DEVELOPMENT OF HA - nAg COMPOSITE COATING FROM GREEN PROCESS FOR HIP APPLICATIONS.

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Abstract

In this study, biological hydroxyapatite was obtained from bovine bones by thermal process. Then 0 and 1 % nano-silver synthesized by Opuntia ficus (nopal) containing biological hydroxyapatite coatings on a Ti6Al4V substrate were developed by atmospheric plasma spray (APS), and their antibacterial efficiency was evaluated in the following bacterial strains: Escherichia coli, Staphylococcus aureus and Pseudomonas aeruginosa according to the JIS Z2801:2000 standard “Antimicrobial product-Test for antimicrobial activity and efficacy”. Ion release test shows that the coating delivered silver ions below 0.05 ppm; as well as calcium, potassium, magnesium and sodium ions (it are cell growing promoters). Scanning electron microscopy (SEM) showed that nano-silver particles (nAg) were distributed on the coating surface. Energy-dispersive X-ray spectroscopy (EDX) shows that apatite deposition occurs day by day, maintaining a Ca/P rate between 2.12 and 1.45. Biocompatibility properties were evaluated with Allium cepa, lymphocytes (fresh blood) and mouse osteoblast cells (MC3T3) by Allium cepa assay, single cell gel electrophoresis assay and Tali image cytometry.

Keywords: Biological hydroxyapatite, coating, silver nanoparticles, atmospheric plasma spray, antibacterial test, citotoxic test.

1. INTRODUCTION.

The calcium phosphates salts (CaP) are the major mineral component of bones and teeth in vertebrates. Bones and other calcified tissues can be considered as natural anisotropic composites of biomaterials inside of a protein matrix, organic material and water. The biomineral phase containing 65-70% of bone approximately (that can contain one or more types of calcium phosphates), water constitute 5-8%, and the rest of the percentage correspond at organic phase, which is mostly collagen. Hydroxyapatite (HA) is the most thermodynamic stable calcium phosphate salt inside the human body fluid, likewise it has the greatest similarity with the mineral part of bone. Naturally, HA is carbonated, this means, calcium deficient; that is why its Ca/P rate is less than 1.67 (Mehdi Sadat-Shojai et al., 2013). HA has been used in coatings to promote bonding of prosthesis through osseointegration (Sebastian Bauer et al., 2012).
It has been reported that infections related to implants play an important role as one of the most common clinical complications. The postoperation infection rates are 5% in primary cases, 6% for revision cases and, 43% for revision of cases previously infected (Yikai Chen et al., 2010). The pathogenesis of the infection is related with microorganism that grow in a biofilm, being so difficult to treat (Daniela Ionita et al., 2011). In order to reduce the risk of infection, prophylaxis is use. Due to the limited blood irrigation in bone tissue, distribution of antibiotic on infected areas is so poor. To reduce infections, concentration of antibiotics are increase. However, too much antibiotics in blood during long time produces intoxication. There are organic and inorganic antibiotics; organic antibiotics form a biofilm on the implant surface, increasing the bacteria resistance to antibiotics. While inorganic antibiotics are antimicrobial agents that do not induce bacteria’s resistance, therefore, there are excellent options for a local antimicrobial treatment (Yikai Chen et al., 2010). Nowadays studies have been developed to improve antibiotic properties in HA coating by the addition of silver nanoparticles (nAg) (Huiliang Cao et al., 2010). In previoud studies, we had developed nAg and HA coating by atmospheric plasma spray (APS) using components processed by chemical way (V. Orozco Carmona et al., 2014).

Talking about biomaterials is talk about materials able to be in contact to living tissue during a time lapse as part of the tissue, with the aim of complete tissue or improve functionality (María Cristina Piña Barba, 2012). The biomaterial science involves the study of physical, biological and chemical characteristics of materials, as well as the respective evaluations and interactions between the material and the receptive body (Buddy D. Ratner et al., 2004). Biocompatibility of biomaterials is classified according their ability to induce cellular dead (cytotoxicity), cancer promoter (carcinogenicity), genetic damage (mutagenesis), etc. The biocompatibility of a medical device covers both compatibility and design (Quizhi Chen and George A. Thouas, 2014). One of the most important characteristics about implant materials is the presence of porosity or cavities that allow tissue growing toward the material (Sebastian Bauer et al., 2012).

In recent years, researches have been directed to develop a HA synthesis, including food waste as precursors or the extraction of HA from animal bones (Mehdi Sadat-Shojai et al., 2013). There are certain differences between synthetic HA and natural or biological HA, the last one have a better metabolic activity and higher dynamic response to environment. That’s why is logic to think that biological HA is better as an implant or coating material (M. Boutinguiza et al., 2011). One of the reasons for this good behavior is that biological HA it’s not stoichiometric own to ion traces incorporated to crystal structure, as Fe$^{2+}$, Mg$^{2+}$, Si$^{2+}$ y F$^{-}$ (P. Kamalanathan et al., 2014).

Aiming at preventing costly therapies or the implant removal, the development of coatings with an excellent antimicrobial activity as well as osseointegration, it’s very important (Daniela Ionita et al., 2011). For its application it’s been used different methods such as sputtering, ionic exchange, sol-gel method, plasma spray, etc. There are some inorganic antibacterial agents, like silver, and its ions, known due to its good inhibitory effects in a wide bacteria spectrum (Yikai Chen et al., 2010). In the other hand, the use of silver was limited due to the toxicity of its ions; however, the industry of nanotechnology has extended the possibilities of its use being that small particles with large superficial area are produced, improving its efficacity but, beyond that, toxicity its reduce (Karla Chaloupka et al., 2010). However, how and what induce cytotoxicity is a subject that is not well understood. It’s been suggested that nAg act like Troy horses, getting inside the cell and releasing silver ions which hurt intracellular functions. However, Kim et al. have argued that nAg cytotoxicity its result of an oxidative stress and completely independent of silver ions toxicity. A better understanding in this issue is crucial for nAg cytotoxicity evaluations (Huiliang Cao et al., 2010). The use of non-toxic and environmentally friendly methods for nanoparticles synthesis is very attractive, especially if there are intended to be use in invasive applications in the medical field. To achieve this, microorganisms, plants and roots tissue, living plants, extract of plants and some marine algae have been use. The biogenic synthesis is useful thanks to its low environmental impact but, more than that, due to the production of free-chemical traces nanoparticles (Abduz Zahir, et al., 2012).

2. MATERIALS AND METHODS.

The natural HA was obtained from bovine bones by a thermal decomposition. The bovine bones were cleaned by hand and water, boiled during 8 hours at 90°C, then the soft bone (bone marrow) was taken away. Bones were treated during 8 hours with a H$_2$PO$_4$ solution maintaining a pH of 2.4. Then, for bones water elimination, they were heated in an oven for 2 hours at 250°C to produce fragility. After that, bones were grill with a blade devise until produce powder.

Powder was calcined at 800°C during 6 hours and then characterized. To analyze crystallinity a PANalytical X’Pert PRO x-ray diffractometer (XRD) was used, with a Cu Kα radiation ($\lambda$=1.5406 Å) over Bragg angle ranging from 5 to 80°. Fourier-transform infrared spectrometry (FT-IR) characterization of the powder was performed on a Perkin Elmer Spectrum 6X with resolution of 4 cm$^{-1}$ in a frequency range of 4000–400 cm$^{-1}$. Surface morphology was studied by a HITACHI SU35000 scanning electron microscope (SEM) equipped with energy dispersive x-ray spectroscopy (EDX). The Cu/P molar ratio was determined either by EDX and inductively coupled plasma optical emission spectrometry (ICP-OES) Thermo Scientific iCAP 600 series. The particle size
distribution (PSD) was determined in aqueous conditions using a laser granulometer Malvern MASTERSIZER 2000.

Silver nanoparticles obtained by a biosynthesis with nopal (Opuntia ficus) extract were used to make the coating with antibacterial properties.

The plasma spray coating was produce by using an atmospheric plasma spray (APS) equipment (PRAXAIR Surface Technologies 3710) with the following operation parameter: 600A, 55psi of primary gas (Ar), 110 psi of secondary gas (He) and application distance of 50 mm (V. Orozco Carmona et al., 2014). The coatings’ morphology was characterized by SEM. Coatings were exposed to simulated body fluid (SBF) solution, to analyze de apatite deposition. Ion release was analyzed by ICP-OES.

Antimicrobial test was performed based on JIS Z 2800:2001. This standard mentioned that “The value of antimicrobial activity obtained by the testing methods of this Standard shall not be less than 2.0 for the antimicrobial efficacy of antimicrobial products”. For a better understanding a converse was made, so, the value of 2.0 is equivalent to a 99% of efficiency.

3. RESULTS.

The XDR analysis (Fig. 1) shows that the result after the thermal process is HA, according to the 00-074-0565 diffraction pattern standard from de ICDD. The pattern also reveals a minor presence of CaO, indexed with the card 00-075-026.

![Fig. 1. XDR pattern of HA obtained from bovine bones at 800°C. *HA °CaO](image)

The FT-IR spectrum from the powder is presented in Fig. 2, exhibiting the OH\(^{-}\), PO\(_4\)\(^{3-}\), CO\(_3\)\(^{2-}\) peaks. This results shown that the HA is carbonated, therefore, it is less stoichiometric and more similar to real bone composition.

According to the chemical formula, the calcium to phosphorous molar ratio is approximately 1.67; however, being a HA obtained from bovine bones is logical to think that the molar ratio is a little under the stoichiometric ratio, due to the CO\(_3\)\(^{2-}\) ions. This is demonstrated analyzing the powders by ICP-OES, and showing that the molar ratio Ca/O is 1.65; the percentage of present elements in the powder was also analyzed: 0.049% K, 0.534% Mg, 0.821% Na, 0.013% Si. The presence of these ions is really important since they have a biological roll according to P. Kamalanathan et al., (2014), Shih-Ching Wu et al., (2013), P.N. Lim et al., (2016), T.A. Grunewald et al., (2015).
The powder has a particle size distribution average of 119.75 µm, this size is a good value to be applied in an APD process, therefore, HA/nAg coatings were applied successfully.

The surface morphology of the coating was observed by SEM. Fig. 3 shows that the coating has texture, also microfractures and the dispersion of silver nanoparticles is good, even when there is an agglomeration tendency. To determine the osseointegration properties, the coatings were submerged in SBF at different times (1, 3, 12 and 24 h, 3, 7, 14 and 30 days) and analyzed by SEM, it showed that apatite is formed on the coating and exhibited a bigger concentration of apatite formation in fracture areas (Fig. 4). According to E. Hatzistavrou et al. (2010), the rapid grow of the apatite on the surface of the samples can be assigned to the presence of the CaO phase in the natural HA.

In transversal section can be seen that the average thickness of the coating is 217.33 µm (Fig. 5). Elemental map (Fig. 6) expose that Ca and P are concentrated almost in the same place, and where have deficit of Ca-P there is presence of Mg; both nAg and Na are present in all coating’s surface.
Allium cepa test was performed in both HA/nAg and HA coating, the last one as a control and which principal aim is to be able to observe the effect of HA on the cells. The results show that the HA coating stimulate the cellular division, this confirms the multiple studies that exhibit HA as an excellent promotor material of cell growing. The nAg coating show a decrement in the mitotic index (MI), which indicates that there is a light alteration in the cellular growing due to the presence of nAg but, it is not significant (Fig. 7); the alteration index (AI) shows that doesn’t exist a significance difference between both coatings, this is, cells doesn’t suffer any more damage attributed to silver nanoparticles, than they suffer by themselves. Single cell electrophoresis was evaluated with the two cell lines mentioned: lymphocytes (that were taken from voluntaries fresh blood and osteoblasts (ATCC: MC3T3)). In both cases is shown cellular damage and the DNA migration occurs but it is not significant compared to negative control and HA coating (Fig. 8).
Fig. 9 shows the results of the Tali image cytometry performed with lymphocytes. It can be appreciate the increment in cell growing, more in HA/nAg than HA. Also there exist apoptosis, which in both cases is bigger than negative, this occurs because apoptosis (natural death cell) is activated when there exist an increment in the living cells so the system can be equilibrated. In the other hand, necrosis is less, even less than negative, this indicates coatings aren’t promoting irreparable damage in cells, so, coatings aren’t harmful agents.

To perform the antimicrobial test, 250,000 CFU had been in contact with each coating. The JIS Z 2800:2001 standard establishes that the antimicrobial value shows the difference in the logarithmic value of viable cells counts between antimicrobial product and the untreated products after inoculation and incubation of bacteria. This value is calculated as falling (Eq. 1):

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R = \log_\frac{B}{A} - \log_\frac{C}{A} = \log_\frac{B}{C} \quad \text{(Eq. 1)}
\]

where: R is the value of antimicrobial activity, A is the average of the number of viable cells of bacteria immediately after inoculation on the untreated test piece; B is the average of the number of viable cells of bacteria on the untreated test piece after 24 h; and C is the average of the number of viable cells of bacteria on the antimicrobial test piece after 24 h. Fig. 10 shows that the three bacteria presented minimum activity when were in contact with the coating for 24 h, all of the values of antimicrobial activity are above 2; this means, the coating has, at least, 99% of efficacy (Table 1).
4. CONCLUSIONS.

Natural hydroxyapatite was successfully extracted from bovine bone using a thermal process, which is cleaner than industrial process and requires fewer raw materials. Characterization results show that this hydroxyapatite has traces of ions that are necessary to cell growing, suggesting that it has better bioactivity and biocompatibility.

Applying the HA/nAg powder mix by atmospheric plasma spray allows handling raw material as one, and HA/nAg coatings had a good performance in all tests, proving that 1% of silver nanoparticles are enough to inhibit bacteria growing but also, not enough to damage human body cells.

This study is the beginning of a serial of studies around the production and optimization of biomaterials obtained by less harm methods.

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