures changes in colour) for measuring cellular proliferation (cell growth). It is used to determine cytotoxicity of potential medicinal agents and other toxic materials.

Yellow MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. A solubilization solution (isopropanol) is added to dissolve the insoluble purple formazan product into a coloured solution. The absorbance of this coloured solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer.

This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion is directly related to the number of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced, through the production of a dose-response curve.

Osteoblast cells were permanent monitored to detect any possible influence due to S1 and S2 thin films that might alter the cell growth, viability and proliferation. This study represents one of the key-step in cell biology, mitochondrial dehydrogenises being essential.

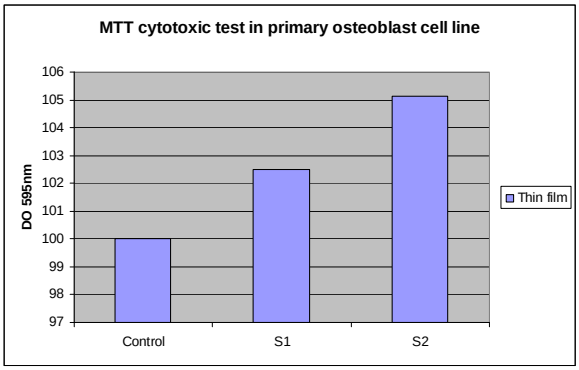
Table 1. Absorbance values at 595 nm.

Samples	DO_{595nm}	Viability (%)
Control	0.1245	100
S1	0.1276	102.49
S2	0.1309	105.1406

The results obtained after MTT assay have revealed (Table 1) as we expected, the fact that control sample present a significant value of absorbance at 595 nm (0, 1245). This value is also established by the high intensity

of the color (deep purple) in control due to the amount of formazan produced by cells.

Table 2. MTT assay in osteoblast cells growing on S1 and S2 thin films.



Our data indicate that osteoblast cells' growing on S2 substrate presented a higher peak (0, 1309), comparing to those incubate on S1 support which shown a smaller peak (0, 1276). Cells growing on S1 (102, 49%) and S2 (105, 14%) thin films shown a significant increase of viability, growth and proliferation, comparing to control (Table 1, Table 2).

4. Conclusions

The hydroxyapatite thin films vacuum deposited on quartz and silver presented a uniform structure, with good adhesion. The typical lines for phosphates compounds are present in FT-IR spectrum as the band 550-600 cm^{-1} attributed to $(PO_4)^{3-}$ ions, and with the most prominent HAp band at 1040 cm^{-1}. The near stoichiometric ratio Ca/P in the upper thin layer is putted into evidence by XPS analysis, for sample S1 (HAp deposited on quartz).

The micro cells configurations (bioceramics) made by thin films could be the suitable support for osteoblast cells adhesion and proliferation without any modification of their structure and function.

The results we obtained using MTT test demonstrate that cells' growing on S1 and S2 thin films can modify growth parameters, leading to an increase of proliferation and viability comparing to control. We can conclude S1 and S2 thin films are excellent support for adherence and cell proliferation.

It is important to conclude that both S1 and S2 thin film supports has a micro cells configuration that allow them to be utilised for obtaining medical biocompatible supports.

Aknowledgements

The authors want to thank to Romanian Scientific Programmes CEEX-24 Viasan and CEEX-28 SESAM for financial support.

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