Adherence and in vitro biomedical properties of calcium phosphate-calcium titanate composite coatings for orthopedic application

Adherencia y biocompatibilidad *in vitro* de recubrimientos de fosfato de calio-titanio de calcio obtenidos por magnetrón sputtering para aplicaciones ortopédicas

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Abstract

The Hydroxyapatite coatings have been used for many years in hip prosthesis stems. However, it has been observed that the coatings detaches leading to the loosening of the prosthesis due to their mechanical properties did not meet the requirements. Since calcium titanate has been proposed as a coating for biomedical applications due to its good *in vitro* biocompatibility and osteoconductivity, adherence and elastic modulus of calcium phosphate-calcium titanate composite coatings were assessed by means of scratch test (ASTM C1624-05) and nanoindentation test (ASTM E2546- 07). *In vitro* biomedical properties such as genotoxicity and hemolysis were evaluated also (ASTM F748-06). 100% calcium phosphate (CP) coating was composed of a mixture of tricalcium and tetracalcium phosphate. Calcium titanate (CT) was added to the coatings, by modifying a Ca10(PO4)6(OH)2- 2xOx□x target, in 25, 50 and 75 volume percentages. For comparison purposes, 100% TC coatings were also obtained. It was found that adherence increases as added 25% of one material of another rising a maximum value in 50-50 composite coating. Besides, the coatings were not genotoxic and not hemolytic.

Keywords: biomaterials; coatings; calcium titanate; calcium phosphate; magnetron sputtering, scratch test.

Introduction

When the hip suffers damage that results in malformations, arthritis or fracture, the daily activities such as walking, can result painful or difficult. One of the Solutions consists in substituting the sick or injured parts with a prosthesis that completely substitutes it or supports it.

The alloys forming the hip prostheses stems are generally made out of inert materials, so, they don't achieve a full adhesion with the surrounding bone tissue, to the point of detachment in any moment. One way of improving its adherence is the application of osteoconductive coatings (Huracek et al., 1994; Andersen et al., 2015), composites, mainly of hydroxyapatite (Chandran et al., 2010; Goyenvallea et al., *2006*). The superiority of these coatings has been observed at early stage of the implants, where the forming of the bone tissue is higher than in stems without coatings. However, these coatings present some inconvenient, since they dethatch or wear down (Porter et al., 2004), decreasing the amount of bone attached to the implant, leading to a long-term adherence loss. Thus, the life span of these prostheses does not exceed the one of the cemented prostheses, which duration is between 12 and 16 years (Chandran et al., 2010).

Since the hydroxyapatite coated prostheses have shown good clinical results and with the goal to extend the life span of these coatings in the device, many studies have been conducted. In these, the collecting methods have been varied (Hong et al., 2007; Bao et al., 2005), different types of substrates have been evaluated (Dinda et al., 2009; Liu et al., 2002) and different seed layers (Nelea, et al., 2000; Zhen-jun, et al., 2006). The hydroxyapatite has been mixed with different materials (Silva et al., 2001; Harle et al., 2006) and different coatings have been produced in the form of multilayers (Ozeki0 et al., 2007). Everything with promising results, but yet without application in commercial prostheses.

In the first stages of nucleation in the apatite titanate alloys, an amorphous layer of calcium titanate has been observed (CT) (Webster et al., 2003). In consequence, this material has gained the attention to be used in biomedic alloys for the activation of osteogenesis (Ohtsu et al., 2008). Some studies have shown that the CT coatings are bio-suitable (Park et al., 2011), protect against corrosion (Tang et al., 2013), are adherent (Stanishevsky et al., 2007) and osteoconductive (Wiff et al., 2007).

On other hand, between the calcium phosphate, the tricalcium phosphate (TCP- $Ca_3(PO_4)_2$) and the tetracalcium phosphate (TTCP- $Ca_4(PO_4)_2O$)) present good osteoconductivity and a medium solubility compared with the hydroxyapatite (HE) (Ozeki et al., 2007). The TCP has been used as a component with bone cements (Grandia et al., 2011). The TTCP mixed with a more stable material can from a porous structure that, at long term, increases the osteoconductivity of the composite material.

Hence, the goal of this work is to carry the adherence and bio-suitability test in TCP, TTCP and CT composites grown over AISI 304 steel substrate previous to the deposition of a titanium layer seed, using the *sputtering* magnetron technique, in order to be applied over hip prostheses stems.

Experimental Details

Coating Collection

The Calcium Phosphate (CP) – Calcium Titanate (TC) coatings were collected from hydroxyapatites (HE) and Calcium Titanate (CT) targets through R.F. Magnetron *sputtering*, as explained in another article (Esguerra et al., 2016). The material proportion in the targets was of 100%HE-0%CT, 75%HE-25%CT, 50%HE- 50%CT, 25%HE-75%CT and 0%HE- 100%CT in volume, obtaining the respective coatings of 100%CP- 0%CT, 75%CP25%CT, 50%CP-50%CT, 25%CP-75%CT and 0%CP-100%CT. The Chart 1 shows the codification of the coatings and their composition. As it can be seen, the 100CP-0TC coatings consist in a β-TCP and TTCP oxygen deficient mixture ($Ca_4(PO_4)_2O_x$); the 0CP-100CT coating consists of 100% CT; and the coatings consisting of a β-TCP, $Ca_4(PO_4)_2Ox$ mixture and TC, in their respective proportions.

Chart 1. Coating codification and composition (% in volume)

Coating Codification	Coating components
100CP-0TC	100% ($2Ca_3(PO_4)_2$+$4Ca_4(PO_4)_2O_x$)
75CP-25TC	75% ($2Ca_3(PO_4)_2$+$4Ca_4(PO_4)_2O_x$) - 25% TC
50CP-50 TC	50% ($2Ca_3(PO_4)_2$+$4Ca_4(PO_4)_2O_x$) - 50% TC
25CP-75 TC	25% ($2Ca_3(PO_4)_2$+$4Ca_4(PO_4)_2O_x$) - 75% TC
0CP-100 TC	100% TC

Source: The authors.

Adherence

The adhesion was evaluated with a scratch-test following the ASTM C1624-05 standard, in a *Scratch Test Microtest* MTR2 system, applying an increasing load of 0 to 40 N in a printing length of 2.6mm. On other hand, in order to co-relate with the adherence, a test of the elastic module was held with the coatings through nanoindentation in a *Hysitron Ubil* nanoindentator, following the ASTM E2546-07 parameter, a depth lesser to the 10% of total coverage. Besides, with the goal to know the 100CP-0TC coating-substrate interface, a study of profile depth was held through, in a SAGE HR100 (SPECS) system with monochromatic font (Al Kα 1486.6 eV), measuring compositional profile depths and high resolution spectrums.

Genotoxicity

For the genotoxicity tests, cryo-preserved NHOst-Osteoblast OGM (Lonza Group) human osteoblasts were used, whose recommended seeding density was of 5000 cells/cm^2 (according to the Lonza protocol). For its cultivation, half basal was used for the osteoblastic cells with cellular growing supplements, such as bovine fetal serum, ascorbic acid and gentamycin sulfate, BulleKit®. The cellular crop was held in cultivating recipients of 25 and 75cm^2 (Corning®), in a humidified incubator at 37 °C and 5% CO_2, for 8 weeks, renovating the cultivation environment every 3-4 days.

For the test, apart from the study samples, titanium was used as negative control (in triplicate), due to its largely reference bio-suitability(Mc Entire, et al.,2015) and 4NQO, as positive control which is a standard solution, that provides the kit.

To make contact between the cells with the study samples, these were sanitized, like the three titanium samples. These were located in culture Wells and then 55000 cells were added to each well with culture medium (1.0 mL in total volumen per well). The same procedure of cellular crop was held for 16 days, making environmental change every 4 days.

The genotoxicity test was held making use of the SOS-Chromo Test® kit. The test used a PQ37 *E.coli* mutant strain that was placed in the cultivation environment with the test materials and the osteoblasts. If the materials dissolves o makes corrosion products that are genotoxic, this will be seen on the bacteria, just like that, a massive amount of DNA damage will show as a response, since these are the emergency mechanisms, which characterize for having superior levels than those of proteins implied in the DNA repairing and recombination. If the amount of injures that there are in the *E. Coli* is way superior than normal, it is induced into a SOS response (this means, the induction of SOS genes that are implied in the DNA reparation). At molecular level, the SOS response consists in the expression induction of a series of genes, which the vast majority are implied in the reparation of DNA damage. This response is related with the β-gal gen, responsible for the production of the β-galactosidase enzyme. Hence, the grade that this cells is working on to repair the DNA damage using this gen complex already mentioned, is directly linked to the amount of produced β-galactosidase, which is measured through a reaction of this enzyme with a blue chromogen.

The genotoxic activity was measured graphing the absorbance (measurement of ELISA readers at 615 nm) according to the dilution order. The slope of the lineal portion of this graphic is the material induction potential to damage the DNA (or SOSIP from SOS induction potential). SOSIP with values near zero indicate non-genotoxic materials.

Hemolysis

The hemolysis test aims to measure the erythrocyte destruction that results in a hemoglobin liberation towards the plasma, and it was done following the ASTM F756-08 parameter. For this, human blood of four healthy non-smoker people was collected. As negative control, polythene (PE) was used and D3 steel was used positive control. A properly prepared mixturewith this blood was put in contact with the other samples and a % of hemolysis was determined. For a value lesser than 2 it is said that the material is no hemolytic, between 2 and 5, is partially hemolytic and higher than 5 is hemolytic.

Results and discussion

Adherence

During the nanoindentation test with raising load along the coating, there was data collected regarding displacement, normal load and friction (or dredging)

coefficient. The Figure 1 shows the curves in function of the dredging coefficient at normal load. After the test, the scratching prints were inspected through optic microscopy, and the modes of failure found, co-related more with the *dredging coefficient – normal load* curves. The identifying of these cohesive and adhesive load, was done departing from this co-relation.

The modes of failure of the coatings are shown schematically on Figure 2. All the coatings displayed semi-circular transversal cracks, splintering and detachment (Holmberg et al., 2003). The semi-circular transversal cracks are related with the cohesive fail, so that the load in which they start to appear, is denominated as cohesive load and is designated as L_{C1}. The material splintering without detachment is displayed with the grey rhombuses and is also a cohesive fail, since the coating splinters itself, but it doesn't completely detach from the substrate. The splintering with coating detachment, displayed with black rhombuses, is related with the adhesive fail between the coating and the substrate. The load when this happens is denominated as *LC2*.

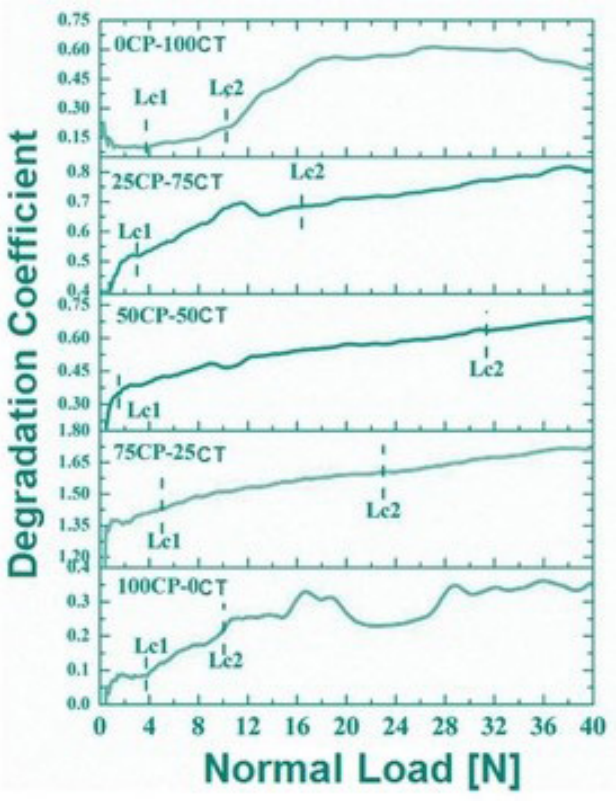

Figure 1. Dredging coefficient in function of the normal load in the scratching test
Source: The Authors.

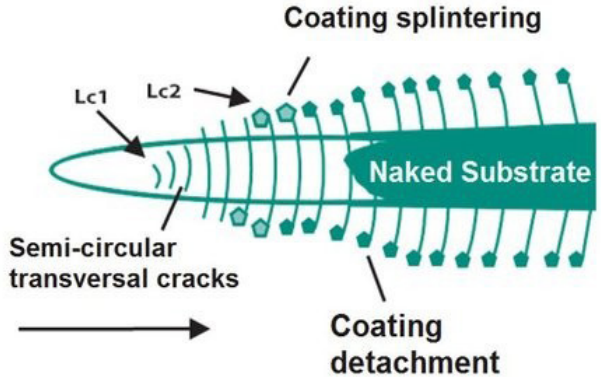

Figure 2. Modes of failure scheme of the coatings put into the scratching test
Source: The authors.

Figure 3 shows the composition profile of the 100CP-0CT coating collected through XPS, where a compositional gradient is observed between the seed layer and the coating. This interface is composed of a Ti, O, P and Ca mix, which makes it Ti richer as it gets closer to the seed layer. The presence of this interface favors the anchoring between both materials, improving the adherence. The formation of this interface seems favored by the deposition temperature, which allows the diffusion of P and Ca atoms in the Ti-O seed layer.

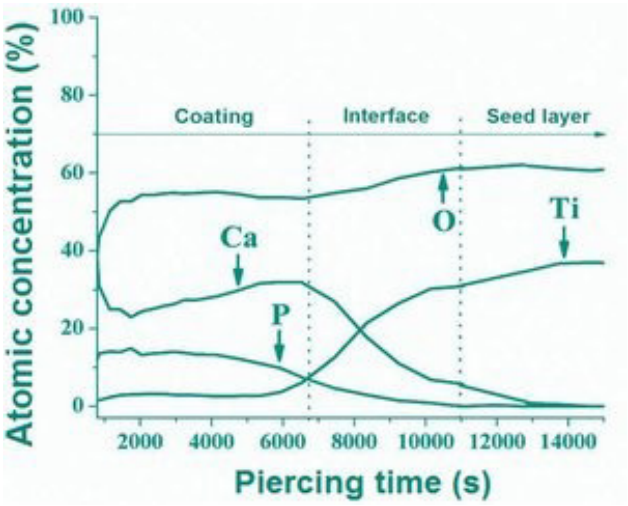

Figure 3. Composite coating profile 100CP-0CT
Source: The authors.

The Figure 4 shows the behavior of the adhesive load of the coatings with CT addition. Same with the case of cohesive load, the 100CP-0CT and 0CP-100CT coatings show similar adherence values, around 10N. Since the adherence values obtained through the scratching test are very uncertain and depend on extrinsic factors such as scratching speed, indenter radius, the indenter wear; and the intrinsic factors,

such as the substrate's properties (hardness, elastic module), the coating properties (thickness, hardness, elastic module, superficial roughness) and the friction coefficient (Bull et al., 2006), a comparison was made between the obtained values for the 100-0 coatings with the literature: it was found that the coating adherence 100CP-0CT is superior than the one reported by Candidato Jr., et al (2015) of 3.3 N for the Calcium Phosphate coatings around a 60 µm thickness, obtained through plasma spray; and superior also to the value reported by Surmeneva et al (2015) of 5.85N for an HE coating of 690 nm thickness placed in a R.F. *sputtering* magnetron, and up to where the authors were able to find, there aren't any references regarding the scratching test upon Calcium Titanate coatings, except for one reported by Stanishevsky et al (2007) but using loads lesser to 1N, which makes it not comparable.

Regarding the discoveries of this mixture, it is observed that there is an increase of adherence as one material is added to another. When adding 25% of CP to the CT coating, the adhesive load increases its value to 16.4N. At adding 25 and 50% CT to the CO coating, the loading adhesive increases its values to 23.1 and 31.2 N, respectively. A similar result was found by K. De-Jung et al., (2012) when adding ZrO_2 to hydroxyapatite in 75HA-25ZrO_2 and 50HA-50ZrO_2 proportions, they obtained 17.5 and 30 N loading adhesives, which they attribute to low residual efforts as the ZrO_2 content increases in the coating and the substrate.

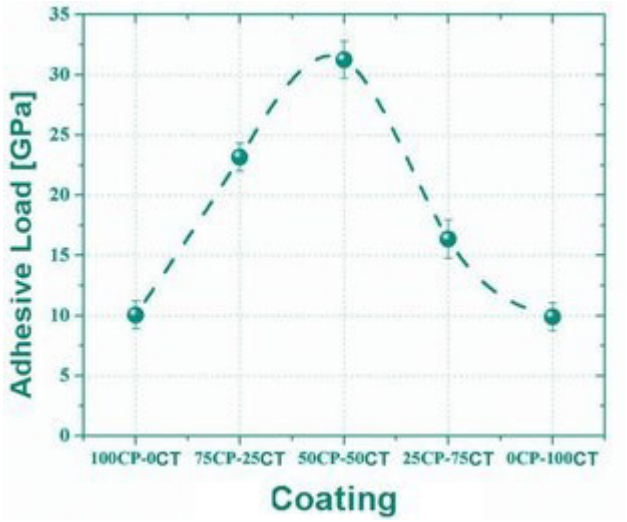

Figure 4. Adhesive load of the CP-CT coatings
Source: The authors.

On Figure 5 there is a correlation between the L_{C1} elastic module with the coatings; a higher cohesive load is given as the elastic module of the coatings increases. This is coherent with the literature (Núñez et al., 2012), since the elastic module is one of the cohesive forces inside a material: the bigger these cohesive forces are, more load must be applied for it to appear the first crack in the material.

Genotoxicity

The Figure 6 shows the genotoxicity of the CP-CT coatings, of AISI 304 steel and the positive (4NQO, as the protocol mentions) and negative (Ti tier 2) controls. The literatura reports that the titanium is not genotoxic (Velasco-Ortega, et al., 2010) and that the AISI 304 and 316L have certain tendency to be genotoxic *in vivo* at long term (Martín-Cameána, et al., 2015). The maximum genotoxicity value is displayed in the graphic is lesser than the ones shown by Kubásek et al (2016), which indicates that, following their reasoning, that none of the materials in the study induces meaningful damage to the DNA, that is to say, aren't genotoxic in *in vitro* conditions. The results of the 100CP-0CT coatings is also a concordance with the scientific community, which reports that these Calcium Phosphates aren't genotoxic *in vitro* (Quan, et al., 2013). About the 0CP-100CT there aren't reports, up to the authors' of this characterization knowledge.

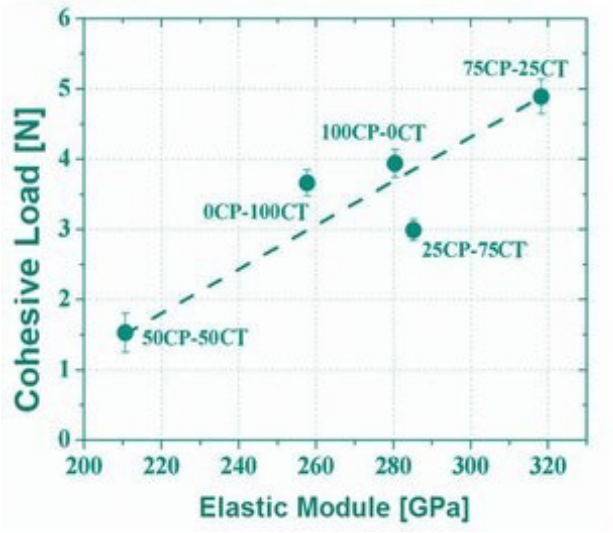

Figure 5. Relation between the coatings' cohesive load and the elastic module
Source: The authors.

When one material to another is added to make a composite, as in this case, but one of them present genotoxicity, it is observed that the genotoxicity in the composite tends to increase at the proportion of the genotoxic material (Quan et al., 2013), however, here the genotoxicity value keeps itself almost constant

and of similar value to the ones of the 100CP and 100CT, since both are not genotoxic (*in vitro*) and with similar genotoxicity values.

Hemolysis

Figure 7 displays the hemolytic index in the coatings, the substrate, the negative control (polythene, PE) and the positive control (D3 steel) after the hemolysis test. The negative control presented a hemolytic index of 1.75 and the positive control a value of 5.79, which means that the PE is non-hemolytic and the D3 is hemolytic, as expected. The AISI 304 stainless steel is non-hemolytic, in fact, it presents a lesser hemolytic index than the coatings. In the coatings is seen the measurement that is added of Calcium Titanate, these make them less hemolytic, being the 100CP-0CT coating slightly hemolytic and the 0CP-100CT non-hemolytic. This can be explained with due to the higher solubility in the Calcium Phospates in comparison to the Calcium Titanate ones (Ozeki, et al., 2007).

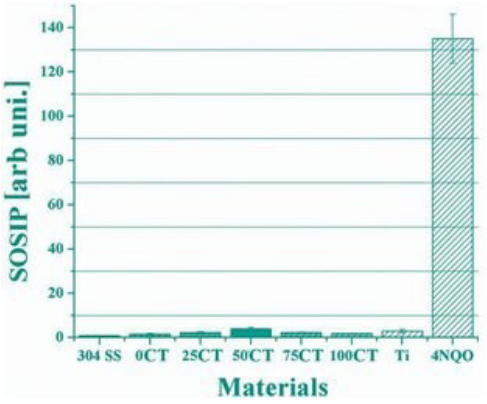

Figure 6. Genotoxicity for each material expressed as SOS
Source: The authors.

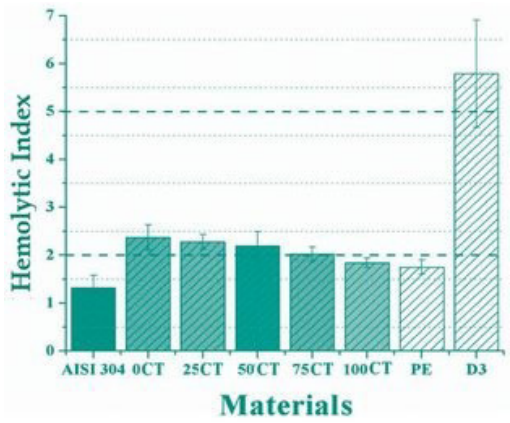

Figure 7. Hemolytic index for each material
Source: The authors.

Conclusions

The adherence, genotoxicity and *in vitro* hemolysis of the Calcium Phosphates – Calcium Titanate, obtained through *sputtering* magnetron.

Regarding the modes of failure in the adherence test through scratching test, it was found that all the coatings display semi-circular transversal cracks, splintering and detachment. It was verified that the higher the elastic module of the coatings, the higher the cohesive load of these.

The adherence values of the 100CP- 0CT coating is superior to the ones reported by other authors for the Calcium Phosphate coatings. The adherence value of the 0CP-100CT was approximately of 10N. Regarding the mixture coatings, it was found that the adherence increases when adding a material in the other one and is maximum in the 50CP-10CT coating. Besides, all the coatings showed to not be genotoxic, and, at most, partially hemolytic *in vitro*. Thus, its *in vivo* bio-suitability test is recommended, in order for it to be used as stems for hip prostheses.

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