The influence of surface chemistry on osteogenic differentiation of Human Mesenchymal Stem Cells

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ABSTRACT

Calcium phosphate (CaP) based ceramics are used as bone graft substitutes in the treatment of bone defects. The physico-chemical properties of these materials determine their bioactivity and osteoinductive potential: macrostructure, microstructure and chemical composition. In previous studies, two tricalcium phosphate (TCP) ceramics were compared, TCP-S (small microstructure dimension) and TCP-B (big microstructure dimension), but several physico-chemical properties were varied. So, it is not known which of them was essential for osteogenic differentiation. Therefore, in the present study, all the conditions were maintained constant for all samples, except one: chemical composition of the coatings. Hereby, three types of TCP-S based discs were compared. All the samples have similar microstructure but different coatings: TCP-S, TCP-S with carbon and gold coatings.

Firstly it was confirmed that surface topography, the ion release and protein adsorption properties were the same regardless the surface chemistry. *In vitro*, both cell proliferation and osteogenic differentiation were enhanced over time. No significant difference was found among the human bone marrow stromal cells (hBMSCs) cultured on different types of TCP-S samples. Regarding cell shape, there were no differences between the three groups of samples. The expression of osteogenic markers, collagen-I, alkaline phosphatase, osteopontin and osteocalcin, was also assessed and the results showed some significant differences among the three groups of samples.

The current *in vitro* data reveal that these surface chemistries do not affect osteogenic differentiation of hBMSCs.

Key words: ceramics, surface chemistry, osteogenic differentiation.

RESUMO

Os materiais cerâmicos à base de fosfato de cálcio são usados como substitutos ósseos no tratamento de ossos. Algumas propriedades físico-químicas destes materiais determinam a sua bioactividade e o seu potencial osteoinductivo: micro e macroestrutura e a composição química.

Num estudo anterior dois materiais compostos por tricálcico fosfato (TCP) foram comparados: TCP-S (pequenos poros) e TCP-B (grandes poros), mas muitas propriedades foram variadas, pelo que não se sabe qual destes factores é essencial para a diferenciação osteogénica. Assim, neste estudo todos os factores foram mantidos constantes e fez-se variar apenas a composição química das superfícies. Aqui foram comparados três discos de TCP-S com a mesma macroestrutura e equivalente microporosidade mas diferentes coberturas: coberturas de ouro, de carbono e discos sem revestimento.

Começou-se por confirmar que os perfis de libertação de iões e a adsorção de proteínas eram idênticas independentemente do tipo de revestimento. No estudo *in vitro*, tanto a proliferação celular com a diferenciação osteogénica aumentaram ao longo do tempo de cultura. Contudo não foram encontradas diferenças significativas entre as células estaminais do mesênquima (hBMSC) crescidas nos diferentes tipos de revestimento. Relativamente à forma das células também não foram encontradas diferenças entre as três amostras. A expressão de Colagénio-I, fosfatase alcalina, osteopontina e osteocalcina, marcadores osteogénicos também foi examinada e os resultados mostraram diferentes tendências.

Assim, os resultados deste estudo revelam que as diferentes composições químicas conseguidas através das diferentes coberturas não induzem diferentes graus de diferenciação osteogénica nas hBMSCs.

Palavras-chave: materiais cerâmicos, composição química, diferenciação osteogénica.

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3D	Three dimensional
ALP	Alkaline phosphatase
BCP	Biphasic calcium phosphate
BM	Bone marrow
BM	Basal medium
BMP	Bone morphogenetic protein
CA	Carbonated apatite
CaP	Calcium Phosphate
cDNA	Complementary DNA
CHA	Carbonated hydroxyapatite
CHAP	Alkoxide-derived hydroxy carbonate apatite
CNT	Carbon nanotubes
Col	Collagen
DAPI	4',6-Diamidino-2-Phenylindole Dihydrochloride
DMEM	Dulbecco´s Modified Eagle´s Medium
ECM	Extracellular matrix
EDS	Energy Dispersive Spectroscopy
ESC	Embryonic stem cell
FBS	Fetal Bovine Serum
HA	Hydroxyapatite
hBMSC	Human bone marrow stem cell
iPSC	induced pluripotent stem cells
MSC	Mesenchymal stem cell
NCP	Non-collagenous protein
OC	Osteocalcin
OPN	Osteopontin
PBS	Phosphate Buffered Saline
Q-PCR	Quantitative polymerase chain reaction
RGD	Arginine-glycine-aspartate tripeptide sequence
rpm	Rotations per Minute
SAMs	Self-assembled monolayers
SEM	Scanning electron microscope
TE	Tissue engineering
US	United States
USA	United States of America
V	volt

1. INTRODUCTION

1.1. BONE

Bone is the component of the skeletal system, which is involved in the protection, support and motion of the body. Bone is a highly vascularized tissue with a unique capacity to heal and remodel without leaving a scar. These properties, together with its capacity to rapidly mobilize mineral stores on metabolic demand, makes of it the ultimate smart material (1).

Bone consists of 60% inorganic components, 30% organic components and 10% water. The inorganic part consists of complexes of calcium phosphates in amorphous (30%) and crystalline fractions (70%) (2) and provides compressive strength. The organic part is composed of 90% type I collagen and 10% proteoglycans and non-collagenous proteins (osteopontin, osteonectin and osteocalcin), being responsible for the bone tensile properties of the bone (3).

Bone is a dynamic tissue that is continuously maintained and renewed by four different kinds of bone cells: osteoclasts, osteoblasts, osteocytes, and lining cells. Osteoclasts are located at the bone surface and remove (resorb) bone tissue by removing its mineralized matrix and breaking up the organic bone. They are giant multinucleated cells and resorb bone via local acidification and secretion of various proteases (4). Osteoblasts synthesize the organic matrix of bone by secretion of a wide variety of extracellular matrix proteins and produce new bone. They also participate in the mineralization process and in the control of osteoclast function. When an osteoblast is in its terminal differentiation stage and resides entrapped in its self-produced bone matrix it is called an osteocyte (5). Osteocytes are the most abundant cells in bones and are believed to maintain the bone by sensing mechanical strains and bone damage (6). They have a typical morphology with long thin cytoplasmic processes, which form a fine network of connections with other osteocytes and with the osteoblasts located at the surface of the bone. Lining cells cover the bone surfaces and thereby separate the bone surface from the bone marrow. However, the exact function of bone lining cells remains unclear (7).

1.2. CLINICAL NEEDS IN THE BONE REPLACEMENT AND REGENERATION FIELD

The economic impact of musculoskeletal conditions in the United States (US) represents $126 billion (8). Bone fracture repairs are one of the most commonly performed orthopedic procedures: about 6.8 million come to medical attention each year and represent 16% of all musculoskeletal injuries in the U.S. annually (9). The most common fracture prior to age 75 is a wrist fracture. In those over age 75, hip fractures become the most common broken bone.

The most common causes of fractures are traumatic injuries, osteoporosis and overuse. Traumatic injuries refer to physical serious or life threatening injuries which require immediate medical attention (10), (11). Osteoporosis is a disorder that weakens bones and makes them more likely to break. Overuse can tire muscles and place more force on bone, it can result in stress fractures (12). In these cases the most

common is to do an invasive surgery to align and stabilize the bone, usually with metallic pins, screws, plates, or rods. Despite the mechanical strength and integrity of metallic implants, there are several drawbacks associated with its use. The induced stress, shielding, stiffness and chronic pain are some of the possible negative side effects. However, for bone deficiencies exceeding a certain size (i.e. critical-sized defects), bone grafting are usually needed (13).

Bone grafting is a big business that generates more than $2.5 billion per year in the USA (14). But in the coming years, with an increasingly aging population, much more people will need to resort to this kind of healthcare treatment (Figure 1A). Possibly, the world won't be prepared for such a demand, and there will be a shortage in the availability of musculoskeletal donor tissue traditionally used.

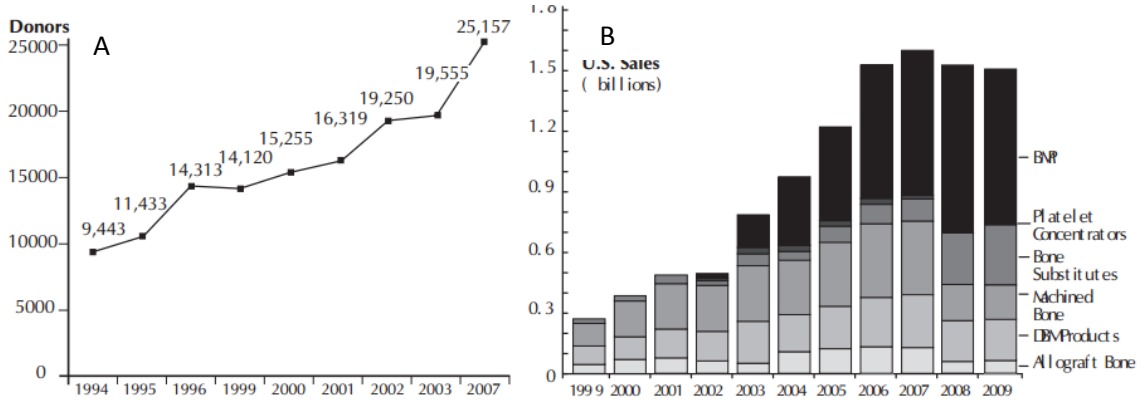

Figure 1 – US trends in musculoskeletal tissue donors. The number of donors needed almost doubled since 1994 to 2007 (A). US sales of bone graft and bone-graft substitutes. It has been noticed a growing market in bone replacement materials (B). (15)

Currently, to improve the quality of life of individuals with bone defects, autografts, allografts, xenografts and synthetic materials are considered (Figure 2).

Autologous bone graft (or autografts), representing bone taken from another part of the patient's own body, has been the gold standard of bone replacement for many years because of their osteoinduction and osteoconductivity capacities (1). Osteoinduction is the ability of inducing bone formation by heterotopic implantation in tissues where bone does not naturally grow while osteoconductivity is defined as bone ingrowth to the material from the surrounding host bone bed (16). In addition, autografts have no immunogenicity and associated risk of viral transmission (17). However, the amount of bone that can be harvested is limited. Furthermore, this method has some disadvantages, such as donor-site pain and morbidity.

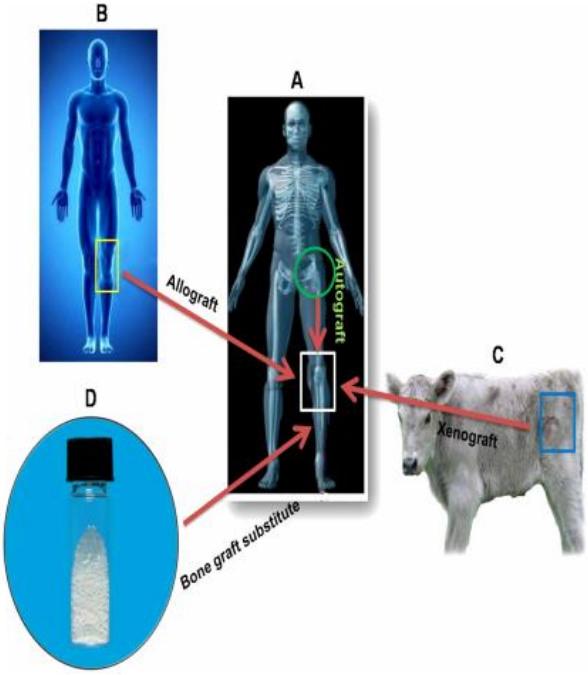

Figure 2 - Types of bone grafts. (A) Autograft: The surgeon harvests bone from another site of the patient's skeleton. (B, C) Allograft and xenograft: Here the bone graft is obtained from a human donor or animal model, respectively. (D) Synthetic bone graft substitute: There are different origins for synthetic grafts (17).

Allografts, that can be obtained from cadavers or living donors, are harvested from one individual and implanted into another individual of the same species. The greatest advantage of allografts is that they can be processed to obtain a given shape or size, avoiding the need to sacrifice host tissues, and presenting no challenges related with donor site. However, bone allografts may induce immunological reactions that interfere with the bone healing process and can lead to rejection of the graft. Moreover, the rate of graft incorporation is lower than with the autografts. To try to solve this problem allogeneic bone has to undergo processing techniques such as lyophilisation, irradiation or freeze-drying to remove all immunogenic proteins in order to avoid any risk of immunogenic reaction. These processing techniques have a negative effect on osteoinductive and osteoconductive potential of the allografts, which consequently decreases their biological performance as compared to autografts (18).

Xenografts are harvested from one individual and transplanted into another individual of a different species. The most common sources of xenografts are natural coral, porcine and bovine (17). For the xenografts to be the best alternative it would be necessary to find a safer way to perform human transplants. But xenografts as well as allografts lose their osteogenic properties during the process of transmission prevention of infection. On the basis of above information, we summarized the advantages and disadvantages of autografts, allografts and xenografts in Table 1.

Table 1 – Advantages and disadvantages of the three most used types of bone grafts (17).

Bone graft	Advantages	Disadvantages
Autografts	Optimal osteogenic, osteoinductive, and osteoconductive properties; without the risks of immunogenicity and disease transmission	Pain and morbidity in the donor site; limited quantity and availability
Allografts	Osteoinductive and osteoconductive properties, without donor site morbidity, high availability	Potential antigenic response and disease transmission, loss of biologic and mechanical properties due to its processing
Xenografts	Osteoinductive and osteoconductive properties, low cost, high availability	Risk of immunogenicity and transmission of infectious

Because of the above-mentioned drawbacks of natural bone grafts, a large number of synthetic grafts have been developed (Figure 1 B). Bone substitute materials are intended to be implanted in a surgical procedure and, over time, become a part of vital bone. These graft alternatives need to be evaluated at the level of safety and effectiveness in patients before their use by orthopaedic surgeons (15). One of the advantages of synthetic bone grafts is that they can easily be tailored to the intended application. However, the biological performance of synthetic bone grafts in terms of initiation and support of bone growth are inferior to those of natural bone grafts. So, further studies are necessary on how to make the biological performance of synthetic biomaterials closer to that of an autologous bone. Bone tissue engineering can be part of these studies.

1.3. BONE TISSUE ENGINEERING

Tissue engineering (TE) is defined by Langer and Vacanti as “an interdisciplinary field of research that applies the principles of engineering and life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function” (19). TE attempts to understand tissue formation and regeneration in order to create new functional tissues. To this end, TE combines knowledge from physics, chemistry, engineering, materials science, and medicine. TE strategies focus mainly on development of scaffolds and/or on the combination of scaffolds with cells.

Bone TE grew out of the increasing need for bone repair due to skeletal diseases, congenital malformations, trauma, and tumour resections. The need for multiple surgeries such as the removal of metallic implants and graft harvesting would be reduced, and thus a quicker recovery time, lower costs and reduced risks are expected. The engineered implant can also be fully integrated into the existing tissue, which avoids some problems associated with other kind of implants, as for instance the induced stress shielding that can result in bone mass loss (13).

For bone TE it is important to know how bone grows and what is required for that growing process. As bone has a three dimensional (3D) structure and cells do not grow naturally in a 3D structure it is necessary, through bone tissue engineering, to search for scaffolds that mimic bone structure.

However, for a successful result, all cell components have to be combined in a well-coordinated spatial and time dependent fashion, which is not an easy task. These scaffolds must be biocompatible to avoid an immune response from the host and should also be biodegradable, ideally at a rate that is in consonance with the formation of new tissue. The mechanical properties of a scaffold for bone tissue engineering are also of extreme relevance due to the mechanical functions of bone and particularly because of the magnitude of the loads subjected in this organ (20). Actually, scaffolds are the most important issue in TE and could be divided into two categories according to the origin of the material used in its construction: natural or synthetic. So, bone TE is an emerging field that may suppress the need for the autologous bone graft in the future.

1.4. OSTEOINDUCTIVE BIOMATERIALS

In addition to the needed requirements for the design and construction of 3D bone scaffolds, an ideal bone scaffold should also be osteoconductive, osteoinductive and osseointegrative (21). Osteoinductive biomaterials arise from the need to create synthetic biomaterials that are able to work as well, or better, than autologous bone grafts, but without the disadvantages characteristic of the use of natural bone grafts. They are called “intelligent” biomaterials due to their ability to instruct the *in vivo* environment to form new bone (22). Hence, these osteoinductive biomaterials hold great potential for the development of new therapies in bone regeneration.

Indeed, over the last years a number of biomaterials have shown the ability to induce bone formation when implanted at heterotopic sites (22) (23). Based on their chemical composition, synthetic bone grafts can be divided into four main groups: polymers, such as poly(methyl methacrylate); metals, such as titanium; ceramics, such as calcium phosphate, alumina, carbon and glass ceramics; and composites of the first three groups, such as calcium phosphate–ceramic coatings on metallic implants and polymer–ceramic composites.

1.5. CALCIUM PHOSPHATE-BASED BIOMATERIALS

Among these materials, those based on calcium phosphate (CaP) ceramics are among the most attractive and also the most reported (23). Due to their chemical composition, these biomaterials are very similar to bone mineral (a CaP in the form of carbonate apatite) and other similarities in some properties of bone include biodegradability, bioactivity and osteoconductivity (24). This latter feature is very useful for a correct osteointegration between the host bone and the implant (25). These biomaterials are considered bioactive due to their active interaction with normal bone surfaces during bone regeneration, allowing osteointegration of the biomaterial and bone interface. This is important in bone tissue regeneration as the bone undergoes dynamic tissue remodelling during regeneration process (26).

CaP biomaterials are not osteoinductive (does not form bone de novo). However, some CaP biomaterials have been described to have 'inherent' osteoinductive property, since several CaP materials, such as synthetic hydroxyapatite (HA) and TCP, have been reported to have the ability to form bone in non-bony sites of different animals without addition of osteogenic factors (24).

CaP are formed by a process called sintering. This is a process in which high temperatures (1100 – 1300°C), pressure, and different apatites are being used to form the final product. The combination of a certain temperature, pressure, and different apatites determines several properties of the final product. For example, pure HA is formed by using an apatite with a Ca/P ratio of 1.7, whereas TCP is formed by using an apatite with a Ca/P ratio of 1.5 (2).

Microporosity of the ceramic material is an important property that is introduced in the manufacture of CaP biomaterials. Microporosity depends on sintering temperature (24), the higher the sintering temperature, the smaller the pore size.

For bone repair, augmentation and substitution, hydroxyapatite (HA), TCP and biphasic calcium phosphate (BCP- mixture of HA and β-TCP of varying HA/ β-TCP ratios) are the most CaP used ceramics (27).

1.6. INFLUENCE OF BIOMATERIALS PROPERTIES ON OSTEOINDUCTION

There are reports concerning the material properties which so far have been suggested to play a role in osteoinduction: chemical composition; macrostructural properties and microporosity (23) (16).

With the help of Figure 3 some researchers have tried to explain how these properties influence osteogenic differentiation. By examining this figure, they show that macro and microstructure are related with geometry and porosity that influence infiltration of nutrients, oxygen and cells and also the surface area available for adsorption. The larger the surface area available, the more proteins can be subjected to a adsorption by the surface. These proteins, like bone morphogenetic protein (BMP), influence osteogenic differentiation. On the other hand, the chemical composition of the used biomaterials is important because it influences the release of ions. The release of ions (e.g. calcium and phosphate ions) from the material into the surroundings may increase the local supersaturation of the biologic fluid – causing precipitation of carbonated apatite that incorporates these ions, as well as proteins, and other organic compounds. These will promote osteogenic differentiation of osteogenic precursors and bone formation in a long term period.

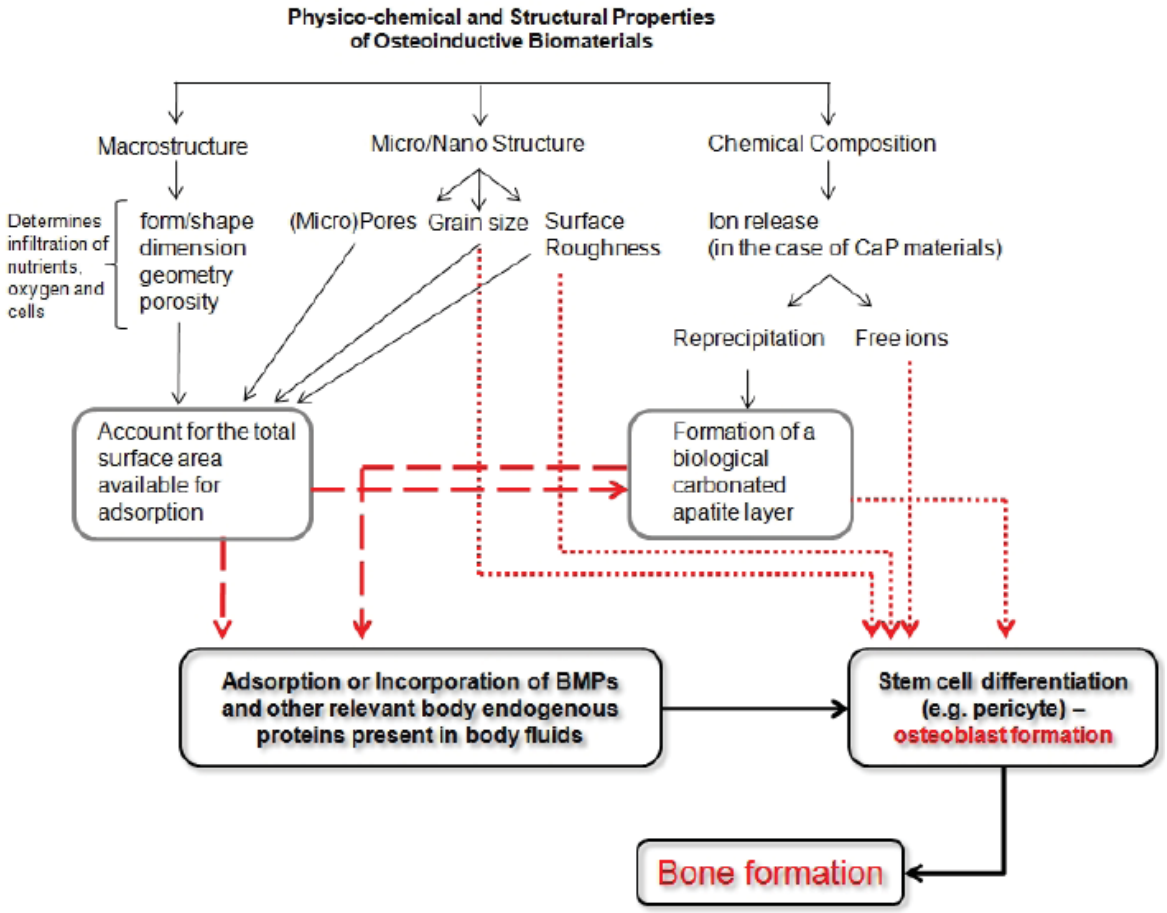

Figure 3 – Physicochemical and structural properties of biomaterials that may influence osteogenic differentiation (23).

1.6.1. CHEMICAL COMPOSITION

1.6.1.1. SURFACE CHEMISTRY

Lately, several researches have been conducted in order to find out if surface chemistry affects hBMSCs differentiation (28), (29). The majority of the groups have been used self-assembled monolayers (SAMs) as model biomaterial surfaces that allow significant control over chemical properties. The results from these studies demonstrate that surface chemistry can, in a long-term, modulate osteoblastic differentiation. This process occurs via adsorbed extracellular matrix proteins which bind to specific integrin adhesion receptors. Surface chemistry-dependent differences in integrin binding differentially regulate focal adhesion composition and signalling, which ultimately leads to changes in initial adhesion and long-term differentiation of osteoblast cells (29).

Other methods were used to study the influence of surface chemistry in hBMSCs differentiation such as NaOH and $Ca(OH)_2$ treatments on the surfaces in order to modify their calcium compositions (30). Modification of titanium alloy (Ti-6Al-4V) with zinc, magnesium (Mg) or alkoxide-derived hydroxy carbonate apatite (CHAP) (31) were also performed. The outcomes were that $Ca(OH)_2$ treatment, in contrast to NaOH, induced osteogenic differentiation of hBMSCs; furthermore, modifying Ti-6Al-4V with CHAP or Mg may contribute to successful osteoblast function and differentiation at the skeletal tissue-device interface.

Carbon-based materials could also be an alternative. They have been used as carbon nanotubes (CNT). These nanotubes have emerged as a promising material for such purposes (physics, chemistry, electronics and materials science), due to their high electrical conductivity, high chemical stability, extremely high mechanical strength and modulus. Their unique electrical and optical properties make CNTs widely used in bio-electronic devices, bio-sensors and bio-probes (32) (33). The biocompatibility and cytotoxicity of CNTs have been researched extensively. It was reported that CNT suspension has good biocompatibility with MSCs and supported proliferation as well as differentiation of MSCs in the presence of induction medium, while CNT substrates show good cell viability and adhesion (34).

In a previous study, Changqing Yi et al. (35) reported that gold nanoparticles enter cells and they may interact with proteins located in the cytoplasm, interfering with certain cellular signalling pathways. In this report, they investigated the cellular effects of gold nanoparticles (AuNPs) on the differentiation of mesenchymal stem cells (MSCs) and the associated molecular mechanisms. The results showed that AuNPs promoted the differentiation of MSCs. AuNPs exerted certain effects in the MSCs that led them to activate an important signalling pathway, which regulates the expression of relevant genes to induce osteogenic differentiation and inhibit adipogenic differentiation.

1.6.1.2. ION EXCHANGE

Calcium ion (mostly calcium phosphate and some calcium sulphate) is the most important and specific element of bone and calcified cartilage. In humans, the total body content of calcium is present mostly in the form of bone mineral (roughly 99%).

It is believed that the main origin of the bioactivity of CaP biomaterials is due to the release of calcium and phosphate ions (22), (36). This release event is followed by the precipitation of a biological carbonated apatite layer that incorporates calcium, phosphate and other ions, as well as proteins and other organic compounds (22). The precipitation of this apatite layer occurs when the concentration of calcium and phosphate ions has reached supersaturation level in the vicinity. This apatite layer may be a factor that triggers the differentiation of the stem cells into the osteogenic lineage. In the case of biomaterials that initially do not contain calcium phosphate, the release of calcium and phosphate ions is missing. However, also in this case, the biological carbonated apatite layer is formed on the surface due to the roughness of the surface, which provides nucleation sites for calcium phosphate precipitation.

Thus, the following questions arise: which of these events is responsible for the osteogenic differentiation? Will be the precipitation of a biological apatite layer, free calcium and phosphate ions, or both? (23)

1.6.2. MACROSTRUCTURAL PROPERTIES

Apart from the chemical composition of the material, the geometry and macrostructural properties have been shown to play an important role in osteogenic potential.

Porosity is the most notable example of the influence of macrostructural properties. Generally, the importance of pores inside bone graft substitutes is associated with the transport of nutrients and oxygen through blood vessels to maintain the correct metabolism of the cells inside the scaffold. In the case of osteoinductive materials, blood vessels can have the added function of bringing along cells with the capacity to differentiate into osteoblasts. A large macroporosity (i.e., 400–600 mm) facilitates infiltration by fibrovascular tissue and revascularization, allowing bone reconstruction (2). When comparing porous scaffolds with scaffolds without pores, one of two things can happen: either no bone formation occurs or the rate of replacement by the newly formed bone is too slow (23). However, the disadvantage of a larger total porous volume is a decrease in mechanical strength. The porosity of a material could enhance the proliferation and remodeling of bone by providing infiltration of fibrovascular tissue in the direction of pores (2). Small particles of a dense biomaterial could avoid these problems. Dense particles could provide the ingrowing tissue a large surface for scaffolding. On the other hand disintegration of porous macrostructure of the ceramic, due to mechanical fracture can be used to avoid bone formation to occur.

Besides the presence of pores with suitable dimensions, geometry of the implant has been shown to be important in osteoinduction. Several studies showed that bone formation is favoured inside pores, concavities or channels because there the cells are not disturbed by high body fluids refreshment or mechanical forces (23).

1.6.3. MICROSTRUCTURAL PROPERTIES

In addition to chemical composition and macrostructural properties, material surface properties at micro- and nanoscale have been demonstrated to be of great importance for osteoinductive potential. In some studies it has been shown that ceramics with different microstructural properties have different performances when implanted heterotopically (23). When microporosity of BCP ceramic was increased by lowering the temperature at which the ceramic was sintered, the number of micropores increased. The number of micropores, together with the grain size, will be reflected in the total surface area. Therefore, several researchers also concluded that by enlarging the surface area of the ceramic, the space available for dissolution/reprecipitation events to occur was greater and mineral deposition from the body fluids was more pronounced, which may be beneficial for osteoinduction to occur (23). However, it has been

demonstrated that there is a limit up to which the osteoinductive potential of a ceramic can be increased by increasing its microporosity, as a mechanically stable surface of the material is needed in order to facilitate new bone formation (22). Microporosity can also influence protein adsorption in calcium phosphate ceramics, which has been considered an explanation of the osteoinductive potential of some ceramics after *in vivo* implantation in ectopic sites. It has been suggested that the adsorption of native bone morphogenetic proteins (BMPs) from body fluids could be a key step in osteoinduction by calcium phosphate biomaterials (37).

The roughness of some osteoinductive calcium phosphate materials caused by both chemical dissolution and osteoclastic resorption of the material can also be a driving force to osteogenic differentiation or bone formation (38). On the other hand, several materials, as glass ceramic or titanium, acquire a rough surface due to their production techniques. Only chemically/thermally treated titanium implants induced bone formation (39).

1.6.3.1. PROTEIN ADSORPTION

Protein adsorption is a special property of biomaterials. When biomaterials are implanted into a living body, proteins, such as fibronectin, laminin and other adhesion glycoproteins, from the surrounding body fluids spontaneously adsorbed onto their surfaces, and then cellular attachment, proliferation and migration occurs (40). The tendency for proteins to remain attached to a surface depends largely on the material properties such as surface energy, texture, and relative charge distribution. Protein adsorption can trigger adhesion of particles, bacteria or cells possibly promoting inflammation cascades, or fouling processes (41). Thus, interactions between proteins and the material surface is an important determinant of the success of an implant.

Microporosity may consequently influence protein adsorption by providing either a larger surface area, thereby increasing the quantity of adsorbed growth factors above a critical level for cell recruitment and activation. Surface texture can also influence protein adsorption, as certain textures are more suitable for specific adhesion of molecules. The specific adhesion results in selective adsorption, leading to enhanced cell anchorage, upregulation, and/or differentiation.

Although it is generally agreed that proteins are important in CaP material bioactivity and biomineralization processes, it is still not clear the role as promoters or inhibitors of proteins in mineral crystal formation (42). Due to the variety of effects of the proteins, it has been difficult to classify them.

Another important aspect that should be considered is that differences in protein adsorption do not necessarily have a causal relationship with osteoinductive potential *in vitro* and *in vivo* (23).

So, the characteristic protein adsorption of biomaterials and the mechanisms involved need further investigation. An intensive knowledge of protein adsorption is not only beneficial to the optimization of the surface structure of biomaterials, but also helpful in determining specific applications within the field of biomedicine.

1.7. CELL SHAPE AND FATE OF HBMSCS

Human bone marrow stem cells (hBMSCs) are a cell source with an immense potential for regenerative medicine due to their capacity for differentiation into a wide range of connective tissue cell types (29).

Connective tissue cells descend from a common mesenchymal stem cell precursor, but they differ greatly in phenotype (43). Several authors have been presented that shape of differentiated cells differ according to the lineage: differentiated adipocytes are usually rounded while osteoblasts have a spread shape. The shape of these cells serve their specialized functions: a round shape allows cytoskeletal for maximal lipid storage in adipose tissue, while cell spreading facilitates osteoblast matrix deposition during bone remodelling. It is thought that these different cell shapes are due to changes in the expression of integrins, cadherins, and cytoskeletal proteins during stem cell commitment, the process by which a cell chooses its fate, and differentiation (44). The clear correlation between cell shape and differentiation leads to the assumption that changes in the assembly and disassembly of the actin cytoskeleton may be critical in supporting osteogenic differentiation (43).

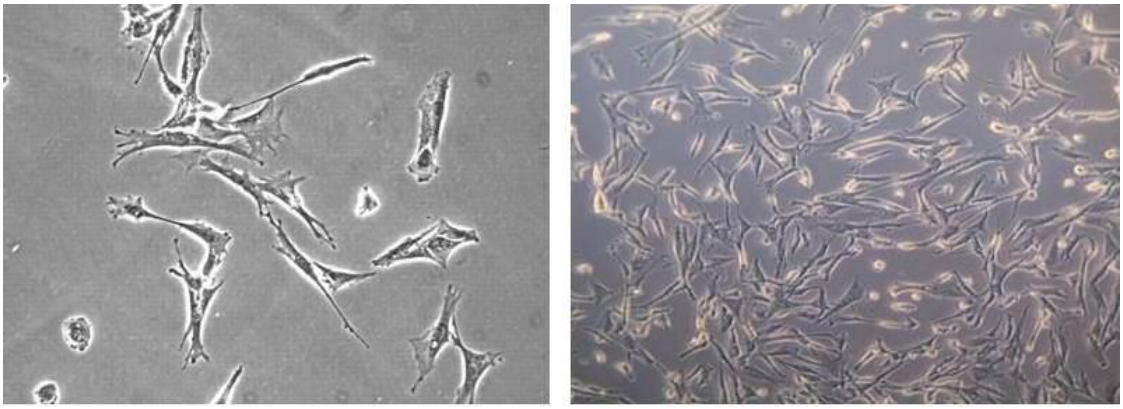

Figure 4 – Fibroblast-like cells (left) and flattened and polygonal shape (right) of hBMSCs.

Thus, the cytoskeleton plays important roles in cell morphology, adhesion, growth, and signaling. Changes in the cytoskeleton of the cell allow the cell to migrate, divide, and maintain its shape. Besides, the cytoskeleton responds to external mechanical stimuli (43).

It has been reported that several surface properties of biomaterials could affect cell cytoskeleton as well as cell fate (45), such as surface roughness and micro- as well as nano-topography are known to influence the activity and behavior of osteoblasts contacting material surfaces *in vivo* and *in vitro* by modifying the integrin expression patterns (45) (46), which may affect cell shape.

Besides surface properties, there are other environmental factors that have an effect on cell behavior. For instance MSCs can be directed towards the osteogenic lineage *in vitro* when cultured in the presence of ß-glycerophosphate, ascorbic acid, vitamin D3 and a low concentration of dexamethasone (45).

1.8. SOURCES OF HUMAN OSTEOGENIC CELLS

There are several considerations when choosing a cell source for bone tissue engineering: efficiency, availability, potential for proliferation, or self-renewing stem cells, the homogeneity of the obtained cell population and controllable induction of the osteogenic phenotype, etc.

In most cases, cells are isolated from a tissue harvest and then expanded *in vitro* to obtain an appropriated number of cells. This is a critical process due to the number of the required cells to build a tissue construct (approximately $70x10^6$ osteoblasts are needed to form 1 cm^3 of new bone). The proliferative rate of a cell type is thus very important for enhanced *in vitro* bone formation (47).

Adult bone tissue and periosteum can be used as sources of primary osteogenic cells. To isolate these, usually an explant culture is prepared from the dissected tissue or through the enzymatic release from the endosteal and periosteal layers. However, due to donor site morbidity and limited proliferation of primary cells, their use in the preparation of large autologous grafts is a real challenge (47). For this reason, the applicability of these cells will strongly depend on developing robust cell preparation procedures from source tissues that are inherently variable due to donor age, gender, health status, systemic conditions and genetic background. Adult mesenchymal stem cells (MSCs) have the potential to differentiate to the mesenchymal lineage tissues of bone, fat, cartilage and muscle. They are the most studied cells in the field of bone tissue engineering. They are believed to be the source of osteoblastic cells during the normal bone growth and remodelling and may be isolated from the bone marrow (BMSCs), among other tissues (47). However, there are some disadvantages related to the use of MSCs. The *in vitro* expansion process is difficult and the cells regenerative potential is lost during *in vitro* passages. Moreover, the available number of BMSCs in bone marrow and their differentiation potential decrease significantly with age. Thus, other stem cells sources have been investigated for bone grafts. Pluripotent human embryonic stem cells (ESCs) are an example of this. They can form any tissue of the body and have exhibited an unsurpassed (possibly unlimited) potential for proliferation *in vitro* (47). Compared to adult stem cells, ESCs grow in colonies and are passaged as small aggregates by mechanical or enzymatic dissociation from the feeder cells. But these cells have some handicaps as the development of abnormal karyotypes and other genetic alterations. This safety issue will always exist because even when ESCs are differentiated into bone cells, there are always some cells that remain pluripotent (i.e. that do not differentiate) and therefore can become tumorigenic. Additionally, ESCs cannot be used in an autologous manner and their use involves the destruction of an embryo (ethic issues). So in order to avoid the destruction of an embryo and take advantage of the beneficial characteristics of ESCs, induced pluripotent stem cells (iPSCs) derived from adult differentiated cells have been used. iPSCs share many characteristics with ESCs, including morphology, proliferation, surface antigens, gene expression, epigenetic status and pluripotency. However, these cells need more studied in order to develop safer alternatives for cell reprogramming.

Each of the sources of osteogenic human cells - primary cells, MSCs, ESCs and induced pluripotent stem cells - have distinct advantages when used for bone tissue engineering, so that the quest for an 'ideal' cell source is still in progress.

1.9. MARKERS FOR OSTEOGENIC DIFFERENTIATION

The potential of cells to differentiate to bone-forming cells relies upon molecular regulation of progenitor cells. Maturation of osteoprogenitor cells is guided by sets of key molecular markers. Known osteoblast markers include alkaline phosphatase (ALP), osteocalcin (OC), osteopontin (OPN) and collagen-I (Col-I); all of them participate in matrix formation and mineralization (48).

The osteogenic differentiation of MSCs *in vitro* has been divided into three stages. The first stage consists of days 1 to 4 where a peak in the number of cells is observed. This is followed by early cell differentiation from days 5 to 14, which is characterized by the transcription and protein expression of ALP. After this initial peak of ALP its level starts to decline. Also found at an early stage is the expression of a collagen type I matrix onto which the mineral is deposited. So, this initial stage is characterized by extracellular matrix deposition and maturation (49). The final stage from days 14 to 28 results in a high expression of osteocalcin and osteopontin, followed by calcium and phosphate deposition (49). These markers are expressed in bones and teeth and are important in regulating mineralization (45) (50) (51).

ALP was the first biochemical marker of bone turnover and is still the one most widely used in clinical practice. This enzyme is primarily found in bone and liver (52) (53). In bone, ALP is produced by osteoblasts and reflects their biosynthetic activity. ALP has been shown to be a sensitive and reliable indicator of bone metabolism (54). The expression of ALP can be measured through mRNA and at the protein level and it is used to describe osteogenic differentiation progression (55).

Osteopontin (OPN) is a highly phosphorylated sialoprotein that is a prominent component of the mineralized extracellular matrices of bones and teeth. Furthermore, OPN is expressed by cells in a variety of other tissues, including kidney, vascular tissues, salivary and sweat glands as well as in activated macrophages and lymphocytes (56). In bone, OPN is produced by differentiated osteoblasts, osteocytes and osteoclasts, working as a key regulator of bone development (57). OPN has an important role in anchoring osteoclasts to bone (resorption process) and it is synthetized during active bone remodeling, which further demonstrates that OPN has a major role in the maintenance of bone homeostasis (58). OPN has an arginine–glycine–aspartate (RGD) tripeptide sequence that promotes cell attachment, is involved in the regulation of osteoclast motility during bone resorption, and has been implicated in the regulation of both osseous and ectopic calcification (59).

Another widely used bone marker is osteocalcin (OC). It is one of the most abundant non-collagenous protein (NCP) in bone, comprising up to 20% of the total NCPs in bone a non-collagenous protein found in mineralized adult bone (57) (59). As osteocalcin is produced by osteoblasts, it is often used as a marker for the bone formation process (60). Furthermore, OC has been shown to be a potent inhibitor of HA formation

by delaying nucleation; its expression is mediated by Ca^{2+} regulated hormones, and recent experiments have demonstrated that OC acts to regulate remodelling through suppression of bone formation by osteoblasts (59).

Collagen is the most abundant protein in mammals and occurs in a number of different connective tissues both calcified and non-calcified. Collagen accounts for 70–90% of the non-mineralized component of the bone matrix and varies from an almost random network of coarse bundles to a highly organized system of parallel-fibred sheets or helical bundles. Bones contain mostly type-I collagen with some type-V collagen. Type-I collagen is the most abundant form, accounting for 90% of the body's total collagen (59). Collagen provides structural and mechanical support to tissues and organs and fulfills biomechanical functions in bone, cartilage, skin, tendon, and ligament (61). It has also been shown that type I collagen affects the expression of bone cell phenotypes (62).

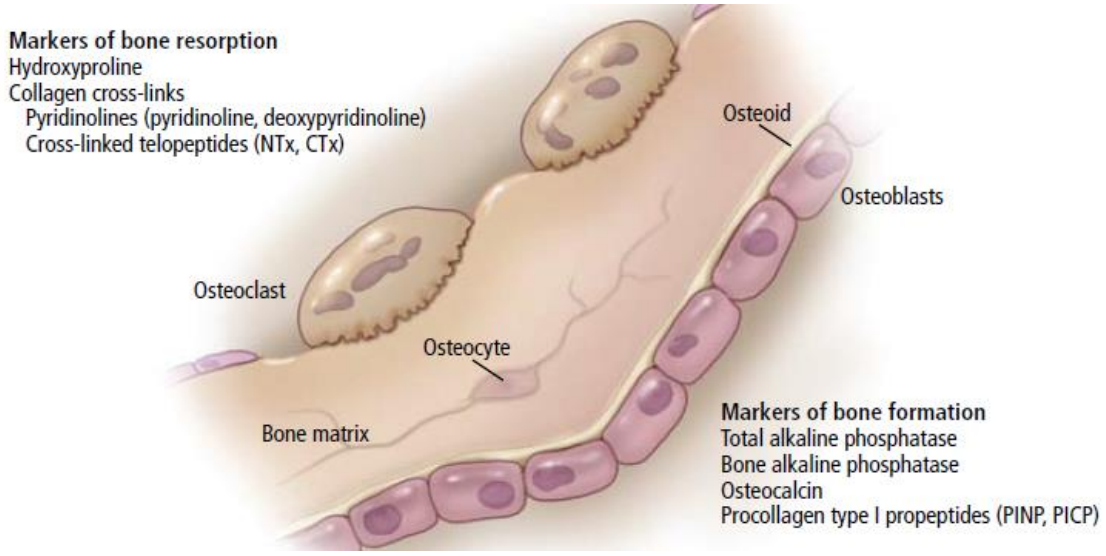

Figure 5 – Markers of bone turnover. The biochemical markers of bone turnover reflect the activity of osteoblast and osteoclast (63).

1.10. AIM OF THE PROJECT

Cells in living tissues reside in a complex three-dimensional viscoelastic environment, the extracellular matrix. Integrated information emanating from the ECM dictates the decision of cells to proliferate, migrate, differentiate, or undergo apoptosis. So, cells that adhere to the ECM can sense and respond to a wide variety of chemical and physical features of the adhesive surface, including the molecular nature of adhesive ligands and their local densities, as well as surface topography and rigidity (64) (65).

An *in vivo* study demonstrated that BMSCs contribute to ectopic bone formation using a sex-mismatched dog model (66). It was shown that BMSCs could migrate from bone marrow via blood circulation to participate in ectopic bone formation of osteoinductive biomaterial. Moreover, it has been previously shown that the response of hBMSCs correlate to different CaP ceramic properties *in vitro* (25) (67). Furthermore, in a recent study, hBMSCs were cultured on two tricalcium phosphate (TCP) ceramics which have the same chemistry but different microstructural dimension, and the study also showed only TCP-S (small microstructure dimension) is osteoinductive *in vivo* and induce better osteogenic differentiation of hBMSCs *in vitro* (16). The result demonstrated that the microstructural dimension played an important role in inducing osteogenic differentiation of hBMSCs *in vitro* and osteoinduction *in vivo*. Bone formation was found only in the TCP-S (canine model). Therefore, we hypothesize that any common used surface for bone graft application having a microstructure comparable to that of TCP-S will have similar capacity to induce osteogenic differentiation of hBMSCs, regardless of the surface chemistry. To test this hypothesis, three TCP ceramics based discs were prepared to have the same surface topography, comparable adsorb protein adsorption, similar ions (e.g. Ca^{2+} and PO_4^{3-}) exchange, but different surface chemistry (TCP-S, TCP-S with carbon coating and TCP-S with gold coating). Cellular behavior of hBMSCs on the three TCP ceramics was then evaluated.

2. MATERIALS AND METHODS

In this section, all the methods used for the production of the discs, culture and characterisation of hBMSCs are described. The handling of cells was performed using aseptic conditions and sterile materials.

Throughout the study for this master thesis three different donors were used. Each of these donors was used in previous studies and was considered the best for a certain type of experiment. Thus, it was used the best donor for each type of experiment.

2.1. PREPARATION OF TCP-S, TCP-S WITH C AND TCP-S WITH AU DISCS

TCP-S discs (diameter of 9 mm, thickness of 1 mm) were kindly provided by Xiaoman Luo (Xpand Biotechnology BV). Prior to the coating process, TCP-S discs were ultrasonically cleaned in water. For carbon coated TCP-S, a carbon coater (Edwards 306) was used at a voltage/current of 36V/60mA with vacuum $10x10^{-5}$ mbar for 4-5 seconds. Gold coated TCP-S (i.e. TCP-S with Au) was done using a sputter coater (Cressington Sputter Coater 108 Auto). Discs were treated with two cycles of 90 seconds each with a current of the 30mA.

2.2. PHYSICOCHEMICAL PROPERTIES OF TCP-S, TCP-S WITH C AND TCP-S WITH AU DISCS

Chemical compositions of TCP-S, TCP-S with C and TCP-S with Au discs were determined using a scanning electron microscope (SEM, Philips XL30) coupled with an energy dispersive x-ray spectrometer (EDS, EDAX, AMETEK Materials Analysis Division). The microstructure was studied with SEM in the secondary electron mode. The surface roughness was analyzed with an atomic force microscopy (AFM; PicoScan Controller 2500 – Quadrexed Multimode; Molecular Imaging). A super sharp TESP cantilever: 42N/m, 320kHz, 2-5nm ROC, No Coatings (Bruker AFM Probes.) was used in tapping mode. Images were taken at a scan rate of 1 Hz on 100 μm^2 area at 3 different surface points for each sample (n=3) with a Nanoscope (612r1®,Digital Instrument Veeco). Obtained images were analyzed with Scanning Probe Image Processor (SPIPTM, version 4.2.2.0) to obtain roughness measurements (Ra, Rq, and Rmax) and high quality 3D images.

2.3. STERILIZATION OF THE DISCS

To find out the best way to sterelize samples, three methods of sterilization were used in this study: physical (UV light), chemical (70% ethanol) and thermal sterilization (autoclave).

Physical sterilization has been performed by UV light (MPXL DUO PS135 UV LAMP). The discs were exposed to 8 μW/cm2 intensity for 30 minutes. To achieve this intensity the lamp was placed to approximately 38 cm distance of the discs. The distribution angle (beam) was approximately 20°.

Sterilization with 70% ethanol was been carried out with alternating cycles of soaking and evaporation of ethanol. In the first two cycles, samples were soaked by 200 μl of ethanol for 10 minutes and then ethanol was removed to evaporate for at least 10 minutes. In the last cycle, the remaining ethanol is allowed to evaporate completely overnight.

Steam sterilization was also executed. Samples were placed in a petri dish covered by aluminium foil and they were steam sterilized at pressure of 1-1.1bar (120 °C) for 20 min by autoclave (Astell Scientific) and dried at 50 °C afterwards.

2.4. ION RELEASE MEASUREMENTS

To evaluate the influence of surface chemistry on the ion release, TCP-S, TCP-S with C and TCP-S with Au discs were immersed in 1 ml of basal medium (BM) and incubated at 37 °C in humid atmosphere with 5% CO2 for 1, 4, 7 and 14 days. Basal medium (BM) consists of Minimal Essential Medium-alpha (α-MEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Lonza Group Ltd), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco), 2 mM L-Glutamine (Gibco) and 0.2 mM L-ascorbic acid 2-phosphate (ASAP, Sigma-Aldrich Co., A8960).

Calcium and phosphate ion concentrations in culture medium containing samples and controls (i.e. BM without ceramics) were measured with Quantichrom™ Calcium Assay Kit DICA-500 (Bioassay systems) and Quantichrom™ Phosphate Assay Kit DIPI-500 (Bioassay systems) respectively, following the manufacturer's guidelines.

Absorbance measurements of the standard solution and samples were performed with spectrophotometer (Thermo Scientific Multiskan GO UV/Vis Spectrophotometer) at 620 nm. Calcium and phosphate ion concentrations were calculated through standard calibration curves and expressed as mean ± SD. Three samples were used per material (n=3).

2.5. PROTEIN ADSORPTION IN THE MEDIUM AND TO THE CERAMICS

To calculate the amount of protein adsorbed by discs, TCP-S, TCP-S with C and TCP-S with Au discs were incubated for 1, 4, 7 and 14 days in 1 mL of basal medium at 37 °C in humid atmosphere with 5% CO2. The protein amounts remaining in the medium and adsorbed on the discs were measured with the Pierce® BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's protocol. Absorbance measurements were performed with spectrophotometer at 562 nm. A calibration curve was prepared using standard BSA solutions.

In order to measure the amount of protein adsorbed by ceramics, 100 µl of RIPA buffer (Thermo scientific) was added to each sample after washing three times with PBS and kept at 4°C for 10 minutes. Thereafter the amount of protein detached from the ceramics into RIPA buffer was measured. The RIPA buffer consists of 10% RIPA buffer (10X) supplemented with protease phosphate and 89% demi water. Three samples were used per material (n=3) and the protein content was expressed as mean ± SD.

2.6. EXPANSION OF HBMSCS

Bone marrow aspirates were obtained from three different donors, at passage 2, with written informed consent. The cells were stored in a liquid nitrogen vessel (Custom Biogenic Systems). Aspirates were thawed directly to the medium and cultured in T-flaks (T175, ThermoScientific). For expansion of hBMSCs, proliferation medium that contained BM and 1% of basal fibroblasts growth factor (bFGF, Instruchemie) was used.

Cells were cultured at 37°C in a humid atmosphere with 5% CO_2, medium was refreshed twice a week. Cells were subcultured until 90% confluence.

2.7. CELL CULTURE ON TCP-S DISCS

To study the influence of surface chemistry on cellular behaviour; hBMSCs were cultured on the TCP-S discs.

The ceramic discs were placed on non-treated 48 well-plates and soaked in basal medium overnight. A cell suspension of hBMSCs (1 ml) was seeded on the materials at a seeding density of 25000 cells/ml for all the experiments except Phalloidin and DAPI in which a cell density of 5000 cells/ml was used.

All the performed experiments are presented in Table 2.

Table 2 – List of the experiments performed.

Experiments	Method	Days of culture
Distribution of cells	Methylene Blue	1, 4, 7 and 14
Metabolic activity/cell viability	Presto Blue	4, 7 and 14
Osteogenic differentiation	ALP staining	7 and 14
	ALP assay	7 and 14
Cell proliferation/ cell amount	DNA assay	1, 4, 7 and 14

Cell shape/morphology	Phalloidin and DAPI	4
	SEM	1, 4 and 7
Gene expression	PCR	4, 7 and 14

2.8. CELL DISTRIBUTION

Cells were fixed with 10% neutral formalin and stained with 1% methylene blue solution (Sigma-Aldrich) in order to observe the adherent cells on the discs, after washing with phosphate-buffered saline (PBS) solution three times.

Cell distribution on the discs was observed with a light microscope (LM; E600, Nikon SMZ-10A, Japan) equipped with KL 1500 LCD camera (SCHOTT).

2.9. CELL SHAPE AND MORPHOLOGY

Cell shape was investigated using SEM and fluorescent microscope after Phalloidin and DAPI staining.

Specimens for SEM were prepared for the high-vacuum imaging environment. Firstly, samples must be fixed to preserve their native structure. In this study, it was used specimens from methylene blue experiment. So, they were already fixed. Following fixation, samples were then dehydrated with graded ethanol (70, 80, 90, 95 and 100%) to ensure that all water was removed. Then they were dried using critical point drier (CPD 030. Balzers), mounted on appropriate stubs and sputter-coated with gold-palladium to prevent charging effects that hinder suitable image formation. Images were collected using SEM (Phillips). This process is illustrated in Figure 6.

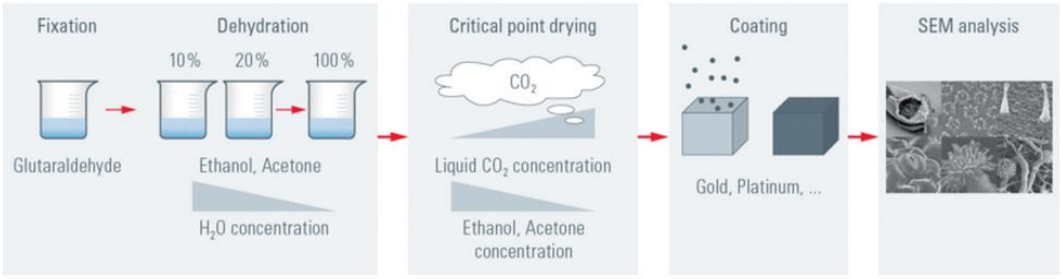

Figure 6 – Example of a schematic diagram of workflow for SEM analysis.

After 4 days of culture, the cells were fixed in 10%formalin for 20 minutes at room temperature. Once fixed, the cells were washed three times with PBS. To permeabilize the cells 1% Triton X-100 in PBS solution was added for 10 min at 4º C. The cells were then washed three times with PBS. Then the samples were

incubated for 1 h at room temperature in 1% BSA/PBS to block the un-specific binding. Following the withdrawal of 1%BSA/PBS, Alexa568-phalloidin (1:60 in 1%BSA, Invitrogen) was added for 1H, avoiding light for actin staining. Then, the samples were stained by DAPI (1:1000, Invitrogen) for nucleus staining during 15 min. After washing three times with PBS, fluorescence images of isolated cells were taken with the fluorescence microscope (NIKON ECLIPSE E600).

2.10. METABOLIC ACTIVITY ANALYSIS

Metabolic activity of the cells was monitored using Presto Blue assay.

A fresh presto blue working solution was made in a concentration of 10% presto blue solution (Invitrogen) in basal medium, avoiding the light.

After aspiring medium from the wells, 1mL presto blue solution was added to each sample and the well plate was placed in the incubator at 37°C for 10 minutes.

Thereafter, 200 µl solution was taken from each well and placed in a white 96-well plate in triplicates, avoiding the light.

The fluorescence was measured using a spectrophotometer (VICTOR3 1420 Multilabel counter Perkin Elmer). The wavelengths of excitation and emission were 540-570nm and 580-610nm, respectively.

2.11. OSTEOGENIC DIFFERENTIATION STAINING

Sigma-Aldrich Alkaline Phosphatase kit was used to study osteogenic differentiation staining. The experiment was conducted following the manufacturer's protocol.

After the 7 and 14 days of cell culture, BM was removed and the samples were washed three times with PBS and fixed for 30 seconds. The fix solution was previously prepared by mixing a citrate working solution (2 ml citrate concentrated solution with 100 ml deionized water) and acetone. Then these samples were rinsed with deionized water and incubated with alkaline dye mixture for 30 minutes, avoiding the light. This alkaline dye mixture was constituted of 2 ml Naphtol AS MX-PO4 and 48 mL prepared diazonium salt solution, which was prepared by dissolving one fast blue salt capsule in 48 ml distilled water, using magnetic stirrer at room temperature.

Finally, the samples were washed 3 times with deionized water and observed under the stereo microscope (LM; E600, Nikon SMZ-10A, Japan).

2.12. OSTEOGENIC DIFFERENTIATION (ALP ASSAY) AND PROLIFERATION ASSAYS (DNA ASSAY)

Cell proliferation was analyzed with a DNA assay (CyQuant® Cell Proliferation Assay kit, invitrogen), while osteogenic differentiation was measured with an alkaline phosphatase (ALP) assay (CDP-*Star* assay kit, Roche).

Before the analyses, the samples were rinsed three times with PBS and stored at -20°C until further use. 500 µl of DNA cell lysis buffer (prepared according to manufacturer's instructions of CyQuant Cell Proliferation Assay kit instructions) was added onto each sample, followed by two cycles of freezing and thawing at -20°C and room temperature, respectively.

After these two cycles, an ALP assay was done immediately according to the manufacturer's instructions. Briefly, 10 µl of cell lysate and 40 µl of CDP *Star* substrate were incubated in white 96-well plate for 20 minutes avoid light. In the end, the luminescence was measured using a spectrophotometer (VICTOR3 1420 Multilabel counter Perkin Elmer). ALP expression was normalized to DNA content. Three samples were used per material (n=3).

For the DNA assay a standard curve that relates the luminescence with DNA content was prepared. DNA standards with the concentrations between 0 and 1000 ng/ml were prepared using λ DNA (100 µg/mL). Then, 100 µl of DNA standards and cell lysate sample were transferred into a white 96-well plate and allowed to incubate in the dark at room temperature for 1h.

Afterwards, CyQuant GR dye was prepared diluting the 200X GR dye (from the kit) 200 times with cell-lysis buffer and incubated to samples at room temperature in the dark for 20 minutes. Fluorescence was measured using a spectrophotometer at an excitation of 480 nm and emission wavelength of 520 nm. The DNA content of the cells was calculated through a standard DNA curve. Three samples were used per material (n=3).

2.13. BONE- RELATED GENE EXPRESSION

Bone-related gene expression was evaluated with quantitative real-time polymerase chain reaction (PCR) assay. RNA isolation was performed using Trizol reagent (Invitrogen) and Nucleospin RNA isolation kit (Macherey-Nagel Gmbh&Co.) according to the manufacturer's instructions. Total RNA was measured using a NanoDrop spectrophotometer (Nanodrop- ND-1000 Spectrophotometer, Fisher Scientific). To synthesize complementary DNA (cDNA), the previously isolated RNA was used with iScriptcDNA Synthesis kit (BioRad) according to the manufacturer's instructions. PCR analysis was performed with the Bio-Rad real-time PCR system (Bio-Rad, Hercules) on markers of alkaline phosphate (ALP), collagen type I (Col I), osteocalcin (OC) and osteopontin (OPN), with beta-2 microglobulin (B2M) as the house-keeping gene for normalization.

The parameters used in real-time PCR run were 95 °C for 10 min followed by 95 °C for 30 s; 60 °C for 1min and 72 °C for 1 min for 40 cycles. Data was analysed using Bio-Rad iQ5 software. The relative amounts of target genes normalized by B2M were calculated by $2^{-\Delta CT}$ method where $\Delta CT = CT_{,Target} - CT_{,B2M}$.

Primer sequences for ALP, Col I, OCN, OPN, and B2M are listed in Table 3. All experiments were done in triplicate (n=3).

Table 3 – Primer sequences of the studied bone-related genes.

Gene	Forward primer	Reverse primer
ALP	ACAAGCACTCCCACTTCATC	TTCAGCTCGTACTGCATGTC
COL-I	AGGGCCAAGACGAAGACATC	AGATCACGTCATCGCACAACA
OCN	GGCAGCGAGGTAGTGAAGAG	GATGTGGTCAGCCAACTCGT
OPN	CCAAGTAAGTCCAACGAAAG	GGTGATGTCCTCGTCTGTA
B2M	GACTTGTCTTTCAGCAAGGA	ACAAAGTCACATGGTTCACA

2.14. STATISTICAL ANALYSIS

Multiple comparisons were performed with two way analysis of variance (ANOVA) followed by Bonferroni post-test comparisons. A $p<0.05$ was considered as a statistically significant difference.

3. RESULTS AND DISCUSSION

3.1. PHYSICOCHEMICAL CHARACTERIZATION OF DIFFERENT TCP-S DISCS

The EDX results of TCP-S, TCP-S with C and TCP-S with Au discs showed all the discs had Ca, P, O elements (Figure 7A). TCP-S with C discs showed an extra peak of carbon and TCP-S with Au discs had an extra peak of gold. Surface microstructure of the discs was observed with SEM (Figure 7B), showing no differences of surface topography among TCP-S, TCP-S with C and TCP-S with Au discs. Therefore, the surface coatings of carbon and gold did not change the surface microstructure. Similar results were confirmed by AFM results (Figure 7C): no differences in surface topography were observed with AFM images (Figure 7C), and the average roughness (Ra) and root mean square (Rq) showed no significant difference among TCP-S, TCP-S with C and TCP-S with Au discs (Figure 7D).

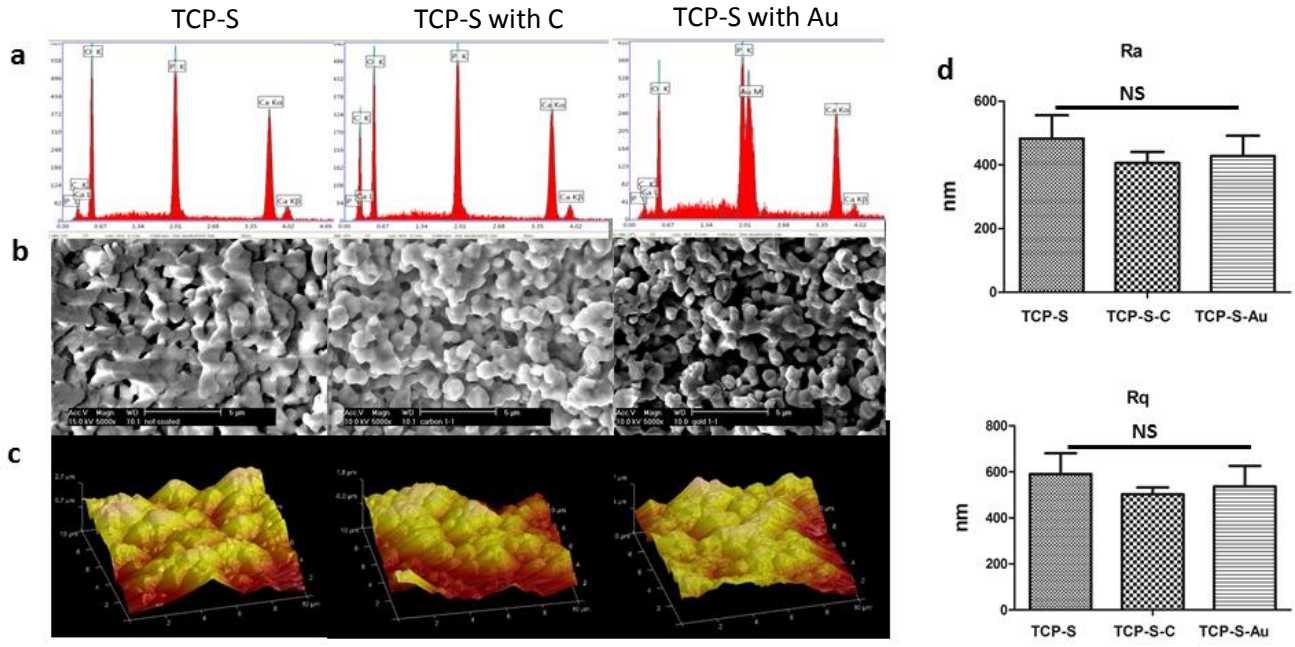

Figure 7 - Chemical compositions (a), SEM pictures of surface (b), AFM topography (c), Average roughness (Ra) and root mean square (Rq) for all types of TCP-S discs (d).

3.2. ION EXCHANGE

Figure 8 depicts the results obtained on calcium concentration: in all solutions with samples, the calcium concentrations slightly decreased from day 1 to day 14 because calcium adsorbs on the surface of the disc. This happened because the calcium concentration reached saturation enabling that precipitation of these ions took place. However, at day 1, as shown in Figure 8, TCP-S released a high amount of calcium meaning that TCP-S are the most dissolved ceramic of this group of samples. On the other hand, all the calcium concentration in the medium with ceramic discs were lower as compared to basal medium (BM) indicating that calcium was not released from the ceramics, but was rather adsorbed in the discs. There was no significant difference between the three samples, which means the coatings did not affect the calcium ions exchange. Overall, the results indicated that there was adsorption of calcium ions since day 1 and that this adsorption increasing over time.

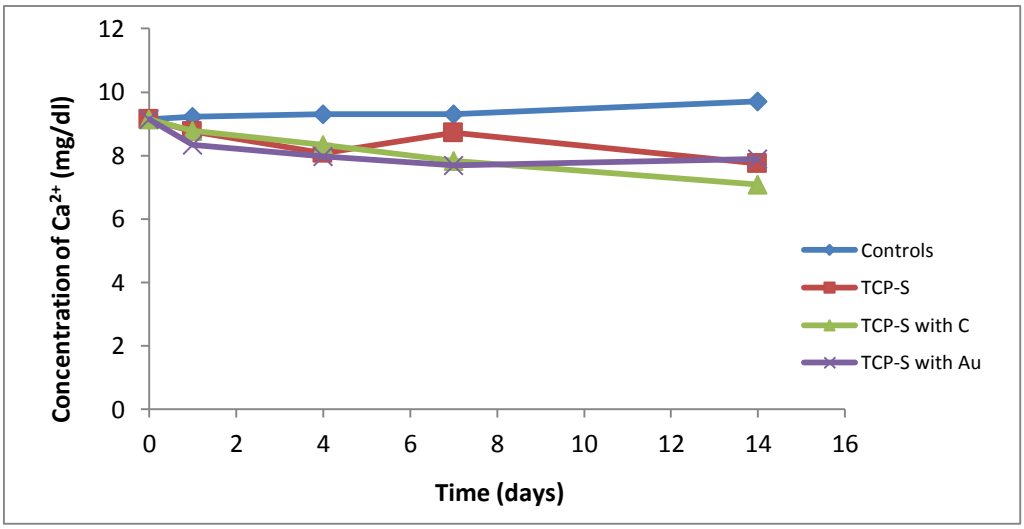

Figure 8 – Calcium release profiles of the TCP-S with different coatings.

In other studies it was assumed that the calcium ions (Ca^{2+}) released from the CaP materials play a role in their bioactivity (68) and that these materials combined with hBMSCs have a positive effect in healing bone defects (69). In addition, the possible role of ion release from CaP ceramics in osteogenic differentiation of hBMSCs has also been presented in literature (68). It is believed that cells may sense the change in the calcium ion concentration on the surface before it precipitates and this is the reason why, in these previous studies, they observed an effect of calcium on the cells.

In the present study, it was observed (Figure 8) a decrease in Ca^{2+} concentration in all the samples, suggesting that precipitation of Ca^{2+} from the culture medium occurred on the ceramics surface, where it could form an apatite layer that would enhance the osteogenic differentiation of MSCs, as assumed by the literature (16) (70).

On the other hand, the analysis of phosphate ions concentration shows that the concentrations of phosphate ion in the medium with ceramic discs were lower than the BM in all the samples. The result showed that the phosphate ions also adsorbed in the ceramic discs, which was correlate to the calcium ions result described above. However, the phosphate ions concentration in the medium with samples decreased from day 0 to day 4, and increased after day 4 until day 14, i.e. phosphate ion precipitated until day 4, but since then, it was released again. Still, it should be noted that the medium was refreshed at day 4 and day 7 twice a week after collecting old medium for ions assay.

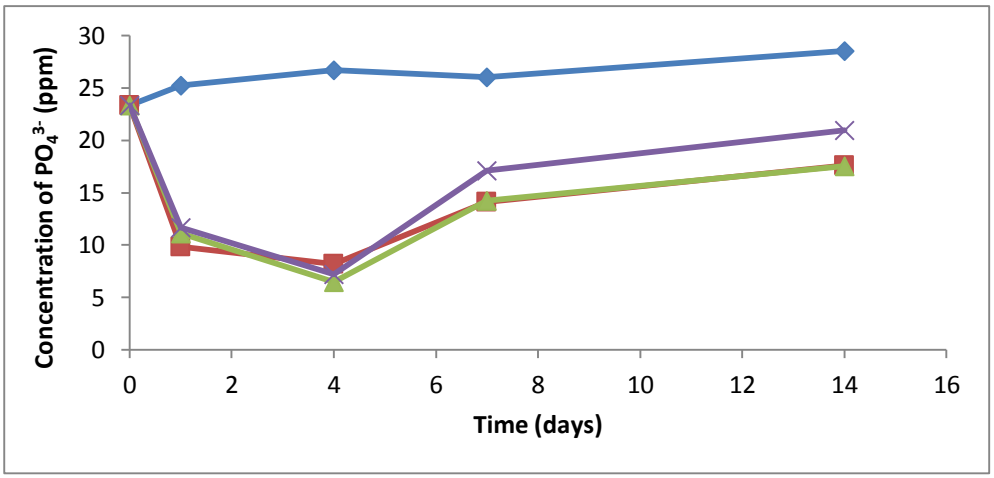

Figure 9 – Phosphate release profiles of the TCP-S with different coatings.

Neither in calcium profile nor in phosphate profile significant difference was found among the different samples. The results showed that all the ceramic discs have similar calcium and phosphate ions exchange profiles respectively. This is an important statement because in the case of CaP containing materials, ion exchange between the material and the medium may significantly modify the composition of the latter, and hence the environment for cells to grow and differentiate. Furthermore, changes that take place in the medium will vary depending on the properties of the material, resulting in a study where same cells were cultured in different environments, making comparisons between them difficult and less reliable.

Figure 8 and Figure 9 showed different release profiles for calcium and phosphate. However, these profiles have a similarity: both ion releases are lower than the control. This is because they precipitate and only small portion of it is released.

In short it is known that the release of ions from these materials will play a role in cell behavior. The response of osteoprogenitors to Ca^{2+} is of great interest for the clinical field of bone regeneration because it uses CaP-based bone void fillers to heal bone defects (68), but it is currently not known how Ca^{2+} released from these ceramic materials influences cells in situ. Understanding how hBMSCs sense extracellular Ca^{2+} and PO_4^{3-} released by CaP biomaterials and respond to it becomes fundamental to improve cell-based therapies for bone tissue engineering.

3.3. PROTEIN ADSORPTION

Under physiological conditions, the adhesion of mammalian cells to a solid surface is mediated mainly by the presence of a protein layer whose properties depend on the substrate and on the composition of the liquid phase (71). These are the facts behind the importance to investigate whether the higher protein content of TCP-S reflects changes in the ratio pro/anti-osteogenic proteins and, if so, which proteins are involved in the process. However, it should be emphasized that differences in protein adsorption do not necessarily have a causal relationship with osteoinductive potential *in vitro* and *in vivo* (67). Besides, it is known the protein adsorbed on the surface has an important role in cellular behaviour.

To measure the quantity of proteins, two methods were used: in the first method proteins in the medium were quantified, while in another method the amount of proteins adsorbed on the discs was measured.

From Figure 10, the amount of proteins in the medium did not change over time. No significant differences in the amount of proteins in the medium were found among the samples and also comparing with the controls at all the time points. However, the result was not evident if there was proteins adsorption on the ceramic discs or not.

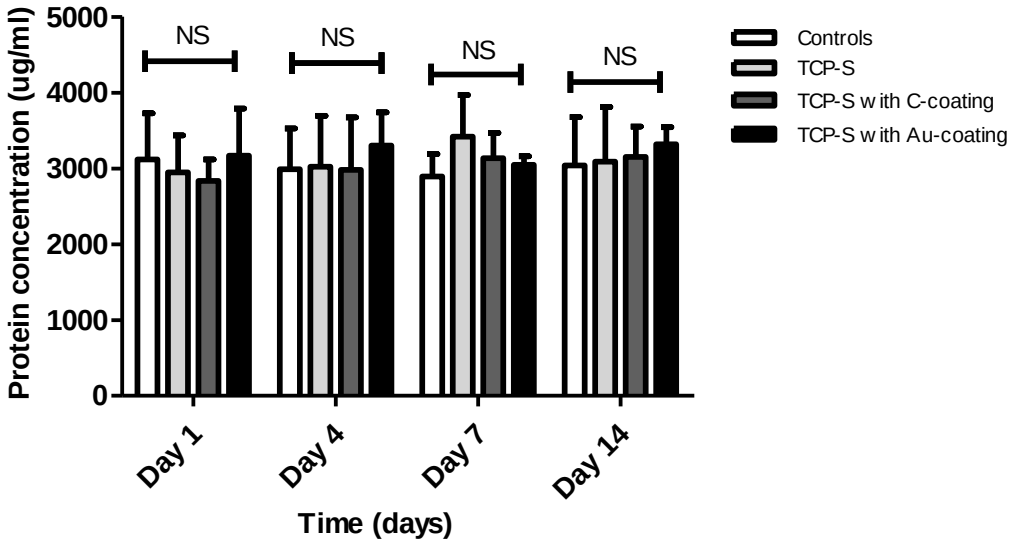

Figure 10 – Concentration of protein in the culture medium.

In order to find out the amount of protein adsorbed on the discs, RIPA buffer was used to separate the proteins from the discs. Then the proteins detached from discs were measured and it was clear that the discs adsorbed proteins from the medium (Figure 11). However, no differences were found between the discs showing that the discs have the similar ability of protein adsorption.

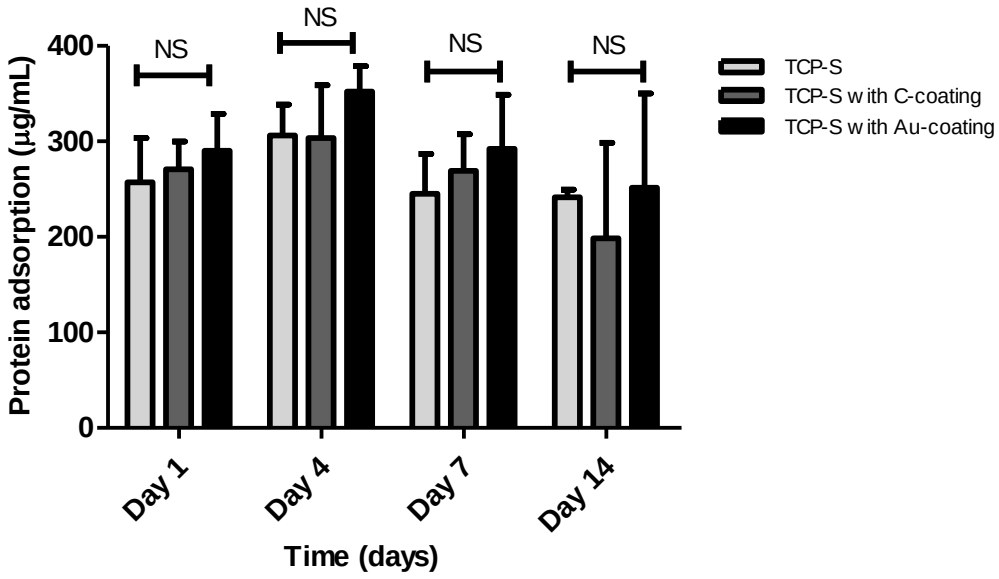

Figure 11 – Concentration of proteins adsorbed on the discs.

The incubation environment is an important factor that impacts on protein adsorption. Differences in the surface charge distribution caused by incubation in the solution have the potential to improve or inhibit protein adsorption on the material surfaces. Therefore, different solutions could induce different protein adsorption behaviors. However, for Ca-P bioceramics, the incubation solutions have similar properties according to the Ca-P application field. In the present study, the incubation environment was the same in all the conditions but the chemical composition of the surface was different. However, these different chemical compositions of the surface had not sufficient influence to create different protein adsorption in each condition. As there were no differences in the amount of proteins, it can thus be concluded that the protein adsorption is a fixed factor in this study. It can be considered that protein adsorption does not vary with the type of coating.

Comparing Figure 10 with Figure 11, it is visible that the amount of proteins in the culture medium is much higher than the amount adsorbed from the ceramic discs. Indeed, only about 10% of proteins from the medium adsorbed on the discs. So, taken together, all the samples showed similar calcium and phosphate ions exchange and protein adsorption potential. Therefore, in this study, it can be assumed that the effect of ions exchange and protein adsorption as a constant parameter in studying the cellular behavior of hBMSCs on the TCP-S, TCP-S with C and TCP-S with Au discs.

3.4. STERILIZATION OF THE DISCS - OPTIMIZATION

Sterilization of scaffolds is a prerequisite for *in vitro* culture as well as for subsequent *in vivo* implantation. The variety of methods used to provide sterility is as diverse as the possible effects they can have on the structural and material properties (40). The sterilization methods (autoclaving, irradiation, and ethanol treatment) can induce changes in the protein chemistry and physical properties, potentially affecting the absorption rate, cell attachment, mechanical strength, or performance (72).

In this study, firstly irradiation with UV light was used to sterilize the scaffolds. The discs were exposed to UV light for 30 min. After this and before seeding the cells, all discs were left in basal medium overnight in order to adsorb some components from the medium in order to promote cell adhesion. To study cells attachment, hBMSC were seeded on the discs and stained with methylene blue after 24h. Figure 12 shows a poor adherence of the cells on the discs, only few cells attached on the surface of all the discs. It is most probable that the UV light is not efficient to sterilize the samples. Therefore, another sterilization method was needed.

As such, a different sterilization method, with 70% ethanol, was used. In this way, the bacteria were killed by the process of dehydration and denaturation of proteins. This surface sterilization requires stability of such agents on the surface for more time, which is not found in absolute ethanol. So, 70% ethanol should be used in order to delay the evaporation. This ensures the proper sterilization of the surface (73). It was clearly observed that a better cell adhesion on the discs which were sterilized with 70% ethanol than using UV light (Figure 12). However, the sterilization with ethanol is time consuming. So, an easier and faster method was desired.

Finally, it was considered to use autoclave for sterilization because it is a method as efficient as 70% ethanol sterilization but easier and faster. However, steam uses both pressure and high temperatures to achieve sterility, which can be very damaging to sensitive biomolecules. In order to prove that this method does not change cell adhesion of hBMSCs, these cells were stained with methylene blue and observed using stereomicroscope. Methylene blue staining result (Figure 13) showed that the cells adhered well to the discs and were uniformly distributed over the entire surface. In addition, no big differences in cell adhesion in discs sterilized with these two methods were found. Therefore, autoclave was chosen to be applied for all the cell culture experiments.

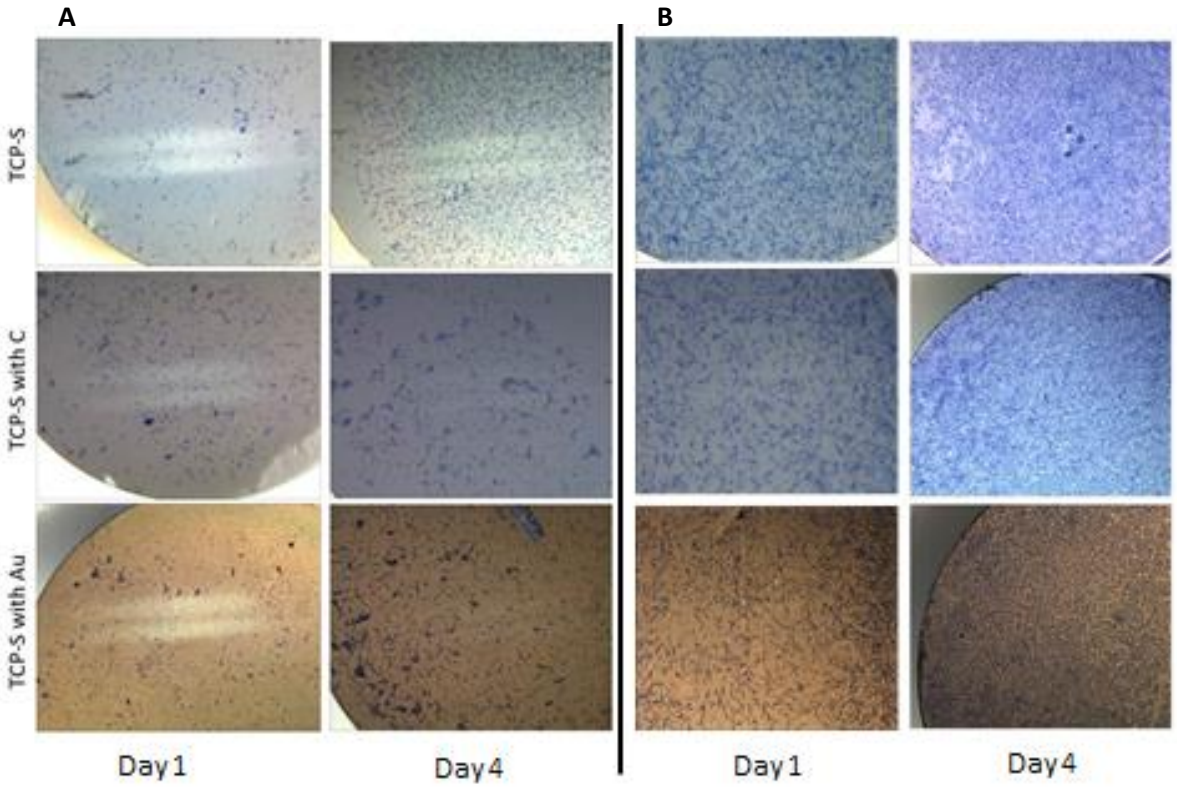

Figure 12 –hBMSCs seeded on discs sterilized with UV light (A) and with 70% ethanol (B).

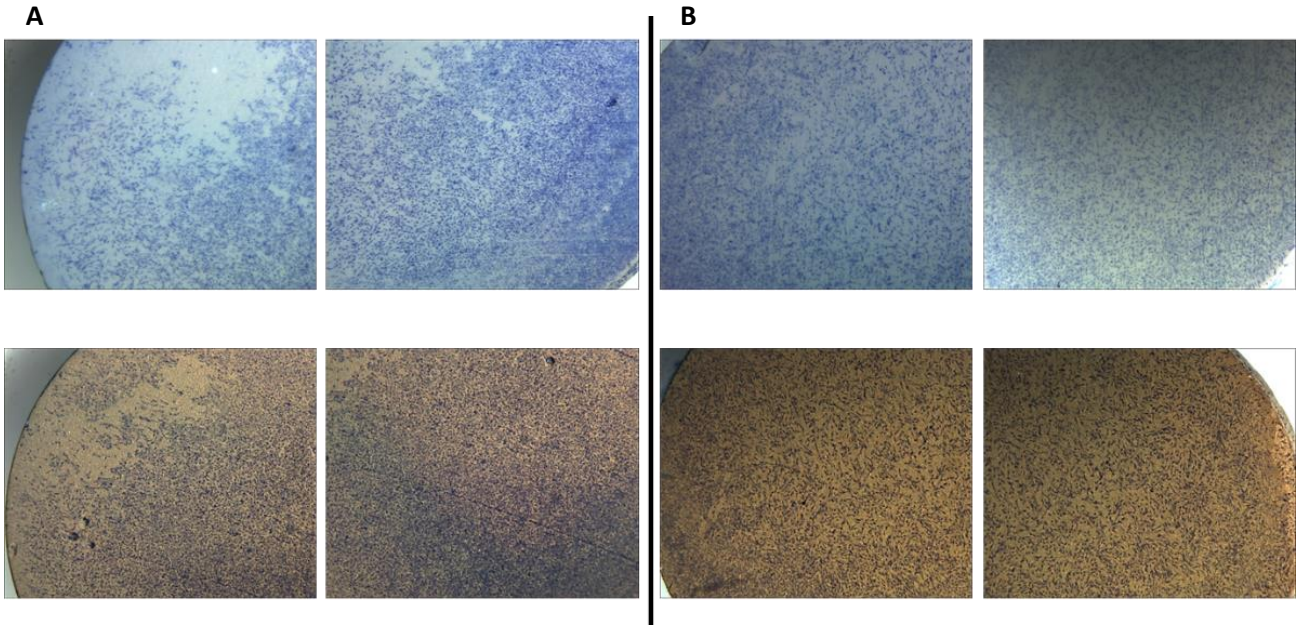

Figure 13 - hBMSCs seeded on TCP-S with C (top) and TCP-S with Au (bottom) discs sterilized with 70% ethanol (A) and with autoclave (B) at day 1 of culture.

3.5. hBMSCs ATTACHMENT AND PROLIFERATION

To evaluate the distribution of hBMSCs on TCP-S, TCP-S with C and TCP-S with Au coatings, cells were cultured for 1, 4, 7 and 14 days and stained with methylene blue.

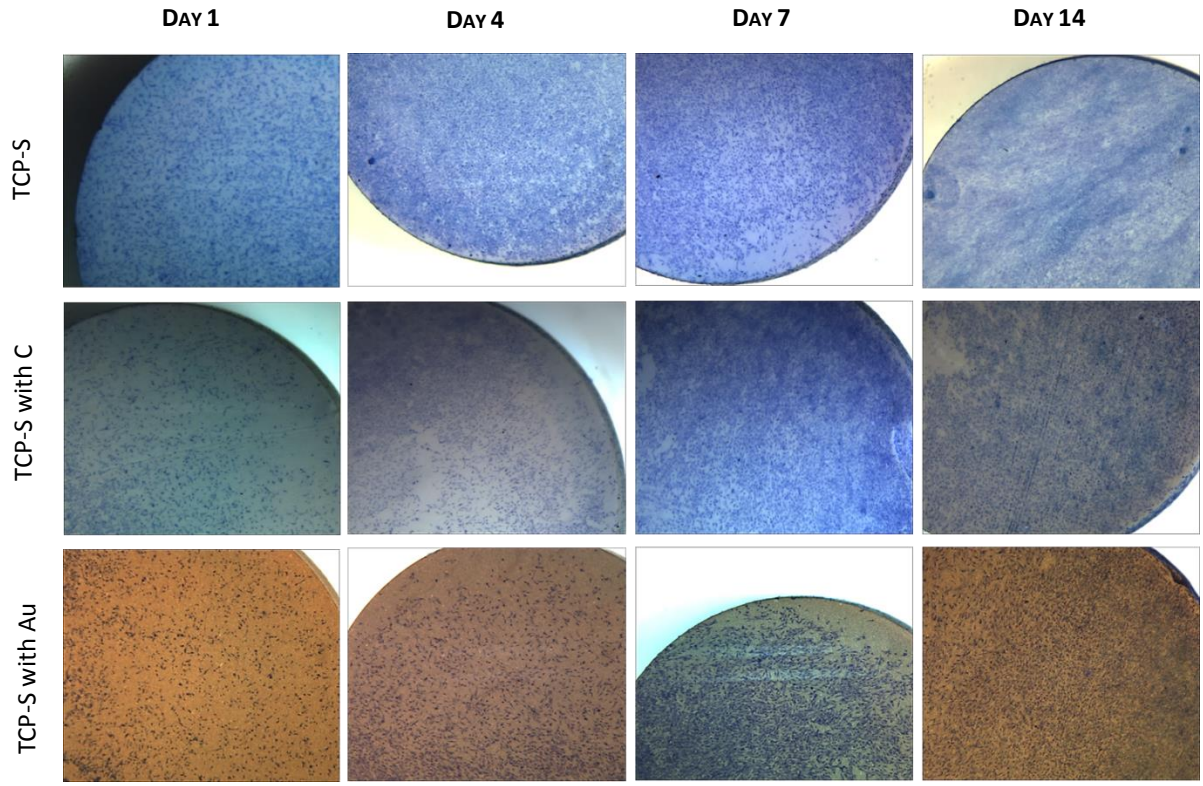

Figure 14 – hBMSCs distribution on TCP-S discs with different coatings.

As observed with stereomicroscopy, hBMSCs were homogeneously distributed on all types of TCP-S discs 1 day after cell seeding and at later time points the staining was more intense (Figure 14). These results indicated that TCP-S discs, regardless of the type of coating, supported hBMSCs attachment, growth and proliferation.

Cell adhesion is a notably complicated process and it is traditionally viewed through at least four major steps that precede proliferation: protein adsorption, cell-substrate contact, cell-substrate attachment, and cell spreading. It is well known that some surface characteristics of biomaterials as hydrophilicity, roughness, chemical composition and electrical properties play an important role in these four steps that influence in cell adhesion and consequently in controlling the activities of cells (74). For instance, the carbonate hydroxyapatite (CHA) layer that forms on the CaP materials after implantation facilitates adhesion of proteins on which the osteoprogenitor cells can attach, proliferate and differentiate (24) (75). Nevertheless, once again there was no significant difference in cell adhesion regardless of the different

chemical composition of the coatings. It means these surface chemistries do not affect cells attachment. There should be other parameters play the dominant role, for example microstructures.

Cell proliferation of hBMSCs on TCP-S was further confirmed by DNA quantification (Appendices 6.1). However, something happened during the experiment since the absorbance values (corresponding to DNA concentrations) were negative. Perhaps the lysis process was not effective and due to that, it was not possible to quantify the DNA.

3.6. CELL SHAPE AND MORPHOLOGY

To understand the role of the surface chemistry on the organization of the cytoskeleton, the shape of hBMSCs cultured on different surfaces was examined.

At day 1 and 4 of culture, the cell shape of hBMSCs cultured on the various types of TCP-S was very similar. The morphology of the cells growing on the ceramics studied initially showed a spreading habit with long and thin extents. After day 7 hBMSCs showed a flattened appearance and presented a close contact with the material. Furthermore, cells increased in number and formed aggregates, partially coating the surface of the ceramics. The cells from this day also presented abundant filopodia and intercellular connections.

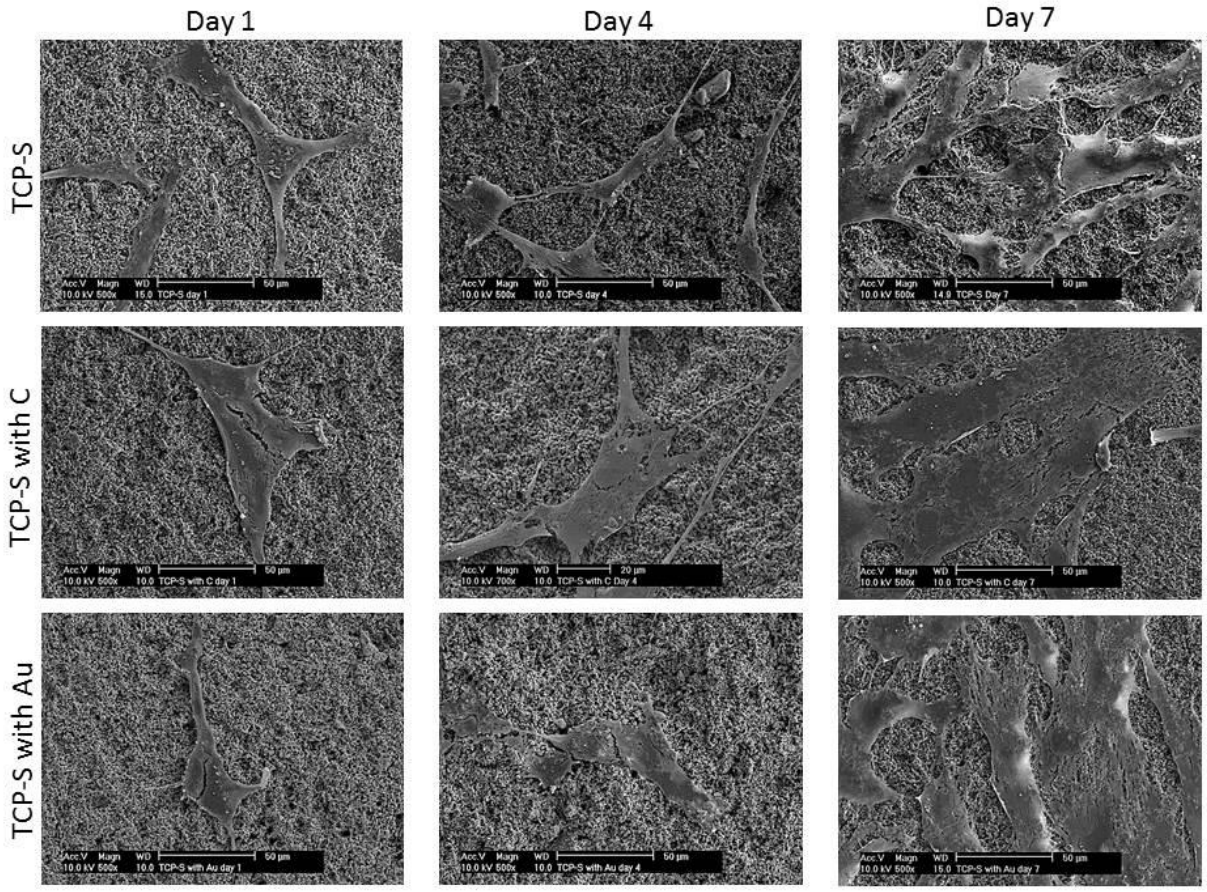

Figure 15 – SEM images of the hBMSCs growing on the TCP-S discs with different coatings after 1, 4 and 7 days.

Although the images from Figure 15 allow an analysis of the cell shape, they were obtained to study cell attachment to these different coatings. So, to confirm that morphologies achieved with SEM are characteristic of this type of cell and of these time-points, an immunofluorescence protocol was used. After 4 days of incubation in basal medium hBMSCs were fixed and stained for actin (Figure 16).

Cells exhibited a characteristic spindle-shape phenotype with actin fibers extending across the cytoplasm arranged in several directions, as revealed by Alexa Fluor 568 phalloidin staining (Figure 16, A-B). During culture time of hBMSCs on different coatings, there were no differences in actin cytoskeleton organisation which then resulted in similar morphologies of cells cultured in basal medium. Cells grown in different discs appeared always with spindle-shape morphology (Figure 16).

Several authors (43) (45) (76) proposed that the hBMSCs shape change from fibroblast-like morphology to a more flattened and polygonal morphology when they are moved from basal medium to an osteogenic medium culture. In this master thesis study, hBMSCs were cultured under basal medium and the main goal was to know if the coating material where hBMSCs were seeded, influenced cells to change their shape. As hBMSCs showed similar morphology when cultured on all the TCP-S based discs, it can be therefore assumed that this surface chemistry alone is not responsible for osteogenic differentiation.

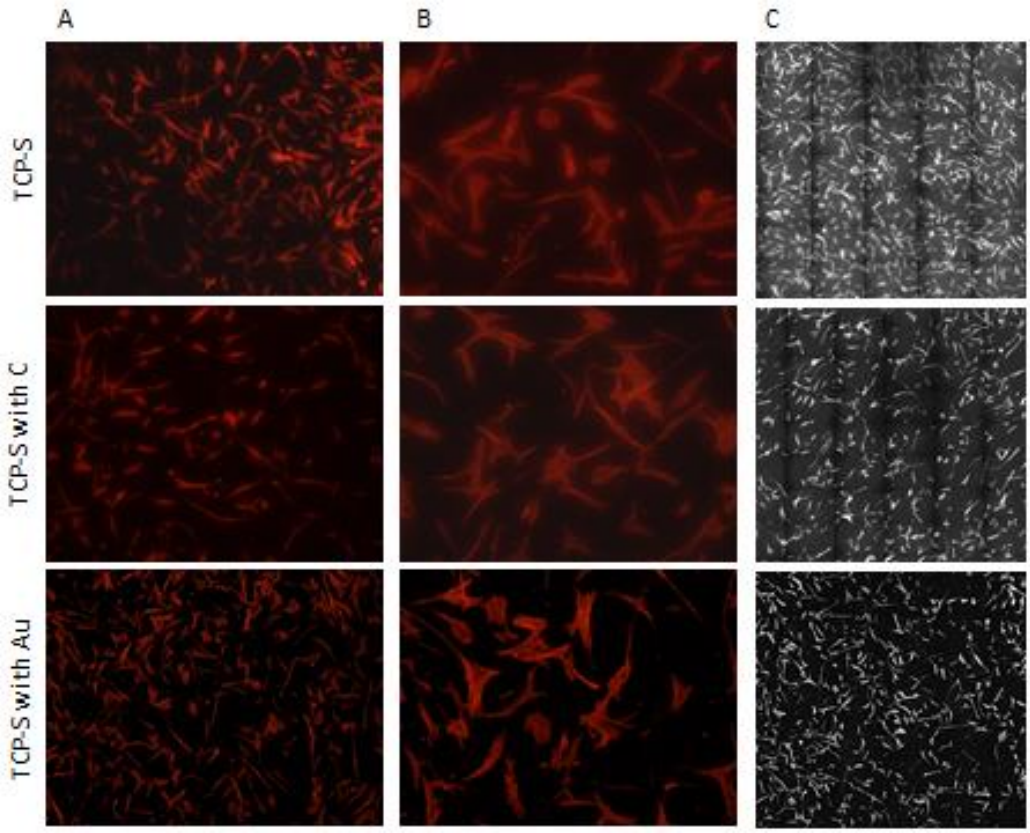

Figure 16 –Immunohistochemical staining of hBMSCs cultivated in BM against actin after 4 days of cultivation. Representative images are presented here with different magnifications 4X (A) and 10x (B). Cells were stained with fluorescent phalloidin and images were converted to grayscale where actin is white (C).

Several studies have also proved the role of topography in stem cell differentiation towards a desired direction for tissue regeneration (77) (78). It is known that microstructure plays a dominant role in cell shape (79) and due to that many of these studies developed scaffolds with different surface characteristics. In some of these, roughness and micro-topography were changed in order to unveil how these surface microstructures influence cell activity and behavior (23) (45) (80). This could explain the similar cell shapes observed on all the TCP-S based discs in this study, because the samples have different surface chemistry but similar microstructure. So, as no differences were present on the cell shape, possibly, no significant osteogenic differentiation of hBMSCs will be observed.

Indeed, previous studies had been proved that integrins establish the connection between cells and ECM and then have a role on regulation of cell growth, differentiation and motility (81). When integrins bind to ECM proteins, they physically link the ECM to the actin cytoskeleton, altering the cell morphology (81). So, due to these changes in expression of integrins and cytoskeletal proteins, it has also been reported that the differentiation of hBMSCs is accompanied by dramatic changes in cell shape (44).

While differentiation may cause changes in cell shape, studies had proved that cell morphology can also alter the differentiation of mesenchymal lineages (44), i.e. changes in cell shape or integrin expression could regulate commitment of mesenchymal cells to different lineages (82) (83). It has been shown that round cells promote adipogenesis while cells with high spreading prefer an osteogenic fate (81) (84). This correlation between cell shape and differentiation leads to the assumption that changes in actin cytoskeleton network may have a crucial impact on osteogenic differentiation (45). In this study there was no evidence that varying the surface chemistry of scaffolds with the specific used coatings altered the cell shape. So, it can be inferred that surface chemistry is not the main responsible for cell shape. On the other hand, microstructure properties of the surface, for example, could be identified as the main originator of cell shape alterations.

3.7. METABOLIC ACTIVITY

Metabolic activity of hBMSCs on TCP-S, TCP-S with C and TCP-S with Au was further studied by PrestoBlue™ analysis (Figure 17). PrestoBlue™ is a trade name for resazurin. Resazurin has a blue color but when entering cells it is reduced to resorufin, which produces very bright red fluorescence. This conversion from resazurin to resorufin is proportional to the number of metabolically active cells and therefore can be measured quantitatively. PrestoBlue™ is one of the most highly referenced substances used for cytotoxicity and viability assays.

Metabolic activity of the cells cultured on TCP-S discs without coating increased along the culture days, as expected, since at least the number of cells increases over time (Figure 14). hBMSC on TCP-S with C or Au coatings had an increase of their metabolic activity only from day 4 to day 7, as between days 7 and 14 there was no increase. At both day 4 and 7, no significant difference in metabolic activity of hBMSCs was found among the three types of coating. However, at day 14 the metabolic activity of hBMSCs cultured on TCP-S discs were significantly higher than the cells cultured on TCP-S with C and TCP-S with Au. A possible explanation for this could be that in the early days hBMSCs are proliferating and as shown in Figure 14 cells seemed to grow equally on all samples. Hence there were no significant differences in the metabolic activity at days 4 and 7. At day 14, on the other hand perhaps some cells begin to differentiate, being this differentiation more evident in TCP-S discs without coating. This differentiation process can therefore explain the significant differences.

It is known that the surface properties of a biomaterial affect metabolic activity as well as affect the cell adhesion and many other biological responses of cultured cells (74). In this study, the difference in surface properties was adjusted by varying the chemical composition of the coating. hBMSCs could not recognize the difference of the surface characteristics on various coatings, and still revealed a similar behavior in the performance of metabolic activity at days 4 and 7. hBMSCs probably could not distinguish those differences

and only revealed slightly different levels of metabolic activities, excluding on day 14 where differences were significant.

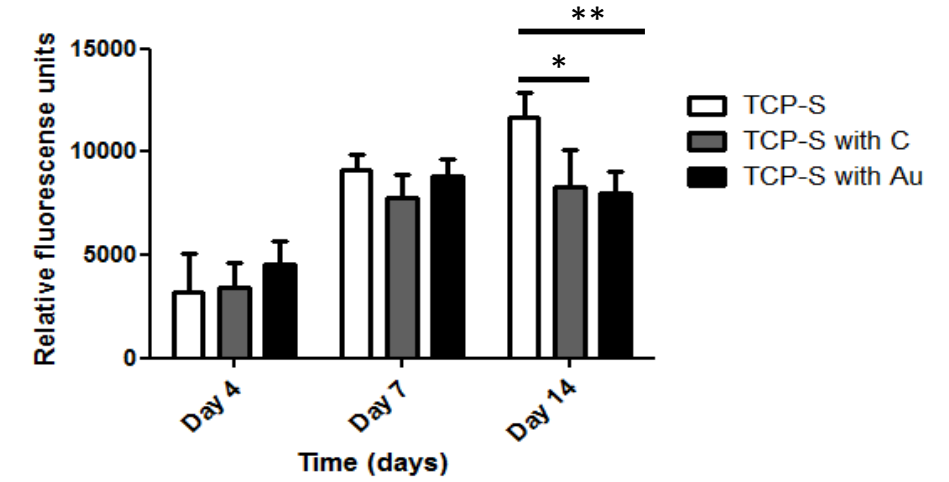

Figure 17 – Metabolic activity of hBMSC on different TCP-S discs over culture time.

The lack of differences may be explained by the fact that roughness is a constant parameter (74). Besides, the metabolic activities of hBMSCs on the TCP-S with various coatings were lower than those on the TCP-S without coating at days 7 and 14 which means that chemical elements of these coatings interfere in some way in cell metabolism, causing a lower metabolic activity. However, this method does not tell us about the type of metabolic activity. It is required to study osteogenic differentiation itself.

3.8. OSTEOGENIC DIFFERENTIATION OF HBMSCS ON THE CERAMICS

Activity of alkaline phosphatase (ALP), a key early marker of osteogenic differentiation, was examined via staining and an activity assay. In humans, ALP is present in many tissues throughout the entire body, but ALP shows particularly high activity in mineralizing bone where it is mainly localized in the plasma membrane of osteoblastic cells (85). That it is why ALP is the most widely used marker for bone turnover.

The osteogenic differentiation was detected by ALP staining at 7 and 14 -days. Undifferentiated mesenchymal stem cells show weak ALP activity, whereas differentiated osteoblasts display very high ALP activity (86). ALP activity was therefore an indication of successful differentiation of hBMSCs into osteoblasts (Figure 18).

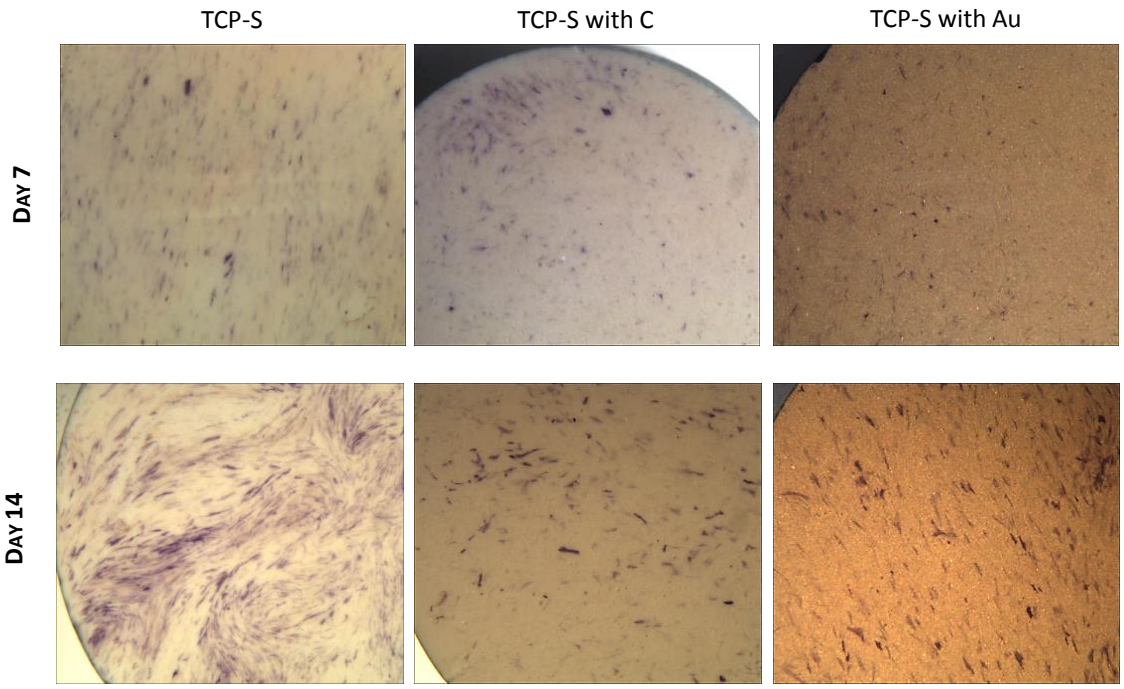

Figure 18 – ALP staining for osteogenic differentiation.

Osteogenic differentiation of hBMSCs was confirmed by a positive ALP staining at day 7 and 14 for all discs (Figure 18). From the images is evident that all showed ALP staining. At day 7 ALP expression is similar for all type of discs. In contrast, at day 14 there seems to be more ALP expression on discs without coating than the others, which may represents an increase on osteogenic differentiation.

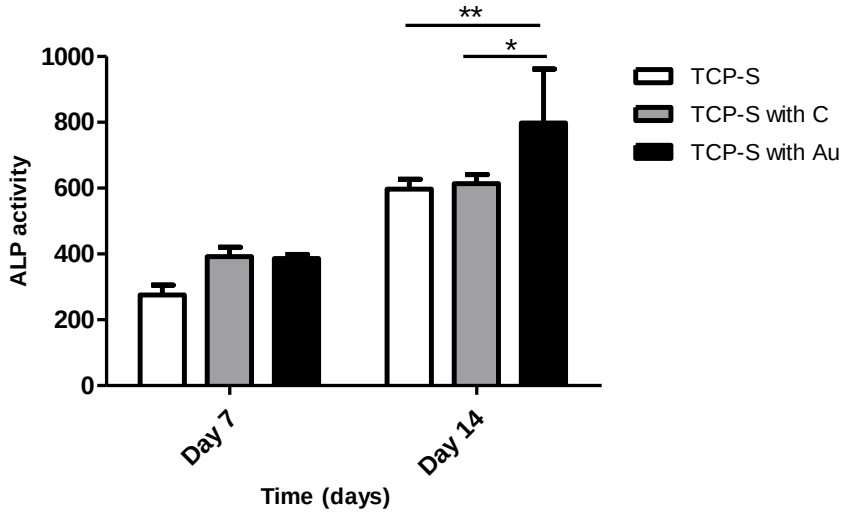

Figure 19 – Total ALP activity of hBMSCs cultured in different types of TCP-S discs.

ALP activity of hBMSCs was then detected and quantified via an enzymatic assay (CDP *Star*) at day 7 and 14 of cell culture. Enzymatic dephosphorylation of CDP-*Star* by ALP leads to the formation of the meta-stable dioxetane phenolate anion which decomposes and emits light at 466 nm (87). The ALP activity of hBMSCs cultured on TCP-S, TCP-S with C and TCP-S with Au is shown in Figure 19, where only on day 14 a significant difference was found among the different types of coating, being the TCP-S with Au discs the one with the highest activity. Most importantly, hBMSCs ALP activity was normalized by presto blue content over time (Figure 20), as a way to eliminate the variability among samples.

Here, no differences in ALP activity of hBMSCs were observed at day 7. However, the hBMSCs on TCP-S with Au discs had significantly higher ALP activity at day 14 time point than TCP-S with C and TCP-S without coating, indicating an enhanced osteoblastic phenotype of hBMSCs on TCP-S with Au.

It should be noted that ALP activity of hBMSC was enhanced from day 7 to day 14 without osteogenic reagents. This may indicate that these cells are moving towards an osteogenic lineage only due to the TCP-S properties. In order to confirm this trend, the analysis of another marker of bone formation like osteocalcin could be relevant.

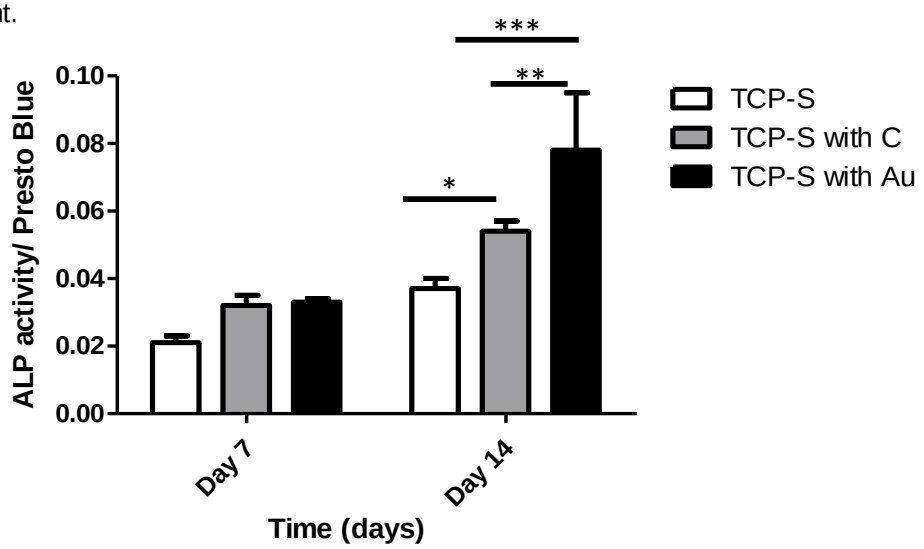

Figure 20 – ALP activity normalized by presto blue results.

On the other hand, if the intent would be the prediction of *in vivo* bone formation, measuring ALP activity alone is not sufficient. Measuring calcium deposition rate, for instance, could be an option since it is an osteogenic differentiation marker that highly correlates with *in vivo* bone formation. This measurement is usually performed using a staining method with alizarin red S solution. However, it is impossible to measure both markers at the same point in time, as ALP is an early marker while calcium deposition rate is a late phenotypic marker. Furthermore, it is also not possible to use the same cell sample to do these two experiments (88). Nonetheless, alizarin red S stains calcium compounds and the discs used in this study are made of calcium. Due to that fact, this process would be ineffective. So, it is necessary to find alternative strategies to measure mineralization and consequently to predict *in vivo* bone formation.

3.9. EXPRESSION OF OSTEOGENIC MARKERS

In this study, quantitative RT-PCR assays (Figure 21) was performed for genes encoding the most typical osteoblast-related membrane and extracellular matrix molecules (i.e. ALP, Col-I, osteocalcin (OC), osteopontin (OPN)). Both OC and OPN are related with osteoblasts that participate in matrix formation or mineralization processes. Collagen is the main component of the organic part of the bone matrix while osteocalcin and osteopontin are non-collagenous proteins that are also secreted in the organic matrix of bone (89). Still, ALP is found in the plasma membrane of osteoblasts (63).

Gene expression of Col-I, ALP and OC were higher at day 7 compared to day 4 and 14; OPN expression decreased from day 4 to day 7 and then slightly increased after day 7 until day 14 on all the samples. No significant differences in gene expression of Col-I were found among TCP-S, TCP-S with C and TCP-S with Au at any time points tested. As for OC and ALP gene expressions it was noted as being significantly higher on TCP-S on day 14 when compared with TCP-S with Au which indicates that surface chemistry had indeed some influence on this expression pattern. As ALP is an early marker and OC a late one, it would be easier to justify if the differences were found in ALP and OC expressions on day 7 and on day 14, respectively. Still regarding the OC, its expression was significantly higher on TCP-S discs when compared to TCP-S with C at day 14. On the other hand, OPN gene expression decreased from day 4 to 7 and increased from day 7 to day 14 on both coating materials and it was significantly lower on TCP-S at day 4 as compared on TCP-S with Au.

In this experiment there seemed to be a small effect of surface chemistry on expression of genes involved in osteogenic differentiation as suggested by ALP activity assay (Figure 20). However, the results found in these two experiments were different. Actually, contradictory results between gene expression and proteins had been presented in some studies (45) (90). With the intent to getting more solid results the experimental procedure could have been repeated with more donors in order to analyse whether the results are consistent or not. Due to the fact that not all the mRNA originates protein, it is usually to rely more in the results at the protein level. So, to study the expression of OC and OPN at the protein level could also be of interest.

In this case it is difficult to affirm with certainty that surface chemistry has influence on the expression of these osteogenic markers since the experimental outcomes obtained were different between gene and protein levels. When a difference in this type of experiments is verified, it is relevant to understand if it is statistically significant or not. Beyond this, it is also important whether they are biologically significant or not.

The ALP and OPN charts (Figure 21) are in accordance with what was presented in literature (49) (91), where it is described that ALP and OPN are easily detected at the end of the proliferation stage and the beginning of the matrix synthesis stage (days 5-14). It is described that ALP activity increases during the differentiation process suggesting that ALP activity may be related to alterations in the matrix in preparation for the mineralization process. On the other hand, expression of osteopontin declines during this time period

but is expressed again at higher levels during the mineralization phase (not presented in Figure 21). Col-I gene expression also corroborate the facts presented in literature (49). Expression of a collagen type I is found at an early stage, between day 5 and 14 of culture, because this stage is characterized by extracellular matrix deposition and maturation (57). This must justify the peak of expression of Col-I observed on day 7. On the other hand, OC gene expression seems to contradict several reports, (49) (57), where it is stated that OC is highly expressed during the last stage of bone formation, i.e. in the final stage from days 14 to 28. Here, a decreased expression from day 7 to day 14 of OC was observed. However, as the period presented on the literature is not shown in Figure 21 it is not possible to conclude if OC results contradicts the literature or not.

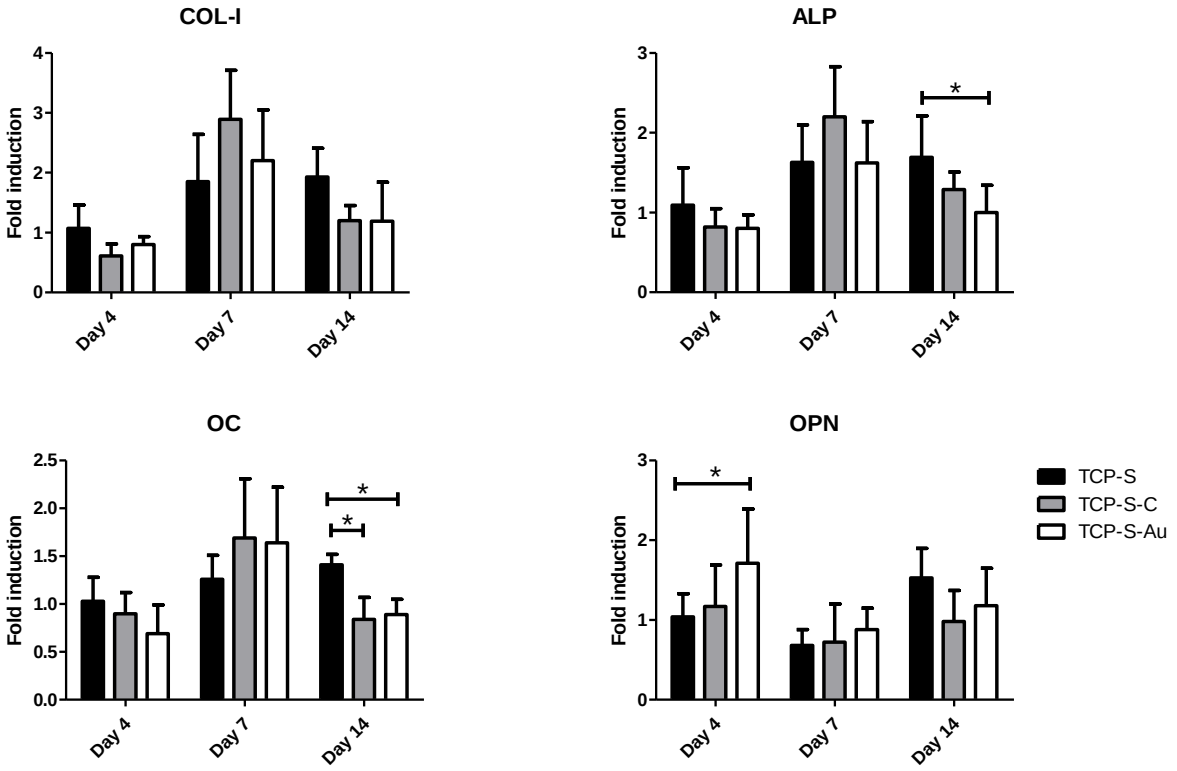

Figure 21 – Osteogenic gene expression of bone specific markers for hBMSCs cultured on TCP-S with different coatings (normalized to the B2M).

Another important aspect to this discussion is related with the lineage-specificity of certain markers. Some recent studies have shown that some of these markers are shared between osteogenic and adipogenic differentiated hBMSCs (57). For this reason, many studies still have to be conducted in order to understand how these proteins and signaling pathways are involved in the differentiation process.

Nevertheless, it is still important to search for new markers that function as tools for evaluating the lineage commitment of hBMSCs.

In addition, relating cell behavior with chemical composition, several studies had reported that hBMSCs may be sensitive not only to the adsorbed osteogenic growth factors, but also to non-osteogenic growth factors (e.g. fibronectin and vitronectin in serum) that induce osteogenic differentiation via a focal adhesion process, for instance. Furthermore, it was also observed that CaP materials adsorb more fibronectin and vitronectin than titanium and steel surfaces (92). It is believed that both Ca^{2+} and PO_4^{3-} act as protein binding sites on Ca-P surfaces and provide the major driving force for protein absorption. In this way, the chemical composition of the material seems to play a major role in the absorption of Ca-P surfaces (33). Thus, the use of CaP materials as bone substitutes is a better choice when compared with other materials since they adsorb more “osteogenic proteins” and consequently they present a major osteogenic differentiation potential.

4. CONCLUSIONS AND FUTURE PERSPECTIVES

In the last years, the role of biomaterials used in medicine changed from biologically passive, more as a structural role, to one in which its properties may be manipulated for a given engineering tissue process. Although many authors have proven that certain features of biomaterials have a positive effect in osteogenesis and proliferation of hBMSCs *in vitro*, autologous bone graft remains the most widely used procedure in bone repair strategies.

Lately, several biomaterials proved to be capable of inducing bone formation when implanted at heterotopic sites, an ability known as osteoinduction. CaP bone substitute materials belong to this group of osteoinductive biomaterials. CaP ceramics have been widely used as bone graft substitutes because their chemical composition is similar to bone mineral and they reveal excellent biocompatibility and bioactivity activity. Indeed, a substantial amount of work has aimed at achieving a better understanding of these two last parameters. It was found that cellular behaviour largely depends on the macrostructure, microstructure and chemical composition of scaffolds. However, the mechanism behind the influence of each of these factors is still an issue being studied worldwide by the scientific community.

In the present study, TCP-S discs with different coatings were used maintaining microstructure and topography constant and varying only surface chemistry. For this, gold and carbon coatings were used and compared with the TCP-S without coating. The main objective was to study if different surface chemistry affect cellular behaviour of hBMSCs. Several experiments were performed concerning cell proliferative capacity, metabolic activity, morphological differences and osteogenic differentiation.

To study the discs properties, measurements on the amount of calcium and phosphate ions in the culture medium and also on the protein adsorption were carried out. In none of these assays significant differences were found between each type of coating, allowing us to conclude that the study was performed under the same culture environments, regardless the type of coating.

Indeed, certain properties of the biomaterial substrate, including surface energy and roughness, have been demonstrated to modulate cell function (24) (81). These surface parameters have been linked to cell behaviours such as adhesion, morphology, and/or proliferation (93). Previous studies also suggest that alterations in these cellular responses may ultimately result in differentiation to different lineages (29) (94). Besides this general observation, it has been difficult to realize what the individual contribution of each surface parameter to the different phenotypes characteristic of each lineage is.

Regarding *in vitro* studies, no differences in cell proliferation nor in cell shape were found. Metabolic activity though was enhanced in TCP-S discs without coating at day 14. Concerning the gene expression, different trends were obtained with ALP, OC, OPN and Col-I, which prevented reaching a conclusion. As the expression of ALP marker at the protein (Figure 20) and gene level (Figure 21) was contradictory. Generally, it can be stated that these coatings did not cause significant differences in the cell behavior, although there were some differences. Although the obtained results may seem scientifically unattractive, it is believed that if these experiments were to be repeated, any significant differences between them would

disappear. Therefore, it can be concluded that possibly any surface having a microstructure comparable to that of TCP-S will have similar capacity to induce osteogenic differentiation of hBMSCs, regardless of the surface chemistries used. Or, in other words, that the osteoinductive character of TCP-S is not due to its surface chemistry or even that the influence of other surface parameters, such as the microstructure, are much more important than the surface chemistry, being its influence negligible. So, the results of the present study are important as they help to decipher the role of ceramics in osteoinduction.

As the main goal of this study was to understand the influence of a single parameter in osteogenic differentiation, only the surface chemistry of TCP-S discs was changed between experiments. It is important to test only one variable at a time because if there is more than one being tested, and the outcome differs between experiments, it would be more difficult to safely assume which variable was responsible for a certain change in the outcome. If only one is being tested though, it is easier to conceptually recognize the cause of a certain change and it can also be easier to measure it independently. In most of the studies using different coatings at least two properties were varied at the same time: surface roughness and surface chemistry (95). Almost all concluded that different coatings imply different cell behaviors. However, as two parameters were varied, it was not possible to identify which of the two parameters was primarily responsible for a certain behaviour. Hence, the importance of the studies that vary only one factor at a time.

Nevertheless, other studies should be carried out to prove that these coatings have no different influences on cell behaviour. Beginning with the study of the discs properties, for instance, a better option would possibly be to perform the coating procedure on the entire disc instead of only on one side, in order to avoid a direct influence of the chemistry of the calcium phosphate material in the culture medium. Because, in this study, the main goal was to study the influence of surface chemistry and not the influence of scaffold chemistry. Also related to coatings, it could be important to know whether these ones are affected by cell culture. Measuring the thickness of the coating before and after cell culture is a way to found out if the coating was affected over time. The type of coating is another important issue. Apart from the fact that they cannot be harmful to the cells, coatings should also facilitate a connection between bone and surface implant *in vivo*. Therefore, the choice of coating material is an important aspect of the study. Additionally, studies on the minimum required thickness for certain materials to have a significant effect on cell behaviour, would also be of interest.

Concerning the protein adsorption study, it could be also interesting to identify which proteins adsorb onto these TCP-S discs in order to know what kind of influence they have on the cells, because there are proteins that promote the biomineralization and others that inhibit it. In order to identify which protein adsorb on TCP-S discs, techniques like Western Blotting could be used.

Moreover, repeating the DNA assays would be appropriate as the results of ALP activity normalized by DNA amount could be compared with the obtained experimental results, i.e. ALP activity normalized by metabolic activity.

Regarding the study of cell shape, it would be much more credible if a quantitative morphological study was carried out. For this optimization, staining with phalloidin and DAPI should be repeated as these staining protocols did not go as expected.

As I mentioned before, the majority of the studied genes could not be lineage-specific, so they may not allow to conclude if these cells were differentiated into bone cells, or not. In order to understand the obtained results, the expression of adipogenic genes should be measured. If their expression became lower than that achieved in the measured genes, or null, it might lead to believe that hBMSCs were in an osteogenic differentiated state.

In addition, it would also be important to study the mineralization process. To study if these cells (because they differentiate into osteoblast cells) are able to induce bone mineralization would be relevant, for example. Nowadays these studies are performed histologically through staining with Alizarin red. It stains free calcium and certain calcium compounds with a red or light purple colour. Due to the nature of this process and the fact that the discs are made of calcium, this process would be ineffective in the present study. So, it is necessary to find alternative strategies to stain for mineralization.

For clinical applications, it is imperative to develop well-defined and efficient *in vitro* protocols for the osteogenic differentiation of stem cells. This will provide the stringent levels of safety and quality control that would make the clinical applications of stem cell transplantation therapy possible to realize. Furthermore, it is necessary to investigate novel techniques to identify biological processes occurring upon implantation of an osteoinductive material *in vivo* because they may be more efficient than the search for predictive *in vitro* assays (23) (93).

A better understanding of how stem cells respond to substrate cues is crucial for a deeper scientific knowledge on the mechanisms regulating stem cell fate decisions and thus for their potential use in Regenerative Medicine. With this master thesis study it can be concluded that the results given by the set of both the topography and the chemistry of TCP is not inseparable, as one can have the same positive influence on cells given by TCP but with certain different chemistries. Even changing the surface chemistry of the TCP, cells are able to attach and proliferate well too. Whether or not having a certain coating (at least the ones tested), the result it will be the same. For materials with similar chemistry and microstructure, similar results are expected. Thus, these results contribute to the development of design principles for the engineering of surfaces that direct cell adhesion for biomedical and biotechnology applications.

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6. APPENDICES

6.1. DNA QUANTIFICATION

Table 4 - Values of the calibration curve.

ng/ml	0	125	250	500	1000
Absorbance	9536	13922	46274	79951	153249
Absorbance - blank	0	4386	36738	70415	143713

Table 5 – Absorbance values obtained directly from the measurement.

	Controls			TCP-S			TCP-S with C			TCP-S with Au		
Day 1	14098	14532	14326	8195	7777	8366	7815	7997	8555	8607	8592	7918
Day 4	20041	19513	22840	8160	8876	8742	8933	10555	9050	8367	9061	9319
Day 7	29125	27260	29512	9466	10104	8781	9203	9337	9715	10086	9964	9976
Day 14	46875	46322	59217	12180	11786	12908	11955	11150	11752	11615	12215	12616

Table 6 - Absorbance values subtracted by the blank.

	Controls			TCP-S			TCP-S with C			TCP-S with Au		
Day 1	4562	4996	4790	-1341	-1759	-1170	-1721	-1539	-981	-929	-944	-1618
Day 4	10505	9977	13304	-1376	-660	-794	-603	1019	-486	-1169	-475	-217
Day 7	19589	17724	19976	-70	568	-755	-333	-199	179	550	428	440
Day 14	37339	36786	49681	2644	2250	3372	2419	1614	2216	2079	2679	3080