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CHAPTER 1: Introduction and objectives

Calcium phosphate materials are widely used in bone tissue repair owing to their close similarity in composition and structure to the mineral phase of bone and find numerous applications in the biomedical field. They are composed of calcium, phosphorus, oxygen and in some cases hydrogen atoms forming crystalline compounds with different physicochemical properties. In spite of the many years of research with these materials, there are aspects that remain to be investigated. For example, in spite of possessing excellent bioactivity, osteoconductivity and biocompatibility *in vivo*, the behavior of calcium phosphates *in vitro* is not always satisfactory. Previous studies with calcium phosphate cements had showed hampered cell proliferation and differentiation during *in vitro* studies.

It is believed that the behavior of calcium phosphates cements during *in vitro* studies is ruled by several factors such as surface topography, specific surface area and/or chemical solubility. All these factors can influence the continuous ion exchange with media environment, which make difficult to clearly determine which of those factors is dominant.

Thus, the objective of this work focuses on the understanding of the cell behavior of two types of calcium phosphate materials, i.e. alpha-tricalcium phosphate (α -TCP) and calcium deficient hydroxyapatite (CDHA), using two different cell types. The two tested materials correspond to the initial (before setting: α -TCP) and the final composition (after setting: CDHA) of a typical calcium phosphate cement. The fact that cements are materials that can be injected into the bony site make this study very relevant as gives the overall cell behavior since an injectable cement is introduced in the body till it fully sets (harden). Cell culture studies are simplified studies that give us an initial screening of the potential reactions that a material might undergo after implantation and become a key tool in the prediction of the behavior of materials prior to implantation. In the present work it will be investigated how the chemical composition, crystalline structure, ion release and the topography of the two materials influences the cell culture behavior.

1.1. General objective

The main objective of this project is to assess the *in vitro* cell behaviour of two different calcium phosphate compounds, one obtained at low temperatures- Calcium Deficient Hydroxyapatite (CDHA), and another obtained at high temperatures-Alpha-Tricalcium phosphate(α -TCP). These materials have the same Ca/P ratio, but they differ in chemical formula, crystalline structure, surface topography and solubility. It is also the goal to assess which of the above-mentioned factors have major implications in cell behaviour.

1.2. Partial objectives

- To establish a protocol of obtaining the above-mentioned calcium phosphates.
- Physicochemical characterization of the materials: composition, microstructure, specific surface area and solubility.
- Assessment of the materials behaviour during *in vitro* study using two different cell lines: SAOS-2 (Sarcome Osteogenic cell line) and rMSCs (rat Mesenchymal Stem Cells).

CHAPTER 2: Background theory

2.1. Bone

According to Moby's Medical Dictionary, bone tissue is a "*hard form of connective tissue composed of osteocytes and calcified collagenous intercellular substance arranged in thin plates*"[1].

In adult skeleton there are two types of bone tissue: the outer layer of the bone is dense and called "compact bone" (also called "cortical bone"), while the inside part of the bone is spongy and named "cancellous bone" (or "trabecular bone") [2][3].

2.1.1. Chemical composition

Bone is a perfect composite material. The organic matrix of bone is composed mostly of proteins with collagen type I as the most abundant one among them. This protein is responsible for the flexibility of bone. However, cell activity and tissue biomineralization is controlled by a number of nanocollagenous proteins[5].

Biological hydroxyapatite is the main mineral component of bone. It is an insoluble calcium phosphate ceramic representing about 65 % of the adult bone mass. The chemical formula of hydroxyapatite is $\text{Ca}_{10}[\text{PO}_4]_6[\text{OH}]_2$. In bone, hydroxyapatite incorporates other elements such as magnesium, sodium, hydrogenphosphate and bicarbonate. Adult bone contains, in addition, approximately 25 % of water [FIGURE 1] [6].

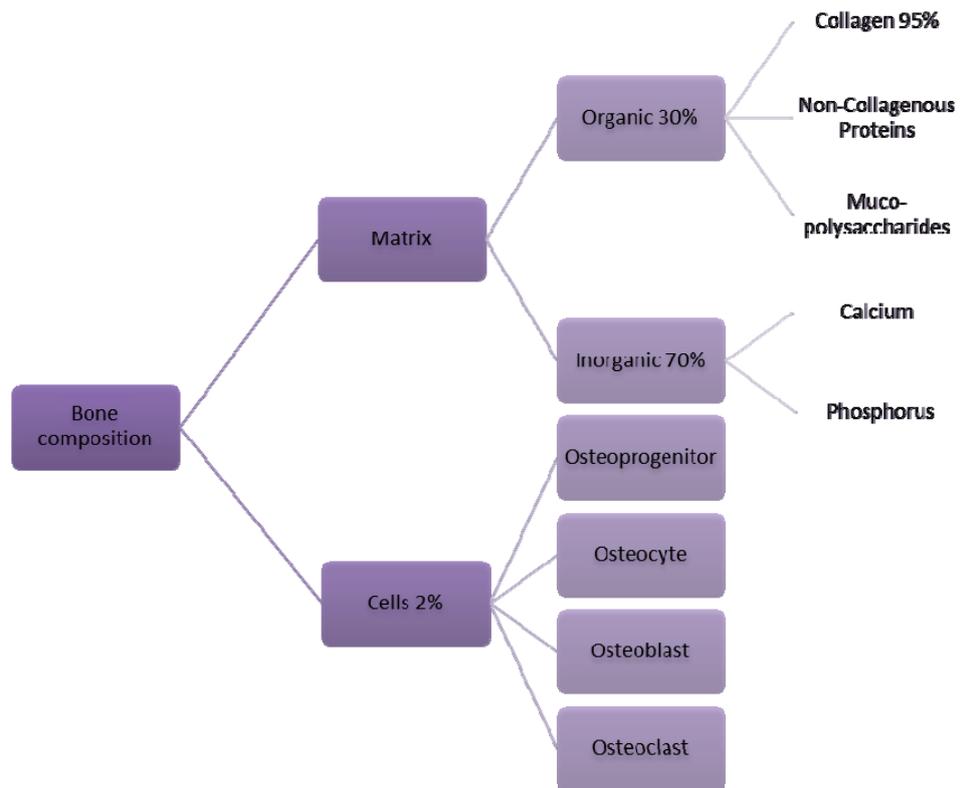


Figure 1 Bone composition [7].

2.1.2. Functional organization of bone cells

We can differentiate four types of bone cells: osteoblasts, osteocytes, osteoclasts and line cells. Osteoblasts are cubical or columnar cells with nucleus placed in the center on the bone surface. They come from mesenchymal stem cells (MSCs can also turn into fat, muscle, cartilage, skin or tendon cells depending on biochemical factors). Their basic function is the production and secretion of organic and inorganic bone extracellular matrix (ECM) called the osteoid. These cells are responsible for the skeletal architecture in two ways, they produce bone matrix and regulate the activity of osteoclasts. They are able to communicate among each other due to gap junctions between them. Osteoblasts have ability to detect some of the hormones: vitamin D, estrogen and parathyroid hormone among other. They can secrete factors which activate osteoclasts. They liberate a protein helping with regulation of the amount of phosphate excreted by the kidneys. After finishing production of the new bone

some osteoblasts get encapsulated in bone matrix and become osteocytes, others stay on the bone surface and differentiate into lining cells. Finally some amount of osteoblasts undergo an apoptosis and disintegrate [8], [9],[10]. Osteocytes are osteoblast that becomes entrapped within the bone matrix and they play a key role in mechanotransduction. their position allows them to sense mechanical strain of the bone. These cells are able to secrete growth factors which in turn activate either lining cells or stimulate the osteoblasts. It is possible that osteocytes can control bone remodeling. They can communicate between each other and with lining cells. [8], [9], Osteoclast is the third cell type in bone. It is a multinucleated cell formed through connection of mononuclear hematopoietic precursors and they share lineage with blood cells. Their precursors circulate in blood, they join and form large cells with many nuclei – osteoclasts. Fusion of precursors is provoked by RANK-ligand activated by osteoblasts or binded by osteoprotegerin. The primary function of osteoclasts is to secrete acids and proteolytic enzymes, which erode bone ECM under the influence of chemical cues. Afterwards when bone is resorbed osteoclasts undergo apoptosis [7],[11]. Bone remodeling is one of the processes influenced by production and resorption of bone ECM by osteoblasts and osteoclasts [5], [8]. As [FIGURE 4](#) shows “*Bone remodelling depends upon a functional interaction between the three major cell types. Bone formation is carried out by the osteoblasts (green) that lie on the cell surface. As the bone forms, it entraps the osteoblasts that gradually transform into osteocytes (blue). These have long extensions that make contact via gap junctions both with themselves and with the other two cell types (red arrows). Bone resorption is carried out by the multinucleated osteoclasts.*” [8]

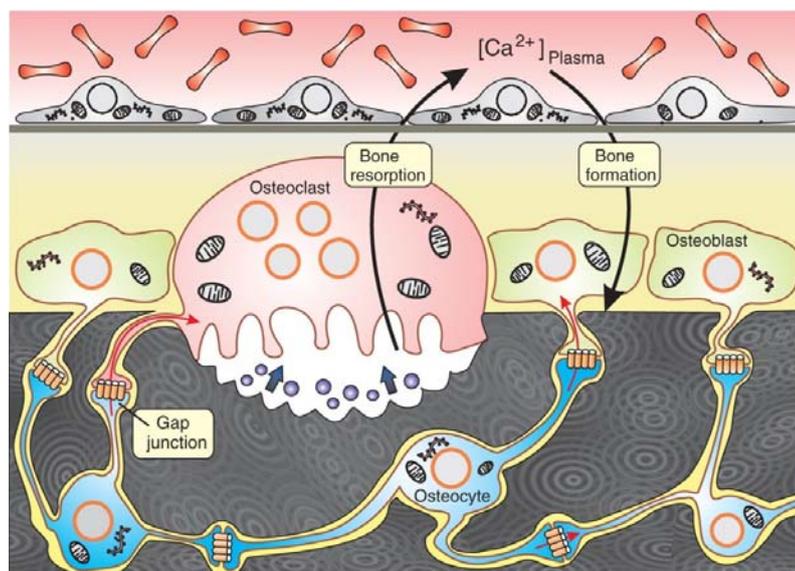


Figure 2 Functional organization of bone[8].

2.2. Calcium Phosphate materials

Calcium Phosphate (CaP) materials play an important role in biomaterials field, in particular, as bone substitutes. The most important parameters are the Ca/P molar ratio which vary from 0.5 to 2.0, basicity/acidity, solubility and composition [1], [11]. All of these factors may affect their *in vitro* and *in vivo* behaviour.

2.2.1. The members of the calcium phosphate family

In the following [TABLE 1](#) are listed the members of the CaP family with their main properties.

Table 1 Existing calcium phosphates [1].

| Ca/P ionic ratio | Compound and its abbreviation | Chemical formula | Solubility at 25°C, log(K _s) | Solubility at 25°C, g/L | Stability in aqueous solutions at 25°C (pH range) |
|------------------|-------------------------------|---|--|-------------------------|---|
| 0.5 | MCPM | Ca(H ₂ PO ₄) ₂ ·H ₂ O | 1.14 | ~18 | 0.0-2.0 |
| 0.5 | MCPA | Ca(H ₂ PO ₄) ₂ | 1.14 | ~17 | [c] |
| 1.0 | DCPD | CaHPO ₄ ·2H ₂ O | 6.59 | ~0.088 | 2.0-6.0 |
| 1.0 | DCPA | CaHPO ₄ | 6.90 | ~0.048 | [c] |
| 1.33 | OCP | Ca ₈ (HPO ₄) ₂ (PO ₄) ₄ ·5H ₂ O | 96.6 | ~0.0081 | 5.5-7.0 |
| 1.5 | α-TCP | α-Ca ₃ (PO ₄) ₂ | 25.5 | ~0.0025 | [a] |
| 1.5 | β-TCP | β-Ca ₃ (PO ₄) ₂ | 28.9 | ~0.0005 | [a] |
| 1.2-2.2 | ACP | Ca _x H _y (PO ₄) _z ·nH ₂ O, n=3-4.5; 15-20% H ₂ O | [b] | [b] | ~5-12 d |
| 1.5-1.67 | CDHA ^[e] | Ca _{10-x} (HPO ₄) _x (PO ₄) _{6-x} (OH) _{2-x} ^[f] (0<x<1) | ~85.1 | ~0.0094 | 6.5-9.5 |
| 1.67 | HA | Ca ₁₀ (PO ₄) ₆ (OH) ₂ | 116.8 | ~0.0003 | 9.5-12 |
| 1.67 | FA | Ca ₁₀ (PO ₄) ₆ F ₂ | 120.0 | ~0.0002 | 7-12 |
| 2.0 | TTCP | Ca ₁₀ (PO ₄) ₆ ^o | 38-44 | ~0.0007 | [a] |

^[a] These compounds cannot be precipitated from aqueous solutions.

^[b] Cannot be measured precisely. However, the following values were found: 25.7 ± 0.1 (pH = 7.40), 29.9 ± 0.1 (pH = 6.00), 32.7 ± 0.1 (pH = 5.28).

^[c] Stable at temperatures above 100 °C.

^[d] Always metastable.

^[e] Occasionally, CDHA is named as precipitated HA.

^[f] In the case $x = 1$ (the boundary condition with Ca:P = 1.5), the chemical formula of CDHA looks as follows: $\text{Ca}_9(\text{HPO}_4)(\text{PO}_4)_5(\text{OH})$.

In spite of the biological and clinical relevance of the listed calcium phosphates, for the purpose of the present project two types of CaP materials were tested and only those are described in detail below: CDHA and α -TCP.

2.2.1.1. Alpha-Tricalcium Phosphate (α -TCP)

The chemical compound calcium phosphate tribasic alfa commonly named α -tricalcium phosphate or simply α -TCP, has the same chemical composition than β -TCP which is $\text{Ca}_3(\text{PO}_4)_2$ but different crystalline structure. α -phase is more soluble, more reactive in aqueous system and has higher specific energy but is less stable than β -phase. To obtain α -TCP, β -TCP is heated above 1125°C and after that quenched to prevent the reverse transformation. Alternatively, α -TCP can be obtained via solid state reaction of CaHPO_4 and CaCO_3 at 1400 °C, following by quenching [Eq. 1].

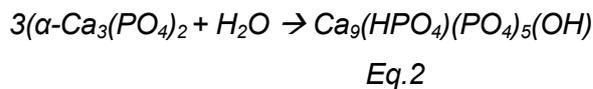


Pure α -TCP is hardly ever applied in biomedical field. Mostly it's readily transformed into CDHA with Ca:P molar ratio of 1.50 in an aqueous solution. This property is used to prepare apatite calcium phosphate cements (CPCs) and α -TCP has become the major component of most apatite cements. [1], [12].

2.2.1.2. Calcium Deficient Hydroxyapatite (CDHA)



Calcium-deficient hydroxyapatite (abbreviation CDHA) is described by the general formula: $\text{Ca}_{10-x}(\text{HPO}_4)_x(\text{PO}_4)_{6-x}(\text{OH})_2$, where: $0 < x < 1$. This compound can be easily obtained by hydrolysis of a metastable CaP (e.g. α -TCP) in aqueous media in what is known as calcium phosphate cement (CPC) setting reaction. In case of hydrolysis of α -TCP, the setting kinetic depends on surface area of reactants, and the Ca/P ratio of the end product correspond to 1.5, equivalent to the chemical reaction showed in equation 2. Furthermore, addition of ~ 2 wt. % pHA (precipitated hydroxyapatite) as a seed in the powder phase is useful to accelerate the kinetics of reaction and faster re-crystallization to CDHA upon contact with water [1], [12] [Eq. 2].



2.2.2. Calcium phosphate cements

Calcium phosphate cements (CPC) consist in the mixture of a calcium phosphate powder with an aqueous liquid to form a paste. The paste is malleable and injectable and for example it can be used to fill bone defects. After implantation the paste set and harden *in vivo*. CPC have three different final products: apatite, brushite or amorphous calcium phosphate (ACP). According to the only study about the third possible end-product ACP is very rapidly changed into pHA. Consequently we divide CaP into two categories: apatitic and brushitic. During the setting the cement powder dissolves and precipitates into a new CaP characterized by a lower solubility. This precipitation process implies the formation of many crystals that they entangle as they grow thus providing mechanical strength to the cement [1]. Due to the fact that CPC are similar to the mineral phase of bone they are widely approved in the biomedical field. In fact, they are excellent materials for bone regeneration *in vivo* as they are bioactive, osteoconductive and biocompatible, however, there *in vitro* behavior is not satisfying [13], [14]. During *in vitro* studies cell proliferation and differentiation seems to be hampered on the calcium phosphate cement surface. It is difficult to determine which factor has greater influence on cell behaviour if topography or ion exchange. Therefore this work focuses on investigating the behaviour of calcium phosphates at the cellular level.

2.2.3. Bioresorption and Replacement of calcium phosphate cements by Bones

Two mechanisms of resorption of CPC are known, active and passive. The first mechanism is mediated by cells, such as, macrophages and osteoclasts among other. Passive mechanism is based on chemical dissolution or, in case of brushite, chemical hydrolysis in body fluids. Biological behavior of CPC is determined by many factors, for example: chemical composition, physical properties and crystal structure. One of the most important properties is solubility in water because the in vivo behaviour of CPC can be predicted based on them solubility. In case of too soluble phosphates, cells do not adhere well what can cause the risk of poor implant fixation. In contrast, CPC with low solubility are not resorbable materials, impairing the complete regeneration of the bone defect.

Numerous types of cells colonize surface of material. In consequence mechanism of bone healing is affected by many factors. Osteoclasts, monocytes degrade bone by phagocytotic mechanisms. Acidic mechanism of bone resorption takes place when proton pump reduces pH and osteoclasts resorb hardened ceramic. In similar manner, CPC can be reabsorbed by those cells because their chemical composition is quite similar to that of bone.

The important advantage of CPC is that before being replaced by a new bone they provide short-term biologically desirable properties. New bone is forming with different speed not only depending on material characteristics but also on age, sex or general metabolic health. Generally it takes between 3 and 36 months for CDHA to be entirely resorbed and replaced by new bone, In contrast for stoichiometric HA it can remain intact for several years. If resorption takes place too fast collapse at the fracture site might occur. To avoid that rate of phosphate resorption and bone formation should be balanced [15].

CHAPTER 3: Materials and methods

2.3. Materials preparation

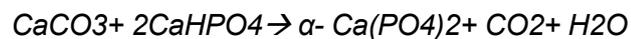
In the sections below will be described the routes that have been followed to prepare calcium phosphate materials: CPC that was pre-set to obtain CDHA discs, used as model for the set composition and sintered α -TCP disks used as a model of the initial composition of the cement when it is implanted

2.3.1. Calcium phosphate cement

CPCs are readily obtained mixing appropriate amounts of a solid phase with a liquid phase. The detailed procedure for the preparation of both phases is described below.

Solid phase

The solid phase was obtained according to the following protocol. First of all α -TCP was synthesized. **150 g of α -TCP** was prepared by sintering a stoichiometric mixture of **48,40 g CaCO_3 (Sigma-Aldrich)** and **131,59g CaHPO_4 (Sigma-Aldrich)** according to solid state reaction [Eq 3.1] To ensure homogeneity of the mixture, both powders were mixed during 15 minutes using a Whip Mix mixer. The mixture was then placed in platinum crucible and was inserted in the furnace to apply thermal treatment according to protocols; the treatment was followed by quenching in air.



Eq. 3

The following [TABLE 2](#) presents steps of thermal treatment which was applied to obtain α -TCP

Table 2 Stages of the synthesis of α -TCP.

| Stage | T_0 [°C] | T_f [°C] | Velocity [°C/min] | Δ Time[min] | Reaction |
|-------|------------|------------|-------------------|--------------------|--|
| 1 | 25 | 300 | 2,5 | 120 | |
| 2 | 300 | 300 | - | 120 | Decomposition of monetite into calcium pyrophosphate and water $2\text{CaHPO}_4 \rightarrow \text{Ca}_2\text{P}_2\text{O}_7 + \text{H}_2\text{O}$ |
| 3 | 300 | 1100 | 2,5 | 320 | Decomposition of carbonate into calcium oxide $\text{CaCO}_3 \rightarrow \text{CaO} + \text{CO}_2$ |
| 4 | 1100 | 1100 | - | 120 | Solid state reaction to obtain β -TCP $\text{CaO} + \text{Ca}_2\text{P}_2\text{O}_7 \rightarrow \beta\text{-Ca}_3(\text{PO}_4)_2$ |
| 5 | 1100 | 1400 | 2,5 | 120 | |
| 6 | 1400 | 1400 | - | 120 | Obtention of the α phase of TCP $\beta\text{-Ca}_3(\text{PO}_4)_2 \rightarrow \alpha\text{-Ca}_3(\text{PO}_4)_2$ |
| 7 | 1300 | 25 | 1275 | 1 | Retention of α phase at low temperature |

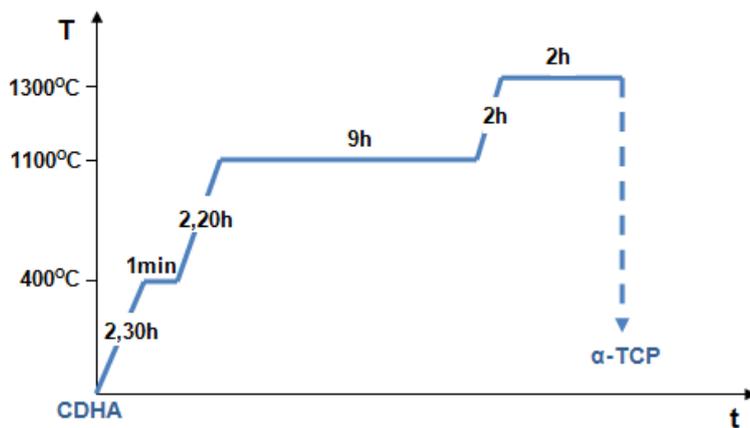


Figure 3 Thermal cycle of synthesis of α -TCP.

After synthesis, α -TCP was milled with a planetary milling system (*Pulverisette 6, Fritsch GmbB*) using agate container and agate balls to obtain the cement powder. The powder was milled with following protocol:

- 10 balls (d=30 mm) for 15 min at 450 rpm.

After milling process to α -TCP was added 2%wt of pHA precipitated hydroxyapatite (*tricalcium phosphate, Merck*). To obtain homogenous mixture, powder was placed at the “Whip Mix” for 10 minutes. The cement obtained was kept under dry conditions.

Liquid phase

The liquid phase consisted of an aqueous solution of 2.5 wt.% disodium hydrogen phosphate, Na_2HPO_4 (*Panreac 131679.1210*), added to accelerate the setting reaction of the cement.

To obtain moldable paste for discs the powder and liquid phase were first mixed in a mortar for about 90 s (L/P ratio of 0.65 ml/g). Then paste was transferred into cylindrical molds 15 mm in diameter and 2.5 mm high.

A CPC was used as starting material to obtain the different phosphates studied in this work.

2.3.2. Obtaining Calcium Deficient Hydroxyapatite discs

To obtain the CDHA discs, either the type I or type II cement discs were kept in 100% relative humidity for 1 h, afterwards they were immersed in distilled water and stored in a heater at 37 °C for 7 days where were allowed to react into CDHA [\[Eq 8.\]](#)



2.3.3. Obtaining alpha-tricalcium phosphate discs

To obtain alpha-tricalcium phosphate discs (α -TCP discs), CDHA discs were placed



in a platinum crucible and were heat-treated up to 1400C following the thermal treatment described in Table 3.

| Stage | T ₀ [°C] | T _f [°C] | Velocity [°C/min] | ΔTime[min] |
|-------|---------------------|---------------------|-------------------|------------|
| 1 | 25 | 400 | 2,5 | 150 |
| 2 | 400 | 400 | - | 1 |
| 3 | 400 | 1100 | ~5 | 140 |
| 4 | 1100 | 1100 | - | 540 |
| 5 | 1100 | 1400 | 2,5 | 120 |
| 6 | 1400 | 1400 | - | 240 |
| 7 | 1400 | 25 | 1375 | 1 |

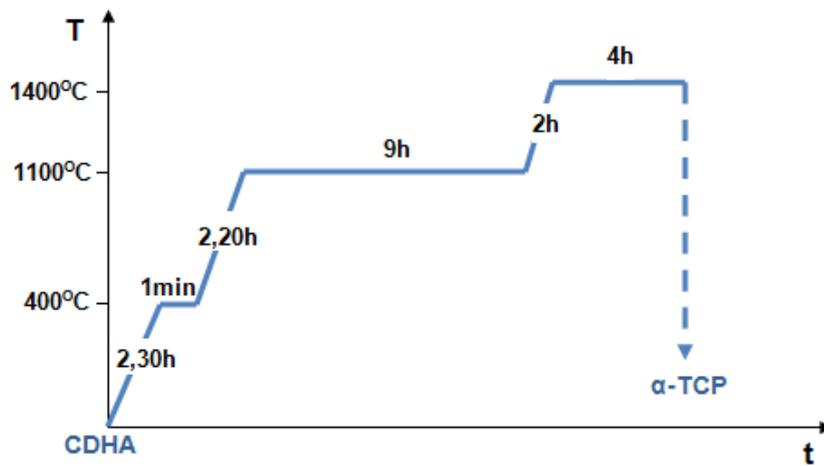


Figure 4 Thermal cycle of α -TCP discs synthesis

2.4. Cell Culture

For the cell culture studies there were used two different cell lines: SAOS-2 (Sarcome Osteogenic cell line) and rMSCs (rat Mesenchymal Stem Cells) which were cultured in direct contact with materials.

2.4.1. Cell culture preparation

All CaP discs used in the study were placed in sterile 24-well plate. Before cell seeding there was applied a protocol of sterilization. It consisted in placing 1 ml of 70% ethanol in each well with the CaP discs. The samples were soaked in ethanol for 3 hours and then, to eliminate the residue of ethanol, they were washed with sterile PBS (phosphate buffer saline) 3 times, each time leaving them in contact with PBS for at least 15 minutes. The same protocol was applied for the tissue culture polystyrene (TCPS) plate which was used as control. To maintain a sterile environment and area free of potential contaminations all work was performed in a laminar flow cabinet.

Two types of cell culture media were used in the study according to the cell type. For SAOS-2 McCoy's 5A Modified Media (*Sigma- Aldrich*) was used and for rMCS Advanced Dulbecco's Modified Eagle Medium (Advanced DMEM; *Gibco*). Both media were prepared following standard preparation [TABLE 4](#), they were additionally supplemented with essential components; L-glutamine, penicillin/streptomycin and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid).

Table 4 Cell culture medium formulations.

| Essential components to obtain complete medium | Advanced DMEM | McCoy's |
|--|---------------|---------|
| Base medium (%) | 86 | 86 |
| L-glutamine (%) | 1 | 1 |
| Penicilin/Streptomycin (%) | 1 | 1 |
| HEPES (%) | 2 | 1 |
| Serum proteins | 10 | 10 |
| FBS (%) | | |

Subconfluent cell cultures were trypsinized in TrypLE (*Invitrogen*), centrifuged at 300

x g during 5 min and plated in flasks. Cells were maintained in a humidified 37°C incubator in 5% CO₂. Cells were used at passage 5.

Before seeding, the discs were kept overnight in the incubator at 37°C with 5 % of CO₂ immersed in cell culture media. The cell culture experiment lasted for 7 days and the cell media was refreshed every day. All withdrawn media were kept at room temperature for further analyses.

2.4.2. Cell seeding protocol

Cells were trypsinized, centrifuged and resuspended in the corresponding medium. Cells were counted using a hemacytometer and 80x10³ cells per disc were seeded. 1ml of media containing the cells was placed on each well containing the discs. Cells were allowed to adhere during 4h (Saos-2) or 6h (rMSCs) in the incubator before cellular assays.

2.4.3. LDH assay

In the present work proliferation assays with the Citotoxicity Detection Kit^{PLUS} (LDH) (Roche) were performed at 4-6h, 3 and 7 days of cell culture. To this purpose, at the pre-set days, the media in contact with the discs was withdrawn and kept for subsequent analyses, and lysis solution (M-PER) was added onto the discs surface to lyse the cells. For cell lysis there was used 500 µL and 300 µL of M-PER for SAOS-2 and rMSCs respectively. To perform LDH assay there was used 100 µL of M-PER and the instructions included in the Kit were followed. The absorbance was determined in a spectrophotometer plate WaveX Power, Bio-Tek Instruments, Inc. at a wavelength about 492 nm. Every assay was made per triplicate.

2.4.4. ALP assay

To detect alkaline phosphate activity SensoLyte® pNPP Alkaline Phosphate Assay Kit was used. The absorbance was determined in a spectrophotometer plate WaveX Power, Bio-Tek Instruments, Inc. at a wavelength about 405 nm. To perform differentiation test there was used 50 µL of M-PER (the same used to lyse cells before proliferation assay) and the instructions included in the Kit were followed. To express results of concentration of alkaline phosphatase a standard curve was created from a solution of p-nitrophenol (Sigma, ref.N7660-100ml) which corresponds to the product formed by the reagent catalysed by ALP



in basic medium. The results were divided by number of cells obtained during LDH assay multiplied by incubation time [16].

2.5. Physicochemical characterization

2.5.1. X-ray diffraction

XRD analysis was performed on the samples previously pulverized in an agate mortar to determine their composition. The assays were realized in *Philips MRD diffractometer*. The working conditions were a potential of 40 KV and intensity of 30 mA. The sweep was realized from 10 to 80 in the 2θ scale, with a pass increment of 0.020° and 150 s/steep.

2.5.2. Scanning Electron Microscope (SEM)

The microstructure of the discs prior to cell culture was asses by SEM. The sample was prepared as follow: The samples were coated with gold-palladium. The images of surface and transversal section of samples were obtained at different augmentations (2KX, 5KX and 10KX). To obtain images of the microstructure of materials, the samples were analyzed by scanning electron microscope- *Zeiss Neon 40*.

Cell morphology was also characterized by means of SEM. For that purpose, the samples were washed in 0.1M Phosphate buffer, pH 7.4, and cells were fixed with a 2.5% glutaraldehyde (*Sigma-Aldrich G400-4*) solution in PBS, washed and maintained in 0.1M phosphate buffer. Osmium tetroxide (*Sigma-Aldrich 201013*) was added after fixation, and graded ethanol solutions were used to dehydrate the samples (50, 70, 90, 96 and 100% ethanol). Finally, hexamethyldisilazane (*HDMS, Fluka 52620*) was used for complete dehydration of the samples, being air-dried afterwards. Finally, the dried samples were covered with a gold-palladium thin film and images were acquired using FIB-SEM (*Zeiss Neon 40*).

2.5.3. Specific Surface Area (SSA)

Brunauer - Emmett –Teller was used to measure the specific surface of the materials, using the equipment *ASAP 2020* [[ERROR! NO S'HA TROBAT L'ORIGEN DE LA REFERÈNCIA.](#)]. Samples do not require previous preparation but they were dried before the analysis. In the case of CDHA,HA, α -TCP, β -TCP cements, the samples were placed at 120 °C, and the

Monetite cement was dried at 60 °C to avoid dehydration.

2.5.4. Inductively coupled plasma (ICP)

To evaluate ion exchange of our materials with media, the concentration of Ca and P ions in the cell culture media was measured by Inductively coupled plasma ICP- optical emission spectrometry (*ICP-OES, Perkin Elmer Optima 3200 RL*). The sample preparation consisted in adding dilute HNO₃ acid (2%) to the samples in the following proportion: 0,5 ml of sample (cell culture media) + 4,5 ml of 2% of HNO₃.

CHAPTER 4: Results and discussion

This chapter is divided in two main parts, the first part describes the results pertaining to the physicochemical characterization of CDHA and α -TCP and the second part is focused on the biological characterization of the materials with the two different cell types: SAOS and rMSC cells.

3.1. Physicochemical characterization

The first aspect that was evaluated of the materials was the weight and dimension of the discs as the thermal treatments led to significant changes. Results are summarized in [TABLE 5](#) where the diameter and average weight of 20 discs with their standard deviation is shown.

Table 5 Average weight and diameter of discs.

| Type of material | Weight + standard deviation [g] | Diameter [mm] |
|------------------|---------------------------------|---------------|
| CDHA | 0,515 ± 0,049 | 15 |
| α -TCP | 0,436 ± 0,046 | 13 |

CDHA samples have the expected dimensions since the setting reactions did not produce any volumetric change of the pre-shaped disks. The shrinking observed for α -TCP was due to the thermal treatment at high temperature that led to densification of the materials. Moreover, the weight loss is due to the release of water molecules from the CDHA after transformation into α -TCP according to the following reaction.



3.1.1. XRD analysis

The results concerning X-Ray characterization of materials are shown in Figures 5-6. For clarity purposes the experimental XRD patterns are shown together with a reference pattern of the same phase obtained from the joint committee of powder diffraction standards. [FIGURE 5](#) shows the diffractogram of the CDHA sample. The pattern in this case was indexed with the standard 09-0432 (JCPDS 09-0432) which corresponds to HA. It is clearly observed

3.1.1. Specific Surface Area (SSA)

The results from the SSA of the samples are compiled in [TABLE 6](#). As can be observed, the material obtained at high temperatures (α -TCP) shows lower SSA than the CDHA which are obtained at low temperatures by hydrolysis reaction. As will be seen in the next section these results correlate very well with the microstructures observed by SEM images.

Table 6 Specific Surface Area, comparison between samples.

| Type of sample | Specific surface area [m^2/g] |
|----------------|---|
| CDHA | $19,13 \pm 0,04$ |
| α -TCP | $0,22 \pm 0,00$ |

3.1.2. Microstructure characterization

[FIGURE 7](#) reveals the surface topography of the investigated materials. The images were taken at magnifications of 2KX and 10KX.

The knowledge of the microstructure of materials is extremely important, especially surface topography because this is the part of the material which interacts with cells and affects the cell adhesion and stimulates them to proliferate and differentiate. All materials present a certain degree of porosity. In the images of CDHA [\[FIGURE 7 A,B\]](#) there are present agglomerates of precipitated crystals (plate- like structure) and their surface is rougher than the surface of α -TCP. In contrast, the surface of α -TCP [\[FIGURE 7 C,D\]](#) is more rounded, smooth and without aggregates. It presents the characteristic morphology of calcium phosphates obtained at high temperature.

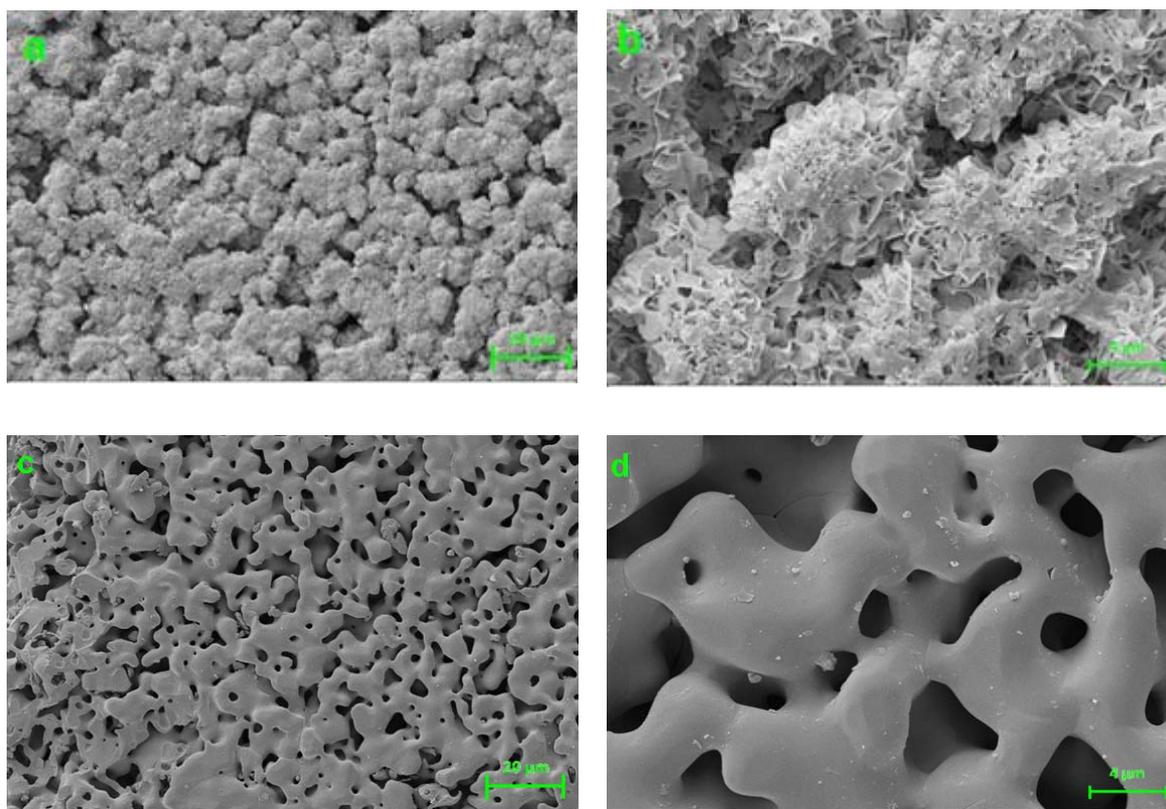


Figure 7 Surface topography of CDHA (a,b), α -TCP (c,d).

3.2. In vitro study

This section presents the results of proliferation and differentiation assays that were performed on the discs of CDHA and α -TCP using two different cell types, i.e. SAOS and rMSC cells. The pH, calcium and phosphate content during cell culture have been monitored to control the solubility of the various materials.

The section is divided into two parts – the first part describes the behaviour of SAOS-2 in the CPC and the second one the rMSCs behaviour on CPC.

3.2.1. SAOS-2 behaviour on Calcium Phosphate Materials

The study of proliferation and differentiation of SAOS-2 was evaluated after 4 hours, 3 and 7 days of cell incubation on the discs. Moreover, the cell culture media in contact with the discs was withdrawn at 1, 3 and 7 days to measure the pH and to quantify the content of Ca^{2+} and P ions. As a control it was used the cell media cultured on a well (TCPS) with cells but without material.

3.2.1.1. Quantification of Ca^{2+} and P^{-} ions content in McCoy's 5A Modified Media

The quantity of Ca^{+} and P^{-} ions the in medium in which the materials were immersed was analyzed after 1, 3 and 7 days.

It was observed that CDHA decreases Ca^{2+} concentration in solution [FIGURE 8]. The ion exchange reflected in the graphics will have an impact on cell behavior. In agreement with previous studies [17], [18], CDHA shows the tendency to uptake Ca^{+} ions which is observed by the lower concentration of Ca^{+} measured in solution. Their high specific surface area provokes that this CPC can easily interacts with the culture media. In contrast, α -TCP is more soluble than CDHA but this material is not deficient in calcium, then do not tend to uptake Ca^{2+} ions from media.

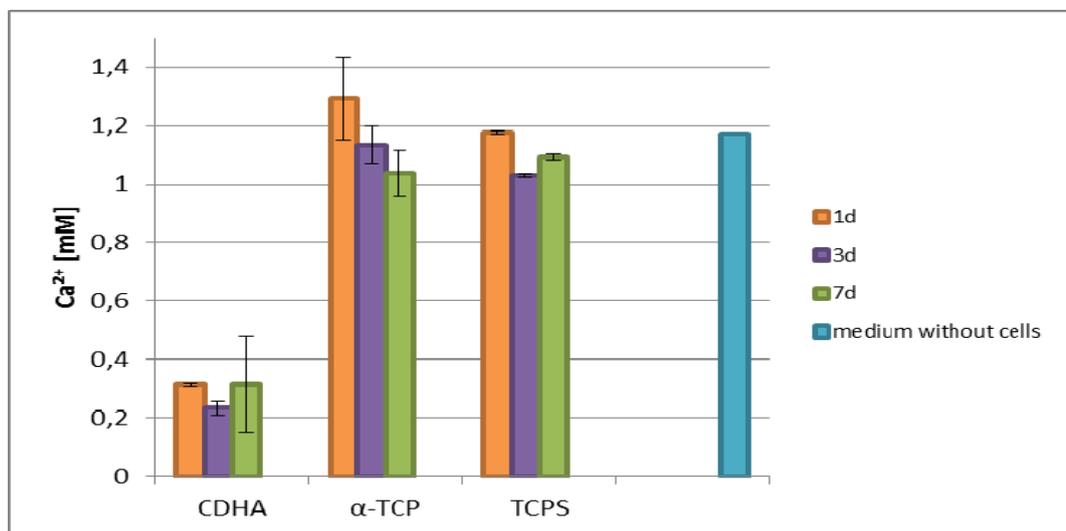


Figure 8 Content of Ca^{2+} ions in Mc Coy's Modified Media.

As was showed Calcium Phosphate Materials did not change significantly the content of P^{5+} ions in culture media [FIGURE 9].

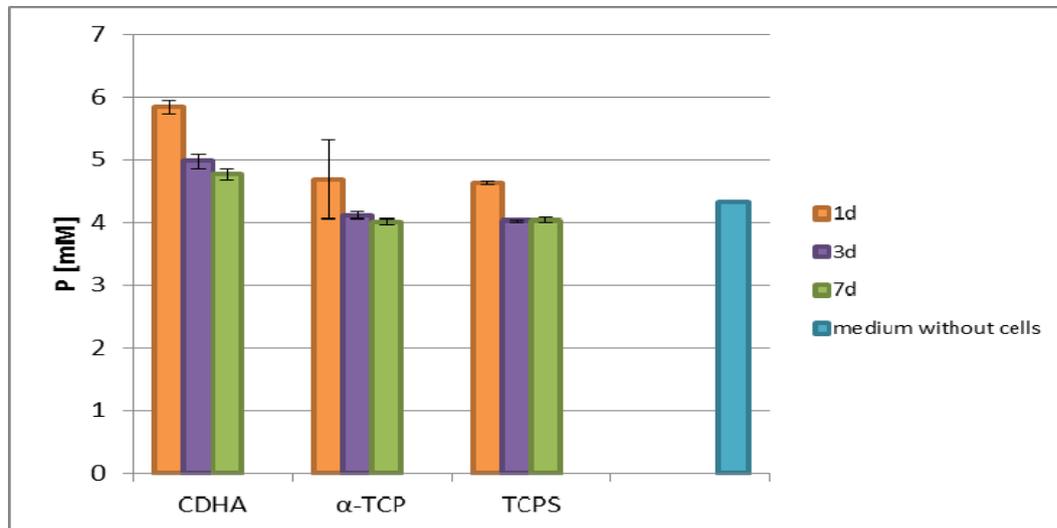


Figure 9 Content of P^{5+} ions in Mc Coy's Modified Media.

3.2.1.2. Proliferation assay

During the study proliferation of SAOS-2 were evaluated after 4 hours, 3 and 7 days. Results on graphics present number of cells per 1cm^2 of disc.

As it can be seen in [FIGURE 10] after 4 hours, cells adhere well to the surface. Results at day 3 and 7 show two trends. On materials obtained at high temperatures with lower Specific Surface Area (α -TCP) the proliferation of cells increase over time. On CDHA which present very high SSA cell proliferation decreases with time as shown in previous studies [17]. Thus, α -TCP is a more favorable material to SAOS-2 proliferation.

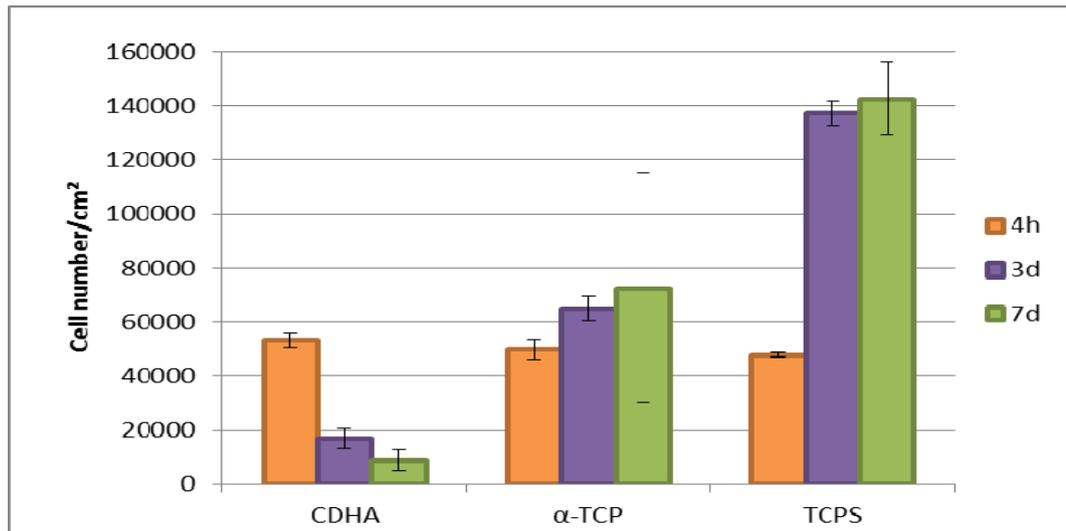


Figure 10 Results of LDH assay for CPC.

3.2.1.3. Differentiation assay

The study of differentiation of SAOS-2 was evaluated after 4 hours, 3 and 7 days. Results obtained during the assay were divided by number of cells obtained during LDH assay* incubation time. Every assay was made per triplicate [FIGURE 11].

Alkaline phosphatase is an enzyme present in the cellular membrane which liberates itself proportionally with formation of bone tissue and promotes mineralization. Results indicate that alkaline phosphatase presents higher activity on CDHA discs and lower activity on the α-TCP synthesized at high temperatures. It was also demonstrated that higher roughness (higher SSA) promotes better cell differentiation. In comparison with αTCP discs formed from CDHA are characterized by higher specific surface area and in SEM images their surface is rougher.

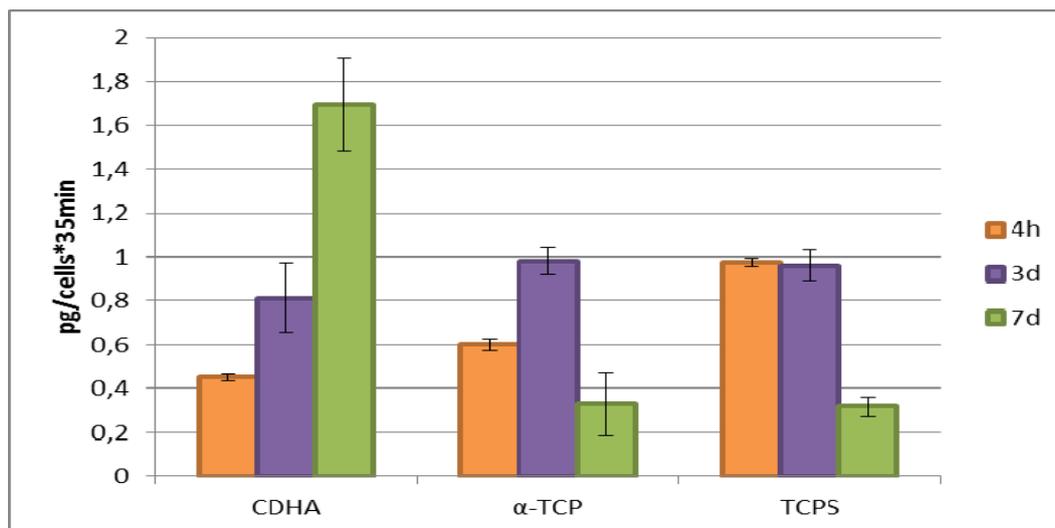


Figure 11 Results of ALP assay for CPC.

3.2.1.4. The Scanning Electron Microscope Images

To complete the study they were taken images of cells attached to the surface of investigated materials in the Scanning Electron Microscope. The pictures were taken after 4h, 3 days and 7 days after cell seeding with magnifications: 500X, 2KX 5KX, 10KX. The following images [FIGURE 12] present cell distribution on discs in 7th day of study with magnification 500X(at left) and 2KX(at right).

The SEM images reflect results obtained during LDH assay. The CDHA [FIGURE 12 A,B] presents a low number of cells on their surface. Moreover, cells are not well-extended, what may mean that it is in process of apoptosis or necrosis.

In case of α-TCP [FIGURE 12 C,D], images clearly show numerous cells on the surface of discs. These pictures reflect the results obtained during the LDH assay which showed that on these materials cells proliferate.

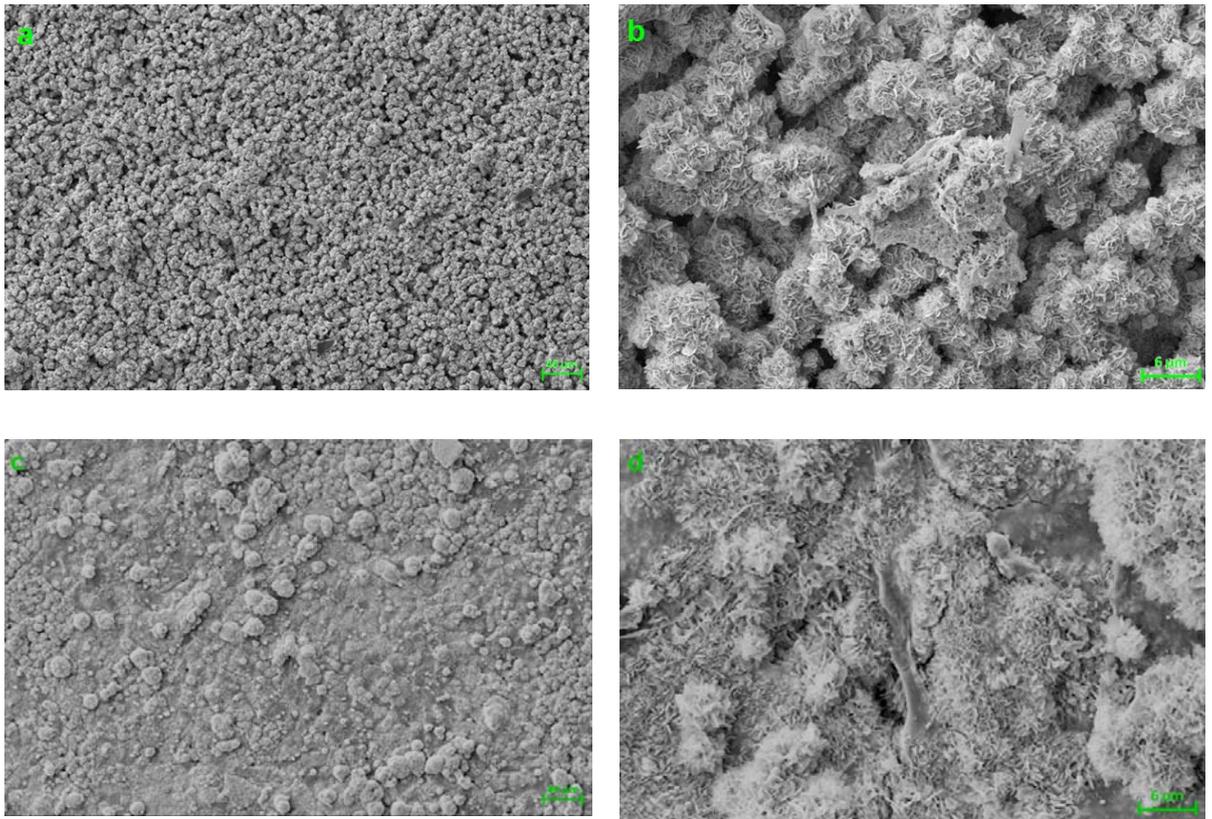


Figure 12 SAOS-2 distribution on CDHA disc (a,b) and α -TCP disc (c,d) on 7th day of study.

3.2.2. rMSCs behaviour on Calcium Phosphate Materials

Apart from SAOS-2, cell behavior in calcium phosphate materials was also evaluated using rat mesenchymal stem cell (rMSCs). These cells are much more sensitive to changes in the ion concentration and surface topography and we had particular interest to assess the mesenchymal cells behavior on cements and contrast the results to those obtained with the other materials.

The study of proliferation and differentiation of rMSCs was evaluated after 6 hours, 3 days and 7 days. The medium was re-used to measure the pH and quantify the content of Ca^{2+} and P ions.

3.2.2.1. Quantification of Ca^{2+} and P^- ions content in Advanced Dulbecco's

Modified Eagle Medium.

The number of Ca^+ and P^- ions in medium in which the materials were immersed was quantified after 6 hours, 3 days and 7 days by inductively coupled plasma.

α -TCP does not alter the quantity of Ca^{2+} ions in medium, while cements (CDHA) clearly tend to uptake calcium from media [FIGURE 13]. This behavior coincides to that observed with the SAOS-2 cells and agrees with the observations made in other works.

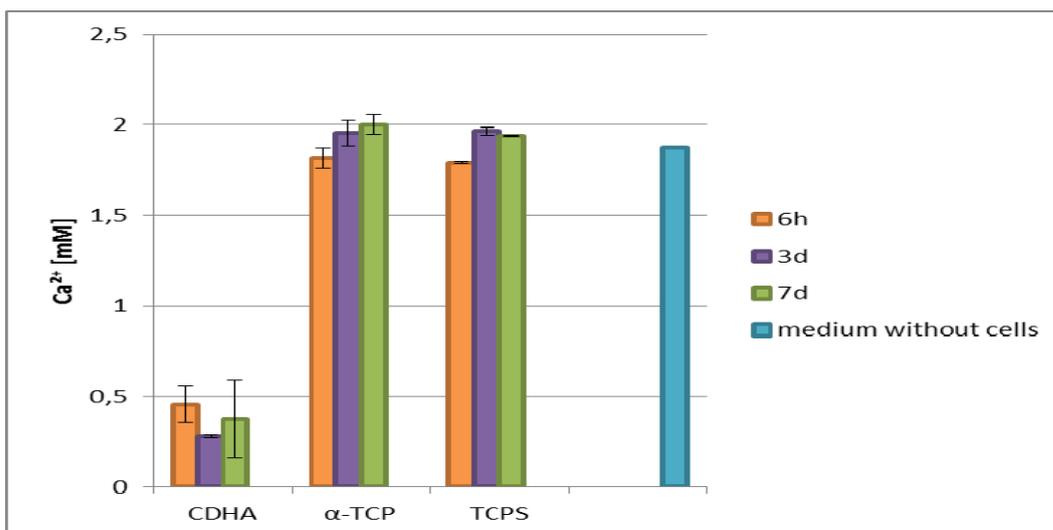


Figure 13 Content of Ca^{2+} ions in advDMEM.

Analyzing the levels of P^{5+} ions they show that α -TCP does not affect number of ions in medium and cement CDHA which tend to release phosphorus to medium [FIGURE 14].

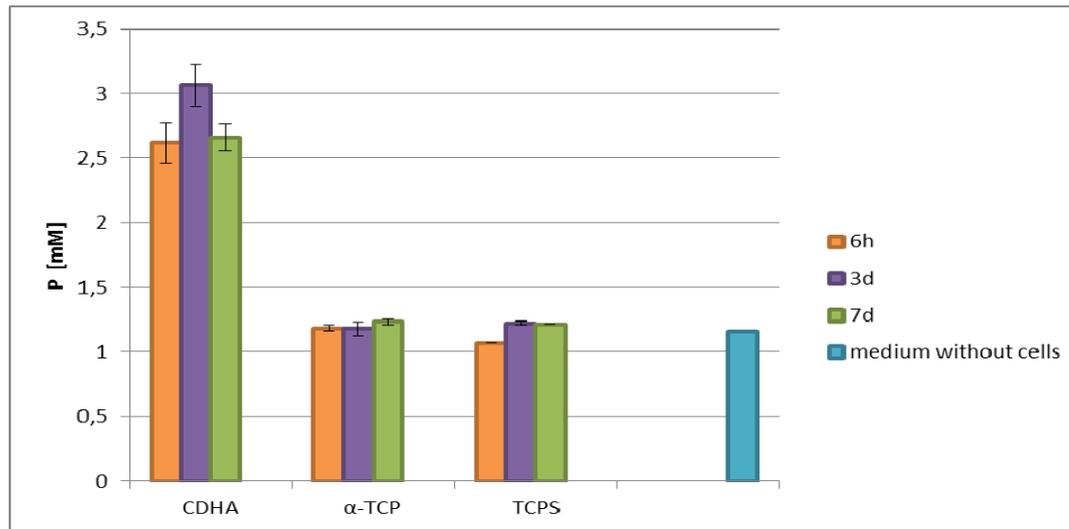


Figure 14 Content of P⁵⁺ ions in advDMEM.

3.2.2.2. Proliferation assay

The proliferation of rMSCs was evaluated after 6 hours, 3 days and 7 days. Results on the graphs present numbers of cells per 1 cm² of discs. Every assay was made per triplicate [FIGURE 15].

The reason for evaluating cell proliferation and differentiation at 6h instead of the 4h for SAOS-2 cells was to increase the adhesion number of cells on the materials.

On α-TCP discs the proliferation of rMSCs increases over time. On CDHA discs the number of cells on the surface decrease with time. This behavior was previously reported and depends of ion exchange with medium, high Specific Surface Area and rough topography.

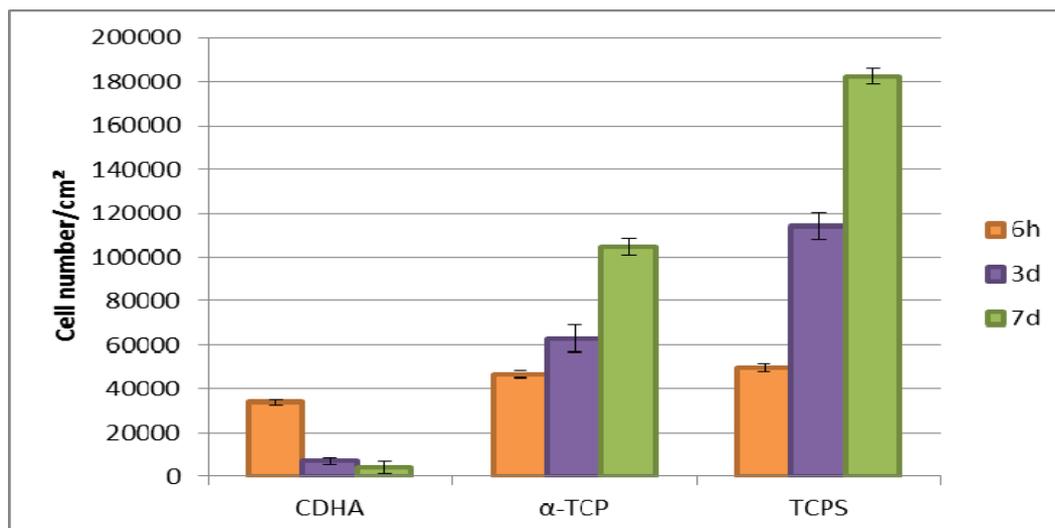


Figure 15 Results of LDH assay for CPC.

3.2.2.3. Differentiation assay

Alkaline phosphatase (ALP) activity of rMSCs was evaluated after 6 hours, 3 days and 7 days. The results obtained of the assay were divided by the number of cells obtained during LDH assay* incubation time. Each result was an average of three independent samples [FIGURE 16].

rMSCs are not differentiated cells thus, they are capable of differentiating into various phenotypes such as the bone phenotype. SAOS-2 are cells extracted from osteosarcoma, with a clear osteoblast phenotype. Therefore, SAOS-2 are able to express ALP more easily while rMSCs should be stimulated by the material to differentiate into the osteoblast phenotype and produce ALP. The measurement of the levels of ALP can give an indication of which material stimulate cell differentiation on their surface. Results indicate that ALP present higher activity on CDHA discs and lower activity on α-TCP. α-TCP discs did not alter the level of ALP suggesting that did not promote the rMSC differentiation into osteoblasts.]. It has to be mentioned that in the control (TCPS) it was observed an increase in ALP activity after 7 days. This could be explained by the fact that cells in TCPS reached confluence and began to differentiate [FIGURE 16].

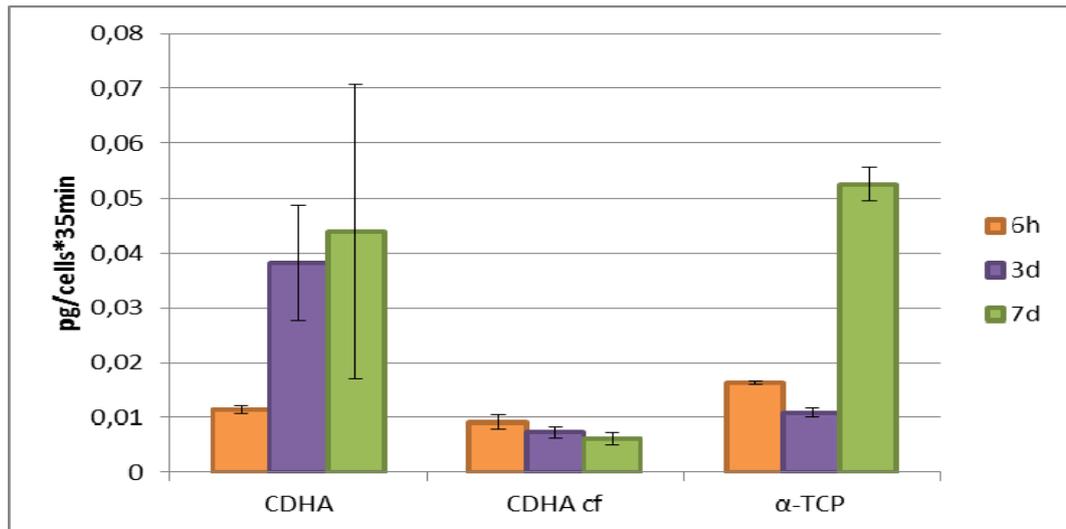


Figure 16 Results of ALP assay for CPC.

3.2.2.4. The Scanning Electron Microscope Images

To complete the study they were taken images of rMSCs attached to the surface of the investigated materials in the Scanning Electron Microscope. The pictures were taken after 4h, 3 days and 7 days after cell seeding with magnifications: 500X, 2KX 5KX, 10KX. The following images [FIGURE 17] present cell distribution on discs in the 7th day of study with magnification 500X (at left) and 2KX(at right).

Discs of CDHA show lower number of cells attached on their surface as compared to the other materials. Moreover, the rMSCs observed on CDHA [FIGURE 17 A,B] were not well extended. A small amount of cells on the surface confirms the results obtained during rMSCs assay. In the case of α-TCP [FIGURE 17 C,D], their surface is almost fully covered by cells. This material seems to be more favorable to rMSCs proliferation in agreement with the results obtained during LDH assay.

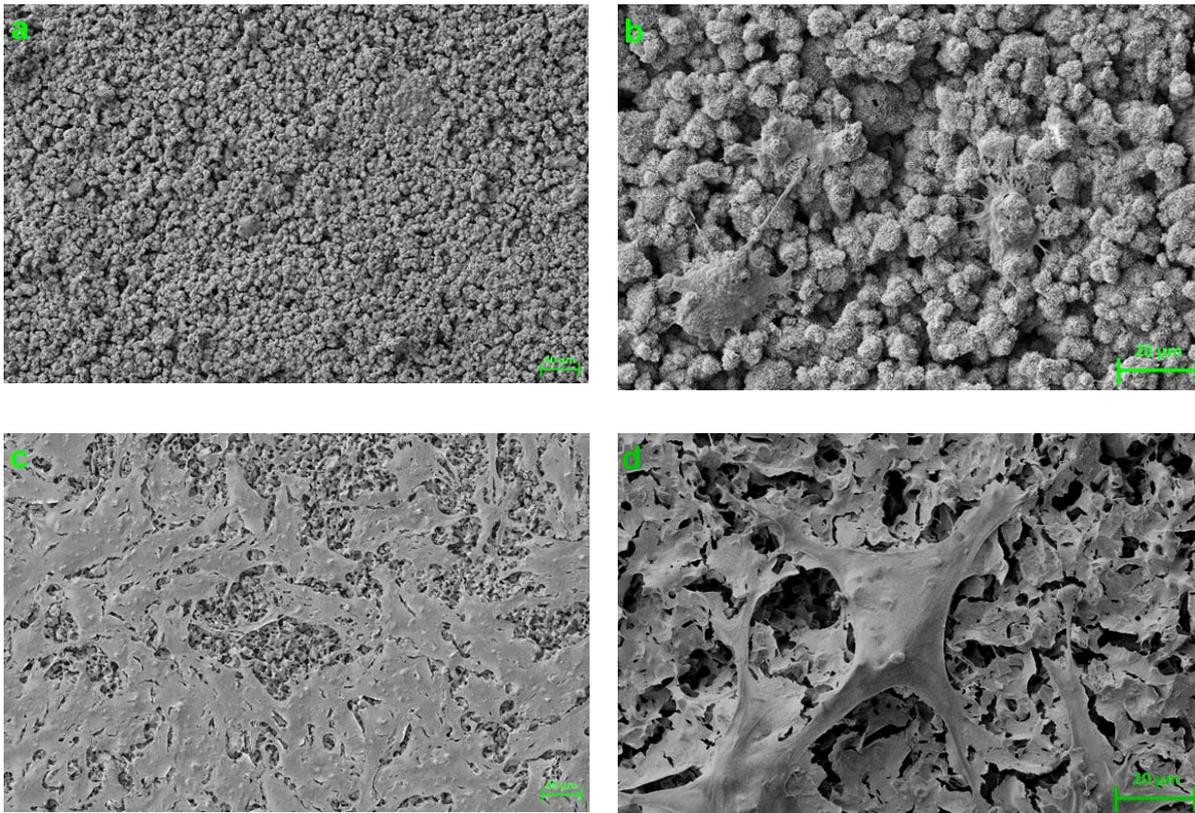


Figure 17 rMSCs distribution on CDHA disc (a,b) and α -TCP (c,d) on 7th day of study.

CHAPTER 5: Summary and Conclusions

The main objective of study was to analyze the cell behavior of two calcium phosphate materials and investigate how the surface topography of the materials and their reactivity (ion release/uptake) influenced cell proliferation and differentiation.

From the two calcium phosphates one was obtained at high temperatures: α -TCP, and the other at low temperature: CDHA. Materials were characterized by X-ray diffraction, B.E.T. and by Scanning Electron Microscopy. DRX confirmed that the obtained CaP materials were pure. BET and SEM images showed that the synthesis route (high or low temperature) had great influence in the Specific Surface Area and surface topography of the materials. CDHA is a phosphate obtained at low temperature, showed agglomerates of precipitated crystals (plate-like structure) and very rough surface. α -TCP obtained at high temperatures showed lower SSA and their topography was more smooth and less porous. It presented characteristics of typical sintered ceramic.

Results obtained by ICP clearly showed that CDHA, probably owing to their deficiency in calcium, tended to uptake Ca^{2+} from media. Its specific surface area was high which facilitated this interaction. In contrast, α -TCP did not alter the ionic concentrations of the cell media. It is interesting to mention that α -TCP powder in contact with water reacts and forms CDHA, but this did not occur when using the α -TCP discs. One of possible explanation is the too low specific surface area of the discs as compared to the powder which would difficult hydrolysis.

During assays with SAOS-2 and rMSCs, proliferation assay presented two trends: on α -TCP proliferation increased over time, while on CDHA cell proliferation decreased with time. Differentiation assay showed that CaP materials with high SSA (CDHA) promoted cell differentiation.

These different cell culture behaviors for α TCP and CDHA are very relevant when implanting injectable cements. The cement is implanted being α -TCP- material which supports the proliferation of mesenchymal and osteoblastic cells. The material with time - hydrolyses to CDHA- thus causing a decrease in cell proliferation but stimulates differentiation.



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