Bioactivity evaluation of commercial calcium phosphate-based bioceramics for bone regeneration

P. Gil Salvador 2015 Award in Bioengineering category. (November 27, 2015 in the Annual General Assembly of the AIQS)

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Evaluación de la bioactividad de materiales biocerámicos a base de fosfato de calcio comercial para la regeneración ósea

Ganador del Premio P. Salvador Gil 2015 en la categoría de Bioingeniería. (Entregado el pasado 27 de noviembre de 2015 durante la Asamblea General Ordinaria de la AIQS)

Avaluació de la bioactivitat de materials bioceràmics de base fosfat de calci comercial per a la regeneració òssia

Guanyador del Premi P. Salvador Gil 2015 en la categoria de Bioenginyeria. (Lliurat el passat 27 de novembre de 2015 durant l'Assemblea General Ordinària de l'AIQS)

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RESUMEN

Las biocerámicas basadas en fosfato cálcico presentan un elevado potencial para su aplicación en la regeneración ósea gracias a su similitud con la estructura química de la biocerámica presente en los huesos y dientes de los mamíferos. Su utilización constituye una alternativa viable a la del uso de trasplantes autólogos u alografos para el tratamiento de enfermedades óseas. Este trabajo estudia la bioactividad de diferentes sustitutos óseos comerciales basados en fosfato cálcico, mediante la medida de su capacidad de intercambio iónico al sumergirlos en fluido corporal simulado (SBF, en inglés *simulated body fluid*).

Palabras clave: Regeneración ósea, biocerámica, bioactividad, intercambio iónico, polimerización por plasma

SUMMARY

Calcium phosphate-based bioceramics constitute a great promise for bone tissue engineering as they chemically resemble to mammalian bone and teeth. Their use is a viable alternative for bone regeneration as it avoids the use of autografts and allografts, which usually involves immunogenic reactions and patient's discomfort. This work evolves around the study of the bioactivity potential of different commercially available bone substitutes based in calcium phosphate through the characterization of their ionic exchange ability when immersed in simulated body fluid (SBF).

Keywords: Bone regeneration, bioceramics, bioactivity, ionic exchange, plasma polymerization

RESUM

Les bioceràmiques basades en fosfat càlcic presenten un elevat potencial per a la seva aplicació en la regeneració òssia gràcies a la seva similitud amb l'estructura química de la bioceràmica present en els ossos i dents dels mamífers. La seva utilització és una alternativa viable a la de l'ús de trasplantaments autòlegs o al·loempelts per al tractament de malalties òssies. Aquest treball estudia la bioactivitat, de diferents substituts ossis comercials basats en fosfat càlcic, mitjançant la mesura de la seva capacitat d'intercanvi iònic al submergir-los en fluid corporal simulat (SBF, en anglès simulated body fluid).

Paraules clau: regeneració òssia, bioceràmica, bioactivitat intercanvi iònic, polimerització per plasma.

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INTRODUCTION

Nowadays, autografts (a patient's own bone) remain the gold standard for bone repair followed by allografts (bone from another subject of the same species). Autografts and allografts have major advantages such being osteogenic or osteoinductive, however they present several disadvantages. As for the autografts, harvesting the tissue requires another second surgical practice, with well documented complications and discomfort for the patient[1]. Moreover, it must be taken into account that the quantity of tissue that can be extracted is limited. Allogeneic bone-grafting constitutes an alternative to autografts, as it overcomes the problem with harvesting and quantity, however, it presents issues of immunogenicity and rejection reactions and possibility of infection transmission[2]. Thus, there is the need for developing synthetic alternative materials to overcome the limitations of both autografts and allografts.

Among all the synthetic biomaterials for bone regeneration, calcium phosphate bioceramics are those which chemically resemble more to the mammalian bones and teeth[3]. **Table 1. Calcium orthophosphates main features4**presents the solubility product constants (Ksp) of the most commonly used calcium orthophosphates in biomedical applications, among others.

Table 1. *Calcium orthophosphates main features[4]*

Ca/P molar ratio	Compound	Formula	-log (K_{sp}) at 25ºC	-log (K_{sp}) at 37ºC
1	Dicalcium phosphate (DCP)	$CaHPO_4$	6,9	7,02
1,33	Octacalcium phosphate (OCP)	$Ca_8H_2(PO_4)_6{\cdot}5H_2O$	96,6	95,9
1,5	b-tricalcium phosphate (a-TCP)	b-$Ca_3(PO_4)_2$	25,5	25,5
1,5	b-tricalcium phosphate (b-TCP)	b-$Ca_3(PO_4)_3(OH)$	28,9	29,5
1,67	Hydroxyapatite (HA)	$Ca_5(PO_4)_3(OH)$	58,4	58,6
2	Tetracalcium phosphate (TTCP)	$Ca_4(PO_4)_2O$	38-44	42,4

It is important to mention that only those compounds which present a Ca/P molar ratio higher than 1 are suitable for biomedical applications, since a Ca/P ratio less than 1 implies a high solubility and acidity when implanted into the body[5]. The majority of available bioceramics are based on HA, β-TCP, α-TCP and/or biphasic calcium phosphate (BCP) which is an intimate mixture of β-TCP+HA or α-TCP+HA. Hydroxyapatite, compared to α-TCP and β-TCP, presents a higher stability under physiological conditions as it has a lower solubility and thus, a lower resorption rate by the surrounding bone tissue. Therefore, BCP formulations aim to present the optimum balance between a more stable HA phase and a more soluble TCP for a specific application. It must be noticed that an increase of TCP/HA ratio increases BCP reactivity, thus its in vivo bioresorbability can be controlled through the phase composition[6]. The control of the bioresorbability of the bone graft is critical, as the dissolution of the bioresorbable material allows the newly formed tissue to grow into the defect site. Thus, bioresorbable materials are able to participate in dynamic processes of formation and resorption occurring in bone tissues and hence they constitute a good option to be used as scaffolds or filling spacers allowing their infiltration and substitution of the tissues[7].

Back in 1990 Kokubo et al. reported for the first time the use of SBF in order to assess the bone-like apatite formation onto a bioactive glass ceramic[8]. Based on their results, in 1991, they proposed that the essential requirement for a material to bond to living bone is the formation of bone-like apatite on its surface when implanted in the living body and that this in vivo apatite formation can be reproduced in SBF[9]however, had explained the bone-bonding mechanism of Ceravital-type apatite-containing glass-ceramic without mentioning formation of the surface apatite layer. In the present study, apatite formation on the surface of one of Ceravital-type glass-ceramics was investigated in vitro as well as in vivo. An apatite-containing glass-ceramic of the composition Na2O 5, CaO 33, SiO2 46, Ca(PO3. Since then, numerous studies have been published which use SBF to evaluate bioactivity[10,11].

This study focuses on the bioactivity study of calcium phosphate-based bone substitute materials that can be used for bone regeneration in dental and medical applications. To do so, based on previous results[10,11], we have measured their ionic exchange ability with SBF. Moreover, we have evaluated the effect of the presence of a pentafluorophenyl methacrylate (PFM) coating over the ionic exchange ability of a commercial hydroxyapatite.

The interest of using PFM coatings arises from its ability to react with amines through a highly reactive ester group[12,13], which constitutes a mechanism to anchor cell-signaling biomolecules (**Figure 2**) enabling the bone substitute to induce a desired cell behavior.

MATERIALS AND METHODS

Five commercial calcium phosphate based-bone substitutes have been analyzed. Table 2 summarizes their properties, the main difference being the composition, shape and particle size..

Table 2. *Main specifications of the studied bone substitutes*

Sample	Particle Size (mm)	Composition
Sample 1	150-500	β-TCP
Sample 2	0,25-1	Bovine HA
Sample 3	500 - 1000	HA/β-TCP (60:40)
Sample 4	--	HA

X-Ray Diffraction (XRD)

Prior to XRD analysis the five samples were grinded into powder. The phase analysis of the HA powders was carried

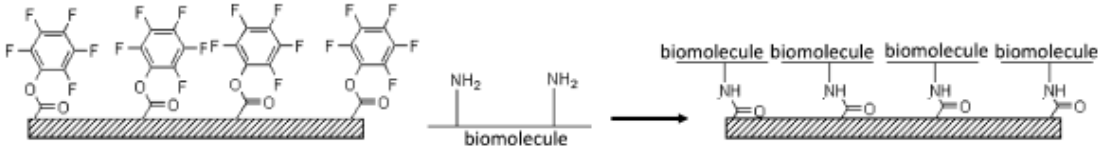

Figure 1. *Biomolecules attachment onto PFM coatings[14]*

out employing an X-Ray diffractometer (Bruker D-5005) using a Cu Ka radiation generated at 40kV and 100 mA.

Bone substitutes interaction with physiologic media

The interaction of the hydroxyapatite – based bone substitutes with physiologic media was determined by soaking the samples in the interest solutions. In analyzed bone substitutes were in form of granules or powder. Using a hydraulic press (10 tones), they were compacted into discs of about 13 mm of diameter and 1,5 mm of height.

The hydroxyapatite discs were immersed at 37ºC into simulated body fluid (SBF), which was prepared by dissolving the following compounds into 500 ml of Milli-Q water, and adjusting the pH with 3.029 g of tris-hydroxymethylaminomethane and HCl to 7.4: 3.273 g of NaCl, 1.134 g of $NaHCO_3$, 0.186 g KCl, 0.0339 g of $Na_2HPO_4 \cdot 12\ H_2O$, 0.152 g of $MgCl_2 \cdot 6H_2O$, 0.184 g of $CaCl_2 \cdot 2H_2O$ and 0.036 g of Na_2SO_4.

pH and calcium concentration measurements were performed at different time points in order to assess the ionic exchange ability of the samples. Changes in calcium concentration were determined by atomic absorption spectroscopy

Atomic absorption spectroscopy (AAS)

Calcium concentration was determined using a 2280 Atomic Absorption Spectrophotometer (Perkin – Elmer). Calibration of the instrument was performed using 0.5, 1, 2.5 and 5 ppm calcium carbonate standard solutions. In order to avoid interferences, both the standards and the samples had to be mixed with lanthanum oxide (La_2O_3). The La_2O_3 stock solution was prepared by mixing 5.865g of the oxide, 50 ml of Milli-Q water and 254 ml of HCl(c) and posterior dilution up to 100 ml in a volumetric flask. The samples and standards where mixed with La_2O_3 maintaining a ratio of 1:2 respectively.

Hydroxyapatite surface modification by PECVD

Before performing the superficial modification the samples were compacted into disks using a hydraulic press following the procedure described above. The hydroxyapatite surface was modified with a coating of Pentafluorophenyl methacrylate (PFM, 95% Sigma-Aldrich) using a cold plasma reactor at low pressure[15]. Briefly, the monomer (PFM) is introduced inside the reactor at nearly constant pressure around 0,02-0,04 mbar. Once pressure is stable the continuous radio frequency power is fixed at 15W and a pulsed plasma polymerization duty cycle (DC) of 10/20 is carried out during 5 minutes.

RESULTS AND DISCUSSION

Soaking the bioceramic bone substitutes with physiologic media

Among all the SBF that have been formulated, we have used the one described by Takadama et al. in 2004[16]. **Table 3. Comparison of ions concentration between blood plasma and SBF**shows a comparison between the ionic concentrations of blood plasma and SBF.

Table 3. *Comparison of ions concentration between blood plasma and SBF*

Ion concentration / mM	Na^+	K^+	Ca^{2+}	Mg^{2+}	HCO_3^-	Cl^-	HPO_4^{2-}	SO_4^{2-}
Blood Plasma	142.0	5.0	2.5	1.5	27.0	147.8	1.0	0.5
SBF	142.0	5.0	2.5	1.5	4.2	103.0	1.0	0.5

When the samples were soaked into SBF, all solutions underwent changes in pH and calcium concentration. As it can be seen in **Figure 1** both the pH and [Ca^{2+}] evolution profile

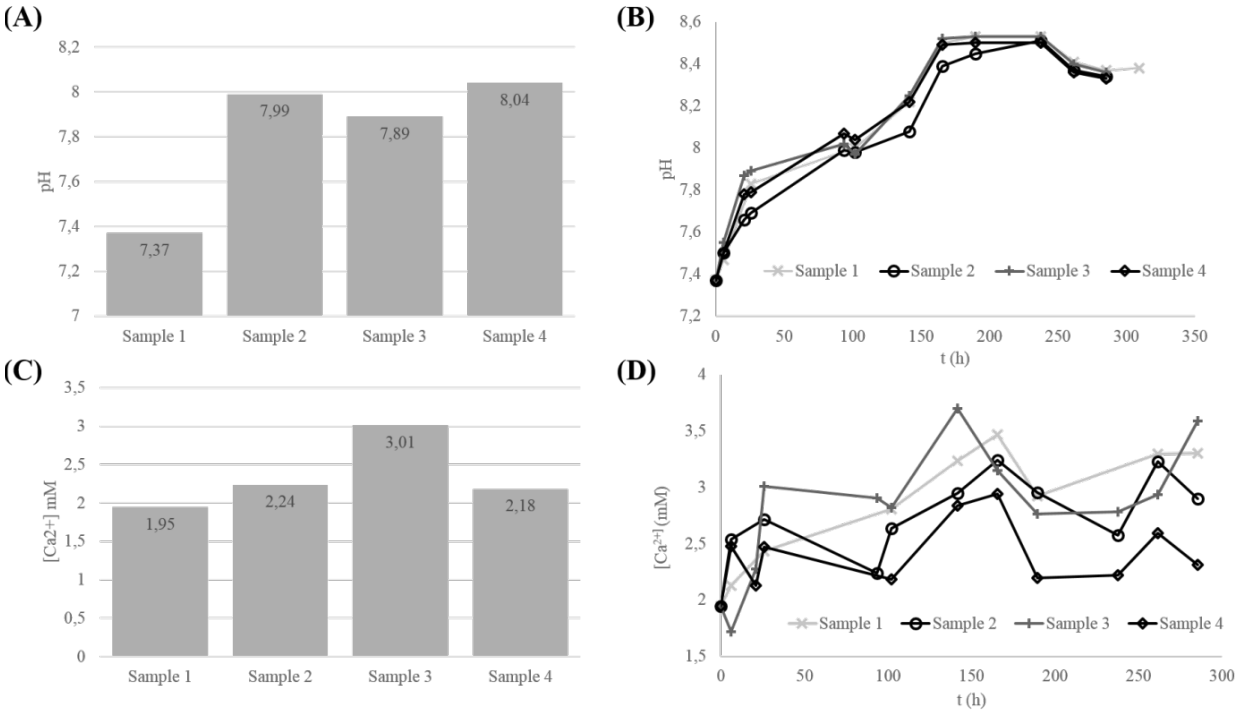

Figure 2. *Calcium phosphate-based samples ionic exchange ability* ***(A)*** *pH evolution of SBF solution during sample's immersion period.* ***(B)*** *pH value of SBF solution after 24 hours of soaking.* ***(C)*** *Calcium concentration at SBF solution evolution during sample's immersion period.* ***(D)*** *Calcium concentration at SBF solution after 24 hours of soaking.*

is very similar for all the samples. It must be noticed how every change of pH occurs simultaneously with a change of [Ca^{2+}], thus, we can be certain that the measured pH changes are due to the interaction between the immersed calcium phosphate disk and the surrounding medium. In order to make comparisons between the studied samples, we will use the pH evolution profile, as it reflects all the events that are taking place during the ionic exchange.

The initial dissolution of β-TCP and HA causes an increase in both calcium concentration and pH. In agreement with previous studies[11,17], we hypothesize that due to the dissolution of β-TCP and HA, there is an initial release of calcium ions to the SBF solution, reflected by an increase of pH caused by the absorption of H_3O^+ from the solution. Following the initial increase, SBF pH value seems to stabilize, however, a third phase in which the pH decreases, can be distinguished. It can be explained considering that the latter is caused by the precipitation of HA or β-TCP, which leads to an absorption of both OH- and HPO^{3-} ions from the solution to the sample disk. As it is shown in **Table 1**, β-TCP presents a higher solubility than HA at 37ºC, so one may think that the initial dissolution would take place faster for sample 1, which is composed of pure β-TCP. However, as said before, the degree of crystallinity has a strong relation with the ionic exchange ability of the sample. A higher degree of crystallinity can be associated with a higher thermodynamic stability, so the most crystalline samples tend to present lower solubilities than those less crystallines. Thus, samples such as sample 3 (less crystallines than sample 1) which consist in a mixture of HA and β-TCP (60:40), show faster initial dissolution rates, achieving the higher pH value and calcium concentration within the first 24 hours of immersion. As it can be seen in **Figure 1 A-C**, the faster release occurs during the first 24 hours of immersion, and the values reached for every studied sample are shown in **Figure 1 B-D**.

For all the bone substitutes, the increase of the pH value that takes place within the first 24 hours represents more than a 30% of the total pH increase. This reflects that the dissolution of the disk surface occurs almost completely during this period.

Next, the effect of a PFM coating on the bioactivity of a commercial hydroxyapatite (Plasma Biotal Ltd.) disk is analyzed when soaked into both SBF and α-minimum essential media (α-MEM). The latter contains proteins and vitamins and provides a more realistic insight of what would occur in the human body. To do so, the HA disks were immersed in the interest solutions during 28 days at 37ºC. To avoid calcite ($CaCO_3$) formation, the medium was changed every two days.

As it can be seen in **Figure 3**, both SBF and α-MEM allow for precipitate formation on the HA surface. The precipitate distribution lacks of homogeneity in all samples, as crystal growth seems to be localized at some points. The latter gives evidence of a mechanism of heterogeneous nucleation, which was expected, as the beginning of apatite formation on calcium phosphate ceramics from SBF solution is usually considered as a process of heterogeneous nucleation[18, 19].

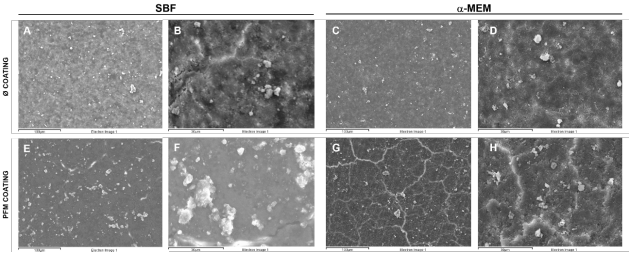

Figure 3. *SEM images of Hydroxyapatite discs after 28 days soaked both in α-MEM and SBF*

Moreover, it can be seen how the PFM coating doesn't block the precipitation events, but instead it seems to provide nucleation points which help the precipitate formation. As said before, the PFM contains a highly reactive ester group which is able to react with the amino acids present in the α-MEM solution, thus the anchorage of some amino acids to the PFM coating will provide the nucleation points and trigger heterogeneous nucleation. By comparing **Figure 3B** and **F**, it can be clearly appreciated that for the PFM-coated disk the formed crystals are bigger and precipitation is more extended.

As for the influence of the soaking solution, differences are more evident for the PFM coated samples. It can be seen how when the sample is soaked into SBF, more crystals are formed over the entire surface. This may suggest that the amino acids present in the α-MEM, attach covalently to the PFM coating through their amino groups and thus, block the nucleation sites which help the precipitation of calcium phosphate salts. It seems that amino acids play an important role in hydroxyapatite precipitation, and different studies reveal how they can play both inhibitory and enhancing effect [20,21]. However, with this assay we have proved the ability of a PFM coating to interact with physiologic medium and to improve salt deposition.

The evaluation of the bioactivity of the different samples has provided insight on the design of a bioceramic suitable for bone grafting. Future work is required to assess effect of the resorption rate over cell growth and tissue formation.

CONCLUSIONS

The comparative study of the ionic exchange ability of different commercial bioceramic bone substitutes and medical grade titanium disks has been performed. Moreover, the effect of a PFM coating over the ionic exchange ability of a commercial hydroxyapatite has been evaluated:

As for the bioceramic bone substitutes the obtained results show how both the chemical composition and the degree of crystallinity have a great influence over the interaction ability of the samples with the surrounding medium. In spite of the higher solubility of the β-TCP with respect to HA, the samples composed by a mixture of β-TCP and HA presented a higher ease of interaction than those composed of pure β-TCP. As the latter presents a higher degree of crystallinity, the initial surface dissolution needed to trigger the ionic exchange events occurs with greater difficulty. Thus, the changes in a bioceramic integration ability can be achieved by modifying the β-TCP/HA ratio to achieve the desired effect.

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