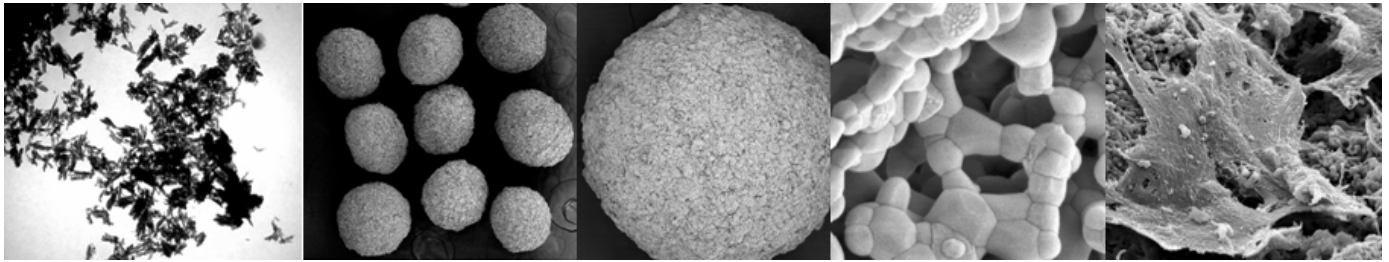


# Development, Characterisation and Application of Calcium Phosphates Nanocrystals Aggregates in a Collagen Matrix to be used as Biomaterial in Bone Regeneration



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# **Development, Characterisation and Application of Calcium Phosphate Nanocrystals aggregates in a Collagen Matrix to be used as a biomaterial in Bone Regeneration**

## **ABSTRACT**

Three-dimensional scaffolds and bone proteins have shown to be important for bone tissue engineering. A scaffold for bone tissue engineering might have several applications and advantages such as: supporting cells for bone regeneration; being part of macromolecule delivery system and act as haemostatic sponge. The goal of this thesis was to develop a biomaterial to be applied for bone regeneration, as a novel means to guide bone cells towards tissue regeneration and/or to allow for local release of therapeutic macromolecules depending on what is the most adequate treatment strategy for each case.

In order to achieve this main goal, several stages were considered in this work. Production of nanometer-sized particles of hydroxyapatite at laboratory scale by chemical precipitation method. This method was selected after a thorough study of other nanotechnology techniques for nanocrystals preparation and characterization. It was shown that CaP nanoparticles are similar to those present in human bone in morphology, dimensions and chemical composition. In collaboration with the Laboratory of Separations and Reaction Engineering - LSRE at Faculdade de Engenharia, Universidade do Porto, a patent on the production of nanohydroxyapatite in a novel mixed reactor was obtained.

Once the nanohydroxyapatite production method in the laboratory was made reproducible, microspheres based on nanohydroxyapatite particles were produced as a potential vehicle for transport and release of enzymes or antibiotics. Thus, *in vitro* studies were carried out to evaluate: nanosized hydroxyapatite performance in microspheres construction, nanohydroxyapatite microspheres as delivery system for antibiotics in periodontitis treatment, and collagen/nanoHA/SPARC (Secreted Protein Acidic and Rich in Cysteine) scaffold biocompatibility as a potential alternative biomaterial for bone cells. Results indicated that SPARC played an important role in the preparation of this new type of scaffold for tissue engineering due to its calcium and collagen binding sites. Its *in vitro* performance suggests a remarkable influence of SPARC on cellular adhesion and proliferation during the process of osteogenesis.

A collagen type I / III matrix supporting hydroxyapatite nanocrystals was synthesised, being a viable porous scaffold for cell proliferation. Additions of SPARC, (Secreted Protein Acidic and Rich in Cysteine) to this biomaterial is a novel approach in Bone Tissue Engineering. SPARC was used because it provides chemical binding between calcium phosphates and collagen fibers in the matrix, which make this biomaterial unique because the protein is not merely placed in the network but chemically attached, due to the SPARC 's calcium affinity domains.

Finally, *in vivo* experiments in rats were conducted to evaluate osteoinductive and osteoconductive properties of the nanoHA/Col/SPARC scaffolds. It was concluded that this biomaterial presented a similar nanostructure to bone in which hydroxyapatite nanocrystals 80x20 nm in size aggregating into microsized clusters, were well dispersed and bound to a collagen matrix through the presence of SPARC. The composite was incorporated into the remodeling

process of bone, resorbed by osteoclastic cells, and new bone was formed by osteoblasts after resorption, as if the composite were grafted into autologous bone. The highly-performing bioactivity of this composite might derive from its similarity in composition and nanostructure to bone. The nanoHA/Col/SPARC biomaterial showed to have the potential to be used as a highly demanding bioactive bone graft material.

# **Desenvolvimento, Caracterização e Aplicação de agregados de nanocristais de fosfatos de cálcio numa matriz de colagénio para ser usada como um biomaterial para Regeneração Óssea**

## **RESUMO**

As matrizes tridimensionais (scaffolds) e as proteínas do osso demonstram ter um papel relevante em engenharia do tecido ósseo. Uma matriz (scaffold) para engenharia de tecido ósseo pode apresentar várias aplicações e vantagens, tais como: Ser capaz de suportar células para a regeneração óssea; fazer parte de sistemas de libertação controlada de macromoléculas ou actuar como esponja hemostática. O objectivo principal desta tese foi o de desenvolver um biomaterial capaz de ser utilizado para a regeneração do tecido ósseo, como um novo meio de guiar células de tecido ósseo para a regeneração e/ou permitir a libertação local e controlada de macromoléculas terapêuticas, dependendo de qual é a mais adequada estratégia de tratamento em cada caso.

Para se atingir este objectivo, várias fases de trabalho foram considerada, incluindo a obtenção à escala laboratorial, por precipitação química, de partículas de dimensão nanométrica de hidroxiapatite. Este método foi seleccionado após um aturado estudo sobre os diversos métodos alternativos para obter este tipo de nanocristais, e da sua caracterização. Verificou-se ser possível obter nanopartículas de fosfatos de cálcio, em particular de hidroxiapatite, semelhantes às presentes no corpo humano, que quanto à sua morfologia, quer em termos de dimensões e composição química. Em colaboração com o Laboratory of Separations and Reaction Engineering - LSRE, da Faculdade de Engenharia da Universidade do Porto, foi obtida uma patente internacional para a produção de nanohidroxiapatite num novo reactor de mistura optimizada.

Quando o processo de obtenção de nanopartículas de hidroxiapatite foi tornado reprodutível, foram desenvolvidas microesferas baseadas em nanohidroxiapatite para funcionarem como potencial veículo para a libertação e transporte de enzimas e antibióticos. Foram realizados ensaios in vitro para avaliar a performance dos agregados de nanopartículas de hidroxiapatite nas microesferas, bem como a sua aplicabilidade a sistemas de libertação controlada de antibióticos para o tratamento da periodontite, e ainda a biocompatibilidade de scaffolds baseados em colagénio/ nanoHA/ SPARC, como potencial biomaterial alternativo para células de tecido ósseo. Os resultados mostraram que a SPARC desempenhou um papel importante na preparação deste novo tipo de scaffold para engenharia de tecidos dada a existência de pontos de ligação de cálcio ao colagénio e a sua performance in vitro sugeriu haver uma influência notável da SPARC na adesão celular e na proliferação durante o processo de osteogénese.

Foi sintetizada uma matriz de colagénio I/III para suportar nanocristais de hidroxiapatite, a qual se mostrou como alternativa viável de scaffold poroso para a proliferação celular. A adição de SPARC (Secreted Protein Acidic and Rich in Cysteine) a esta matriz é uma inovação em termos de aproximação à engenharia de tecido do osso. A SPARC foi utilizada por fornecer ligação química entre os fosfatos de cálcio e as fibras de colagénio, o que torna este biomaterial como

singular, dado que a proteína não está meramente presente na rede, mas antes está quimicamente ligada devido aos seus domínios de afinidade para o cálcio.

Por fim foram realizadas experiências *in vivo*, em ratos, para avaliar o potencial de osteocondução e osteoindução dos scaffolds de nanoHA/Col/SPARC. Concluiu-se que este biomaterial apresentava uma nanoestrutura semelhante à do osso, na qual os nanocristais de hidroxiapatite com 80x20 nm se reúnem em agregados de dimensões micrométricas, bem dispersos e ligados a uma matriz de fibras de colagénio, através da presença da SPARC. O compósito foi incorporado no processo de remodelação óssea, re-absorvido pelos osteoclastos e formou-se osso novo através da acção dos osteoblastos após a reabsorção, como se o compósito estivesse integrado em osso autólogo. A elevada performance em termos de bioactividade deste compósito poderá também ter origem na sua semelhança com o osso quer em termos de composição quer de nanoestrutura. O biomaterial compósito nanoHA/Col/SPARC demonstrou ter potencial para ser utilizado como um material adequado ao preenchimento de defeitos ósseos.

# **Développement, caractérisation et application d'agrégats de nanocristaux de phosphate de calcium inclus dans une matrice de collagène pour être utilisés comme biomatériau pour la régénération osseuse**

## **RÉSUMÉ**

Les matrices tridimensionnelles (scaffolds) et les protéines de l'os ont un rôle important dans l'ingénierie du tissu osseux. La matrice (scaffold) peut présenter plusieurs applications potentielles et avantages tels que la capacité de structure-support aux cellules afin de promouvoir une régénération osseuse, faire partie d'un système permettant une libération contrôlée des macromolécules ou présenter un comportement similaire à celle d'une éponge hémostatique.

Le principal objectif de ce travail de thèse a été de développer un biomatériau capable de guider les cellules osseuses vers une stimulation de la régénération de l'os et/ou permettre la libération contrôlée et localisée de macromolécules thérapeutiques.

Pour atteindre ce but, plusieurs étapes ont été considérées. Tout d'abord, des nanoparticules d'hydroxyapatite ont été obtenues et caractérisées au sein du laboratoire par un procédé de précipitation chimique après une étude de plusieurs méthodes alternatives pour obtenir ce type de nanocristaux. Il a été vérifié que ces nanoparticules de phosphates de calcium, en particulier d'hydroxyapatite, ressemblaient à celles présentes dans le corps humain, en termes de morphologie, de dimensions et de composition chimique. Grâce à une collaboration avec le Laboratory of Separations and Reaction Engineering - LSRE, de la Faculté d'Ingénierie de l'Université de Porto, un brevet international a été obtenu décrivant la production de nanoparticules d'hydroxyapatite au sein d'un nouveau réacteur de mélange optimisé.

Puis, des microsphères ont été développées à partir de ces nanoparticules d'hydroxyapatite, obtenues de manière reproductible, afin d'être utilisées comme véhicule pour la libération contrôlée d'enzymes ou d'antibiotiques. Des essais *in vitro* ont été réalisés afin d'évaluer la performance des agrégats de nanoparticules d'hydroxyapatite dans la synthèse des microsphères, et d'étudier leur application comme système de libération contrôlée d'antibiotiques pour le traitement de la périodontite. La biocompatibilité de scaffolds composés de collagène/nanoHA/SPARC (Secreted Protein Acidic and Rich in Cysteine) a été déterminée après contact avec des cellules de l'os. Les résultats ont démontré que la SPARC a un rôle très important dans la préparation de ce type de scaffold dû de l'existence de points de liaison entre calcium et collagène, et leur performance *in vitro* a paru indiquer qu'il y avait une influence remarquable de la SPARC sur l'adhérence et la prolifération cellulaires lors du procédé d'ostéogénèse.

Permettant la prolifération cellulaire, une matrice poreuse composée de collagène de type I/III ainsi que des nanocristaux d'hydroxyapatite a été synthétisée. Le fait d'associer SPARC à ce biomatériau est une nouvelle approche en ingénierie du tissu osseux. La protéine SPARC n'est pas seulement séquestrée dans le réseau du biomatériau mais est chimiquement attachée grâce à son affinité particulière avec le calcium permettant de créer des liaisons chimiques entre le

phosphate de calcium et les fibres de collagène de la matrice.

Enfin, des expériences *in vivo* sur rats ont été réalisées afin d'évaluer les propriétés d'ostéoinductivité et d'ostéoconductivité des scaffolds composés de collagène/nanoHA/SPARC. Il a été montré que ces biomatériaux présentaient une nanostructure similaire à l'os dans lequel les nanocristaux d'hydroxyapatite, de taille de 80x20 nm, sont agrégés en microclusters, bien dispersés et liés à la matrice de collagène grâce à la présence de SPARC.

Après implantation, ce composite a été intégré comme s'il s'agissait d'un os autologue et soumis au processus de remodelage osseux, résorption par les cellules ostéoclastiques puis formation d'os par les ostéoblastes. Cette performance bioactive est probablement due à la forte similarité en termes de composition et nanostructure de ce composite avec l'os. Ainsi, il a été démontré que le biomatériau composé de collagène/nanoHA/SPARC peut-être utilisé comme matériau bioactif permettant la reconstruction osseuse.

A  
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## TABLE OF CONTENT

|                                                                                                              |           |
|--------------------------------------------------------------------------------------------------------------|-----------|
| <b>INTRODUCTION .....</b>                                                                                    | <b>23</b> |
| <b>CHAPTER I – BONE AND BIOMATERIALS BASED ON COLLAGEN AND<br/>HYDROXYAPATITE FOR BONE ENGINEERING .....</b> | <b>24</b> |
| <b>1. BONE.....</b>                                                                                          | <b>25</b> |
| 1.1 CONCEPT OF BONE.....                                                                                     | 25        |
| 1.2 BONE FUNCTIONS .....                                                                                     | 26        |
| 1.2.1 Mechanical support .....                                                                               | 26        |
| 1.2.2 Protection of vital structures.....                                                                    | 26        |
| 1.2.3 Hematopoiesis.....                                                                                     | 26        |
| 1.2.4 Mineral homeostasis .....                                                                              | 28        |
| 1.2.5 Locomotion and others .....                                                                            | 28        |
| 1.3 BONE COMPOSITION.....                                                                                    | 28        |
| 1.3.1 Compact bone .....                                                                                     | 28        |
| 1.3.2 Trabecular bone.....                                                                                   | 29        |
| 1.3.3 Periosteum .....                                                                                       | 29        |
| 1.3.4 Endosteum.....                                                                                         | 29        |
| 1.4 BONE CELLS.....                                                                                          | 29        |
| 1.4.1 Osteogenesis .....                                                                                     | 29        |
| 1.4.2 Osteoblasts.....                                                                                       | 30        |
| 1.4.3 Osteoclasts .....                                                                                      | 30        |
| 1.4.4 Bone-lining cells .....                                                                                | 30        |
| 1.4.5 Osteocytes .....                                                                                       | 31        |
| 1.4.6 What are Stem Cells?.....                                                                              | 31        |
| 1.4.7 Mesenchymal Stem Cells.....                                                                            | 32        |
| 1.5 ANGIOGENESIS .....                                                                                       | 33        |
| 1.5.1 Growth Factors.....                                                                                    | 34        |
| 1.5.2 Hormones.....                                                                                          | 37        |
| 1.5.2.1 Parathyroid hormone or PTH.....                                                                      | 37        |
| 1.5.2.2 Calcitrol and Vitamin D.....                                                                         | 37        |
| 1.5.2.3 Calcitonin.....                                                                                      | 38        |
| 1.5.2.4 Sex hormones .....                                                                                   | 38        |
| 1.5.2.5 Other important hormones.....                                                                        | 39        |
| 1.5.3 Chemotaxis.....                                                                                        | 40        |
| 1.6 MOLECULAR STRUCTURE OF BONE .....                                                                        | 40        |
| 1.6.1 Organic matrix.....                                                                                    | 40        |

|                                                            |           |
|------------------------------------------------------------|-----------|
| 1.6.1.1 Glycoproteins.....                                 | 41        |
| 1.6.1.2 Proteoglycans .....                                | 42        |
| 1.6.2 Mineral phase and water .....                        | 42        |
| 1.7 BONE FORMATION .....                                   | 43        |
| 1.7.1 Intramembranous ossification.....                    | 43        |
| 1.7.2 Endochondral Ossification .....                      | 43        |
| 1.8 FRACTURE HEALING .....                                 | 44        |
| 1.8.1 Reactive Healing.....                                | 45        |
| 1.8.2 Reparative Healing .....                             | 45        |
| 1.8.3 Remodeling .....                                     | 46        |
| 1.9 BONE DISEASES .....                                    | 46        |
| 1.9.1 Osteoporosis .....                                   | 46        |
| 1.9.1.1 The problem .....                                  | 47        |
| 1.9.1.2 Symptoms and diagnosis .....                       | 47        |
| 1.9.1.3 Clinical Management.....                           | 48        |
| 1.9.2 Other bone diseases .....                            | 49        |
| 1.9.2.1 Rickets and osteomalacia.....                      | 49        |
| 1.9.2.2 Paget's disease of bone (Osteitis deformans) ..... | 50        |
| 1.9.2.3 Osteogenesis imperfecta .....                      | 50        |
| 1.9.2.4 Bone Cancer.....                                   | 50        |
| 1.9.2.5 Oral health and bone disease.....                  | 51        |
| 1.9.2.6 Osteonecrosis.....                                 | 51        |
| <b>2. SCAFFOLDING FOR BONE ENGINEERING.....</b>            | <b>53</b> |
| 2.1 BIOCERAMICS .....                                      | 54        |
| 2.1.1 Calcium phosphates (CaP) .....                       | 54        |
| 2.1.1.1 Dicalcium phosphate dehydrate(DCPD) .....          | 55        |
| 2.1.1.2 Dicalcium phosphate anhydrous (DCPA).....          | 56        |
| 2.1.1.3 Tricalcium phosphate (TCP).....                    | 56        |
| 2.1.1.4 Hydroxyapatite.....                                | 56        |
| 2.1.1.5 Hydroxyapatite in nanometric scale .....           | 57        |
| 2.1.1.6 Nanohydroxyapatite Synthesis Review.....           | 58        |
| 2.2 COLLAGEN .....                                         | 61        |
| 2.2.1 Structure and function.....                          | 63        |
| 2.2.2 Collagen Porous scaffolds preparation .....          | 65        |
| 2.2.3 Collagen scaffolds Cross-linking .....               | 65        |
| 2.3 NANOCOMPOSITES .....                                   | 66        |
| 2.3.1 Synthesis of collagen porous scaffolds.....          | 66        |

|                                                                                                                                                                        |            |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| 2.3.2 Nanohydroxyapatite association to Collagen .....                                                                                                                 | 68         |
| 2.3.3 Bone Tissue Engineering using Col/HA composite scaffolds.....                                                                                                    | 69         |
| REFERENCES .....                                                                                                                                                       | 70         |
| <b>CHAPTER II – NANOPARTICLES OF HYDROXYAPATITE: PREPARATION, CHARACTERISATION, AND CELLULAR APPROACH – OVERVIEW .....</b>                                             | <b>85</b>  |
| 1. INTRODUCTION .....                                                                                                                                                  | 85         |
| 2. HYDROXYAPATITE PARTICLES .....                                                                                                                                      | 86         |
| 3. NANOHA APPLICATIONS.....                                                                                                                                            | 87         |
| 4. NANOHA PREPARATION METHODS .....                                                                                                                                    | 87         |
| 4.1 MECHANOCHEMICAL PROCESSING.....                                                                                                                                    | 87         |
| 4.2 SOL-GEL ROUTE.....                                                                                                                                                 | 88         |
| 4.3 BIOMIMETIC PROCESS .....                                                                                                                                           | 88         |
| 4.4 HYDROTHERMAL METHOD .....                                                                                                                                          | 89         |
| 4.5 EMULSION TECHNIQUE .....                                                                                                                                           | 89         |
| 4.6 CHEMICAL PRECIPITATION.....                                                                                                                                        | 90         |
| 5. MATERIALS AND METHODS .....                                                                                                                                         | 91         |
| 5.1 NANOHA SYNTHESIS .....                                                                                                                                             | 91         |
| 5.2 NANOHA CHARACTERISATION .....                                                                                                                                      | 91         |
| 5.2.1 Physical Characterisation .....                                                                                                                                  | 91         |
| 5.2.2 Chemical Characterisation.....                                                                                                                                   | 92         |
| 5.2.3 In vitro performance.....                                                                                                                                        | 92         |
| 6. RESULTS .....                                                                                                                                                       | 93         |
| 7. DISCUSSION .....                                                                                                                                                    | 95         |
| 8. CONCLUSIONS.....                                                                                                                                                    | 96         |
| REFERENCES.....                                                                                                                                                        | 97         |
| ANEX I.....                                                                                                                                                            | 102        |
| <b>CHAPTER III - MICROSPHERES BASED ON HYDROXYAPATITE NANOPARTICLES AGGREGATES FOR BONE REGENERATION.....</b>                                                          | <b>114</b> |
| 1. INTRODUCTION .....                                                                                                                                                  | 114        |
| 2. MATERIALS AND METHODS .....                                                                                                                                         | 115        |
| 3. RESULTS .....                                                                                                                                                       | 116        |
| 4. DISCUSSION .....                                                                                                                                                    | 118        |
| 5. CONCLUSIONS.....                                                                                                                                                    | 118        |
| REFERENCES.....                                                                                                                                                        | 118        |
| <b>CHAPTER IV– NANOHYDROXYAPATITE MICROSPHERES AS DELIVERY SYSTEM FOR ANTIBIOTICS: RELEASE KINETICS, ANTIMICROBIAL ACTIVITY, AND INTERACTION WITH OSTEOBLASTS.....</b> | <b>120</b> |
| 1. INTRODUCTION .....                                                                                                                                                  | 121        |

|       |                                                                                                      |     |
|-------|------------------------------------------------------------------------------------------------------|-----|
| 2.    | MATERIALS AND METHODS .....                                                                          | 122 |
| 2.1   | MATERIALS PREPARATION AND CHARACTERIZATION .....                                                     | 122 |
| 2.1.1 | <i>Preparation of HA granules.....</i>                                                               | 122 |
| 2.1.2 | <i>Synthesis of nanosized HA .....</i>                                                               | 122 |
| 2.1.3 | <i>Alginate/ hydroxyapatite microspheres preparation.....</i>                                        | 122 |
| 2.1.4 | <i>Materials characterization.....</i>                                                               | 123 |
| 2.2   | DRUG LOADING.....                                                                                    | 123 |
| 2.3   | RELEASE KINETICS AND BIOACTIVITY .....                                                               | 123 |
| 2.4   | CYTOTOXICITY .....                                                                                   | 124 |
| 2.4.1 | <i>Growth of human osteoblasts - Qualitative cytotoxicity .....</i>                                  | 124 |
| 2.4.2 | <i>Direct growth of MG63 cells on the surface of materials loaded with antibiotics.....</i>          | 125 |
| 3.    | RESULTS .....                                                                                        | 125 |
| 3.1   | MATERIALS CHARACTERIZATION.....                                                                      | 125 |
| 3.2   | RELEASING PROFILE AND BIOLOGICAL ACTIVITY .....                                                      | 125 |
| 3.3   | CYTOTOXICITY .....                                                                                   | 132 |
| 3.3.1 | <i>Growth of human osteoblasts - Qualitative cytotoxicity .....</i>                                  | 132 |
| 3.3.2 | <i>Direct growth and adhesion of MG63 cells on the surface of materials loaded with antibiotics.</i> | 132 |
| 4.    | DISCUSSION .....                                                                                     | 133 |
| 5.    | CONCLUSION.....                                                                                      | 135 |
|       | REFERENCES.....                                                                                      | 136 |

## **CHAPTER V - COMPARATIVE STUDY OF NANOHYDROXYAPATITE MICROSPHERES FOR MEDICAL APPLICATIONS.....138**

|       |                                                         |     |
|-------|---------------------------------------------------------|-----|
| 1.    | INTRODUCTION .....                                      | 139 |
| 2.    | MATERIALS AND METHODS .....                             | 140 |
| 2.1   | SYNTHESIS OF NANOSIZED HYDROXYAPATITE (HA).....         | 140 |
| 2.2   | PREPARATION OF HA MICROSPHERES .....                    | 140 |
| 2.3   | CHARACTERISATION OF NANOSIZED HA .....                  | 140 |
| 2.3.1 | <i>Infrared spectroscopy .....</i>                      | 140 |
| 2.3.2 | <i>Transmission electron microscopy – TEM.....</i>      | 140 |
| 2.4   | CHARACTERISATION OF NANOHA MICROSPHERES.....            | 141 |
| 2.4.1 | <i>Inverted stand optical microscope analysis .....</i> | 141 |
| 2.4.2 | <i>Infrared spectroscopy .....</i>                      | 141 |
| 2.4.3 | <i>Scanning electron microscopy analysis – SEM.....</i> | 141 |
| 2.4.4 | <i>Mercury porosimetry method.....</i>                  | 141 |
| 2.4.5 | <i>X-ray diffractometry – XRD.....</i>                  | 141 |
| 2.5   | BIOLOGICAL ASSESSMENT .....                             | 142 |

|                                                       |     |
|-------------------------------------------------------|-----|
| 2.5.1 Cytotoxicity tests ( <i>Neutral Red</i> ) ..... | 142 |
| 2.5.2 Cell morphology ( <i>SEM and CLSM</i> ) .....   | 142 |
| 3. RESULTS .....                                      | 143 |
| 4. DISCUSSION .....                                   | 147 |
| 5. CONCLUSIONS .....                                  | 151 |
| REFERENCES .....                                      | 151 |

## **CHAPTER VI - SPARC (SECRETED PROTEIN ACIDIC AND RICH IN CYSTEINE) IN COLLAGEN/CALCIUM PHOSPHATES SCAFFOLDS FOR BONE ENGINEERING .....155**

|                                                                                                                |     |
|----------------------------------------------------------------------------------------------------------------|-----|
| 1. INTRODUCTION .....                                                                                          | 155 |
| 2. MATERIALS AND METHODS .....                                                                                 | 156 |
| 2.1 SYNTHESIS OF HA .....                                                                                      | 156 |
| 2.2 PREPARATION OF COLLAGEN SCAFFOLDS .....                                                                    | 157 |
| 2.3 PHYSICOCHEMICAL CHARACTERIZATION .....                                                                     | 158 |
| 2.3.1 Analysis of pore structure .....                                                                         | 158 |
| 2.3.2 Trace Element Analysis .....                                                                             | 158 |
| 2.3.3 Mechanical compression testing .....                                                                     | 159 |
| 2.4 IN VITRO CELL CULTURE STUDIES .....                                                                        | 159 |
| 2.4.1 Seeding of GBMSCs on scaffolds .....                                                                     | 159 |
| 2.4.2 Diameter contraction .....                                                                               | 160 |
| 2.4.3 Cell viability .....                                                                                     | 160 |
| 2.4.4 Cell proliferation evaluation .....                                                                      | 160 |
| 2.4.5 Histology studies .....                                                                                  | 160 |
| 2.4.6 Statistical Analysis .....                                                                               | 161 |
| 3. RESULTS AND DISCUSSION .....                                                                                | 161 |
| 3.1 ANALYSIS OF PORE STRUCTURE .....                                                                           | 161 |
| 3.2 TRACE ELEMENT ANALYSIS .....                                                                               | 161 |
| 3.3 MECHANICAL COMPRESSION TESTING .....                                                                       | 163 |
| 3.4 DIAMETER CONTRACTION .....                                                                                 | 164 |
| 3.5 CYTOTOXICITY ASSESSMENT .....                                                                              | 164 |
| 3.6 CELLULAR PROLIFERATION .....                                                                               | 167 |
| 3.7 HISTOLOGY STUDIES .....                                                                                    | 167 |
| 4. CONCLUSIONS .....                                                                                           | 168 |
| REFERENCES .....                                                                                               | 170 |
| ANEX II .....                                                                                                  | 172 |
| HISTOLOGICAL STUDY OF GOAT BONE MARROW STEM CELLS CULTURE (GBMSCs) AND SCAFFOLDS BASED ON COLLAGEN I/III ..... | 172 |
| 1. INTRODUCTION .....                                                                                          | 172 |

|                                                                                                                                                |                                                                                                |            |
|------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------|------------|
| 2.                                                                                                                                             | MATERIALS AND METHODS .....                                                                    | 172        |
| 2.1                                                                                                                                            | ISOLATION AND CELL CULTURING OF GBMSCs .....                                                   | 172        |
| 2.2                                                                                                                                            | COLLAGEN SCAFFOLDS PREPARATION .....                                                           | 173        |
| 2.3                                                                                                                                            | SEEDING GBMSCs ON SCAFFOLDS AND CELL CULTURE .....                                             | 173        |
| 2.4                                                                                                                                            | HISTOLOGY AND HISTOCHEMICAL STUDIES .....                                                      | 173        |
| 3.                                                                                                                                             | RESULTS .....                                                                                  | 174        |
| 4.                                                                                                                                             | DISCUSSION .....                                                                               | 175        |
| 5.                                                                                                                                             | CONCLUSIONS .....                                                                              | 177        |
|                                                                                                                                                | REFERENCES .....                                                                               | 177        |
| <b>CHAPTER VII - BONE REGENERATION USING A SCAFFOLD BASED ON COLLAGEN I/III, SPARC AND HIDROXYAPATITE NANOPARTICLES: AN IN VIVO STUDY.....</b> |                                                                                                | <b>179</b> |
| 1.                                                                                                                                             | INTRODUCTION .....                                                                             | 179        |
| 2.                                                                                                                                             | MATERIALS AND METHODS .....                                                                    | 180        |
| 2.1                                                                                                                                            | NANOPHASED HYDROXYAPATITE AGGREGATES/TYPE (I/III) COLLAGEN COMPOSITE SCAFFOLDS PROCESSING..... | 180        |
| 2.2                                                                                                                                            | ANIMAL EXPERIMENTATION AND OPERATIVE PROCEDURE.....                                            | 181        |
| 2.3                                                                                                                                            | HISTOLOGY.....                                                                                 | 181        |
| 2.3.1                                                                                                                                          | <i>Microscopic studies</i> .....                                                               | 182        |
| 2.3.2                                                                                                                                          | <i>Statistical Analysis</i> .....                                                              | 182        |
| 3.                                                                                                                                             | RESULTS .....                                                                                  | 182        |
| 3.1                                                                                                                                            | HISTOLOGICAL FINDINGS .....                                                                    | 182        |
| 3.2                                                                                                                                            | CONTROL SAMPLES HISTOLOGY .....                                                                | 182        |
| 3.3                                                                                                                                            | SUBCUTANEOUS IMPLANTED SAMPLES - HISTOLOGY .....                                               | 183        |
| 3.4                                                                                                                                            | INTRAOSSEOUS IMPLANTED SAMPLES - HISTOLOGY .....                                               | 187        |
| 4.                                                                                                                                             | DISCUSSION .....                                                                               | 190        |
| 5.                                                                                                                                             | CONCLUSIONS .....                                                                              | 192        |
|                                                                                                                                                | REFERENCES .....                                                                               | 192        |
| <b>GENERAL CONCLUSIONS .....</b>                                                                                                               |                                                                                                | <b>195</b> |
| <b>ERRATA .....</b>                                                                                                                            |                                                                                                | <b>196</b> |

## TABLE OF FIGURES

### CHAPTER I

|                                                                                         |    |
|-----------------------------------------------------------------------------------------|----|
| Figure 1. Bone composition: compact and spongy tissues. ....                            | 25 |
| Figure 2. Hematopoiesis pathway.....                                                    | 27 |
| Figure 3. Distribution by weight of the constituents of whole cortical bone [12]. ....  | 41 |
| Figure 4. Collagen structure.....                                                       | 63 |
| Figure 5. Collagen type I/III scaffolds following the above procedure. (Own work) ..... | 67 |

### CHAPTER II

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |    |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 1. SEM (left) and TEM (right) micrographs. NanoHA particles have been studied using these methods to characterize morphologically these nanocrystals that are presented in agglomerates of few microns. Nanometric particles of HA exhibit in both micrographs bar and needle-like shapes with 80x20nm dimensions in mean. (SEM, scale bar=500nm; TEM, 12000x).....                                                                                                         | 94 |
| Figure 2. XRD spectra of nanoHA en powder. It is indicated that results (blue line) fit the most of peaks (vertical lines) that exhibits natural hydroxyapatite. ....                                                                                                                                                                                                                                                                                                              | 95 |
| Figure 3. SEM (left) and Confocal (right) micrographs of seeded microspheres with MG-63 osteoblast cells after 7 days of culture. In the left, osteoblasts proliferation was observed on nanoHA microspheres means scanning electron spectroscopy using backscattered electron imaging. In the right, a confocal laser scanning microscopy image shows aligned osteoblasts proliferating on microsphere surface where nuclei is stained in red and cytoplasm stained in green..... | 96 |

### CHAPTER III

|                                                                                                                |     |
|----------------------------------------------------------------------------------------------------------------|-----|
| Figure 1. SEM images from HA-1 and HA-2 powders .....                                                          | 116 |
| Figure 2. SEM images of HA-1 (up) and HA-2 (down) microspheres.....                                            | 117 |
| (a, b: x50; c,d: x190 and e,f: x30000).....                                                                    | 117 |
| Figure 3. XRD spectra from HA powders and microspheres .....                                                   | 117 |
| (a) HA-1 powder, (b) HA-1 microspheres, (c) HA-2 powder, (d) HA-2 microspheres, ↓: monoclinic HA, *:β-TCP..... | 117 |

### CHAPTER IV

|                                                                                                                                                                                                                            |     |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 1. SEM micrograph of HA-1 microspheres (a, x50 and b, x30000) .....                                                                                                                                                 | 126 |
| Figure 2. Bacteria inhibition zones around the wells that were initially filled with 70 µL of amoxicillin released from HA-1 microspheres after different elution periods.....                                             | 126 |
| Figure 3. Bacteria inhibition zones diameter (A) and the mean cumulative concentration of release amoxicillin (B) as a function of release time. The variation of the released amoxicillin (µg/h) is inserted in (A) ..... | 128 |

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |     |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 4. Bacteria inhibition zones diameter (A) and the mean cumulative concentration of release amoxicillin + clavulanic acid (B) as a function of release time. The variation of the released amoxicillin + clavulanic acid ( $\mu\text{g/h}$ ) is inserted in (A).....                                                                                                                                                                                        | 129 |
| Figure 5. Bacteria inhibition zones diameter (A) and the mean concentration of released erythromycin (B) as a function of release time. The variation of the released erythromycin ( $\mu\text{g/h}$ ) is inserted in (A).....                                                                                                                                                                                                                                    | 130 |
| Figure 6. Effects of the presence of different concentrations (4, 10, 25 100 and 200 $\mu\text{g/ml}$ ) of amoxicillin (A), amoxicillin + clavulanic acid (B) and erythromycin (C) on the growth of MG63 cells. MG3 cells were grown on TCPS with and without (control) antibiotics supplement, as described in materials and methods. Results are expressed as the average increase in the number of cells relative to the numbe present initially on day 0..... | 131 |
| Figure 7. Effect of amoxicillin (A), amoxicillin + clavulanic acid (B) and erythromycin (C) adsorbed on HA granules, HA-1 and HA-2 microspheres, relative to the level expressed by control cells, grown on the same material without adsorbed antibiotic (defined as 100%) .....                                                                                                                                                                                 | 131 |

## CHAPTER V

|                                                                                                                                                                                                  |     |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 1. FTIR spectra of HA-1 and HA-2 samples (powders and microspheres).....                                                                                                                  | 144 |
| Figure 2. TEM images of HA-1 (a: x20000) and HA-2 (b:x20000).....                                                                                                                                | 145 |
| Figure 3. SEM micrographs of of HA-1(a,c,e) and HA-2 (b,d,f) microspheres: a,b (x50) c,d (x190) and e,f (x30000).....                                                                            | 145 |
| Figure 4. X-ray diffraction of powders and microspheres of HA-1 and HA-2. (a) HA-1 powder, (b) HA-1 microspheres, (c) HA-2 powder, (d) HA-2 microspheres. ↓: monoclinic HA, *: $\beta$ -TCP..... | 146 |
| Figure 5. MG-63 cells culture on microspheres based on hydroxyapatite nanosized ....                                                                                                             | 147 |
| Figure 6. SEM micrographs of 1 to 7 days of MG-63 cells culture on microspheres HA-1 and HA-2 (x180 and x1000). ....                                                                             | 148 |
| Figure 7. SEM micrographs (left x110 and right x1000). Notice that osteoblast cells built bridges between neighbors microspheres. This image corresponds to HA-1 construct after 3 days. ....    | 148 |
| Figure 8. CLSM images of HA-1 (left, x100) and HA-2 microspheres (right, x100) respectively) after 5 days of MG63 culture. ....                                                                  | 149 |

## CHAPTER VI

|                                                                                                                                                                                                                                                           |     |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 1. Set-up nanoparticulate hydroxyapatite synthesis. ....                                                                                                                                                                                           | 157 |
| Figure 2. Multi-step process for Col/SPARC/CaP scaffolds preparation G4* and G7* groups were prepared for ICP analysis. ....                                                                                                                              | 158 |
| Figure 3. Microtomed section stained with Aniline Blue of 6 $\mu\text{m}$ of thickness, fom collagen scaffolds (G1). 4x, Pore diameter 208 $\mu\text{m}$ and apparent density of $2\text{E}-5 \text{ g/mm}^3$ , that corresponds to 89% of porosity ..... | 161 |
| Figure 4. Calcium and phosphorous content into scaffolds (n=3). Controls: G1, G4* and G7*.....                                                                                                                                                            | 163 |

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |     |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 5. Different zones in the characteristic strain-stress curve (a). Linear elastic modulus values ( $\pm$ SD) for all groups of samples ( $n=5$ ) (b). .....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | 165 |
| Figure 6. Diameter contraction in percentage of G1-G7 scaffolds ( $n=3$ ).....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | 166 |
| Figure 7. Cell viability after 1 day and 2 weeks of GBMSCs in osteogenic medium ( $n=6$ , mean $\pm$ SD) .....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | 167 |
| Figure 8. DNA content of GBMSCs cultured on the scaffolds 2 weeks of culture in osteogenic medium ( $n=6$ , mean $\pm$ SD) .....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 168 |
| Figure 9. Light micrograph of central region of goat bone marrow stem cells seeded on (a) G1 and (b) G7 scaffolds at 14 dys. H&E stain; scale bars, 100 $\mu$ m.....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   | 169 |
| Figure A1. Scheme about goat bone marrow stem cells' culturing. After isolation of 6 goats, cells were culture to primary culture (P0) and sub-cultured until osteoblasts differentiation (P2) and finally they were seeded on sterile scaffolds and TCPS, used as controls in a cellular density of $4 \times 10^6$ cells/scaffold.....                                                                                                                                                                                                                                                                                                               | 174 |
| Figure A2. Light micrographs of osteoblast-seeded scaffolds following 3 weeks (a, b) and 5 weeks (c, d) culture period. (a) osteoblasts only growing on scaffold surface, (b) cell concentration on scaffold surface, (c) and (d) higher population of cells conforming a expanded mass on scaffold surface where a oriented growth in layers parallel to surface is observed. In any case, cells could not colonize the inner portions of the scaffold due to the high crosslinking density (5:2:5) chosen for this test. ....                                                                                                                        | 175 |
| Figure A3. Light micrographs of histology and immunohistochemistry of osteoblast-seeded scaffolds following 3 5 weeks. (a) Alizarin Red technique indicated positive result and it is observed calcium phosphate deposits inside the scaffold, (b) ALP by immunohistochemistry where extracellular matrix formation is observed, (c) H&E staining indicating inside and superficial cell proliferation, (d) Negative Safranin-O staining to verify that stem cells did not differentiate into chondrocyte cells and did not produce cartilage tissue. Images were taken from central regions of the constructs, which appeared uniform throughout..... | 176 |
| Figure A4. Light micrographs where different types of bone cells over 5 weeks of cell cultre in ostegenic medium are observed. OB: osteoblasts, OC: osteoclasts, OCy: osteocytes, LC. Bone lining cells.H&E, Scale bar: 100 $\mu$ m. ....                                                                                                                                                                                                                                                                                                                                                                                                              | 177 |

## CHAPTER VII

|                                                                                                                                                                                                                                                                              |     |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 1. Collagen I/III control scaffolds were studied using Masson's Trichromic acid stain differentiating collagen I to III fibers in the network, where fine and thick fibers that stain green and red, representing col I and col III respectively, were observed. .... | 183 |
| Figure 2. Small and well dispersed aggregates of small particles (black arrows) containing calcium (probably nanoHA aggregates) are observed along the collagen matrix. Mineralized and crosslinked control scaffolds (Collagen I/III + nanoHA) stained with Von Kossa.....  | 184 |
| Figure 3. Foreign body giant cells formation (black arrow) announcing the initial stages of foreign body reaction (FBR) of mineralized scaffold (Col I/III+nanoHA) after 7 days of subcutaneous implantation (H&E). ....                                                     | 184 |
| Figure 4. Foreign body giant cells formation (black arrow) announcing the initial stages of foreign body reaction (FBR), after 7 days of implantation subcutaneously. Scale bar: 40mm (H&E). ....                                                                            | 185 |

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |     |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 5. Firm collagen layer surrounding the mineralized scaffold created by neighbouring cells as a protective measure against the foreign body after 7 days of subcutaneous implantation (H&E).....                                                                                                                                                                                                                                                                                                 | 185 |
| Figure 6. Fibroblasts proliferation conforming granulation tissue near new blood vessels, after 7 days of subcutaneous implantation (H&E).....                                                                                                                                                                                                                                                                                                                                                         | 186 |
| Figure 7. Processes of fibrosis and scar formation are fully visible after 15 days of subcutaneous implantation (H&E).....                                                                                                                                                                                                                                                                                                                                                                             | 186 |
| Figure 8. At later healing stages after implantation, the collagen persistence is evident as well as the foreign body reaction (FBR) after 15 days of subcutaneous implantation (TC).<br>.....                                                                                                                                                                                                                                                                                                         | 187 |
| Figure 9. Mineralized sample (col I/III+nanoHA) implanted intraosseously after 45 days. There are three regions of the wounds: a deeper area where osteoclasts and osteoblasts coexist in large quantities, with osteoblasts depositing calcium phosphates on collagen fibers (A1), an intermediate area (A2) where the same process is occurring but delayed, and a top area where the inflammation reaction is severe, suggesting that the regeneration process has not yet occurred (A3)(RET) ..... | 188 |
| Figure 10. Typical new bone formation for uncrosslinked mineralized samples implanted intraosseously for 45 days. the presence of osteoclasts and osteoblasts in well-organized structures, divided by fibrosis trabecula (TC) is observed. ....                                                                                                                                                                                                                                                       | 189 |
| Figure 11. Structured bone between fibers with new blood vessels for uncrosslinked mineralized samples implanted intraosseously for 45 days (H&E).....                                                                                                                                                                                                                                                                                                                                                 | 189 |
| Figure 12. Old bone in the presence of increasing osseous medulla and newly organized bone and newly formed collagen fibers for uncrosslinked mineralized samples implanted intraosseously for 45 days. (H&E).....                                                                                                                                                                                                                                                                                     | 190 |

## TABLE OF TABLES

### CHAPTER I

|                                                                                         |    |
|-----------------------------------------------------------------------------------------|----|
| Table 1. Comparison between structures that compose skeleton [9].....                   | 25 |
| Table 2. Growth factors - Overview .....                                                | 35 |
| Table 2. Growth factors - Overview .....                                                | 36 |
| Table 4. Apatitic and Non-apatitic calcium phosphates. [200,201].....                   | 55 |
| Table 5. Comparison of mechanical features of human bone and bioceramics [228]. ....    | 57 |
| Table 6. Nanohydroxyapatite processing methods - Review.....                            | 59 |
| Table 7. Various collagen types and their distribution in the body. Adapted from [283]. | 62 |
| Table 8. Tensile strength performance of different tissues - Comparison .....           | 65 |
| Table 9. Tensile elastic modulus of various porous scaffolds. ....                      | 68 |

### CHAPTER II

|                                                                                            |    |
|--------------------------------------------------------------------------------------------|----|
| Table 1. Ion concentrations of SBF solution and “human plasma” .....                       | 89 |
| Table 2. Binding energies (eV) of core electrons and surface atomic ratios of apatite .... | 94 |

### CHAPTER V

|                                    |     |
|------------------------------------|-----|
| Table 1. Mercury porosimetry ..... | 146 |
|------------------------------------|-----|

### CHAPTER VI

|                                                                                                                            |     |
|----------------------------------------------------------------------------------------------------------------------------|-----|
| Table 1. Groups of scaffold used in the presen study. G4* and G7* groups were fabricated as controls for ICP analysis..... | 159 |
|----------------------------------------------------------------------------------------------------------------------------|-----|

### CHAPTER VII

|                                      |     |
|--------------------------------------|-----|
| Table 1. Design of experiments. .... | 182 |
|--------------------------------------|-----|

## INTRODUCTION

Transplantation provides hope for many patients with tissue loss or organ failure, but inherent limitations such as donor shortage and the required immunosuppression therapy seriously hinder the potential benefits that such an approach can provide for many patients. The problems of current transplantation therapy have stimulated research for alternative solutions. An emerging interdisciplinary and multidisciplinary field aims to recreate biologically functional tissues and organs. This field is either called tissue engineering or regenerative medicine, depending on what aspects are more deeply focused. Man-made materials, not necessarily originally designed for body part replacements, are being used as implants and prostheses. These foreign materials' lack of biological function imposes serious health risk in patients.}

Scaffolds play a critical role in tissue engineering by acting as temporary artificial extracellular matrices for cell accommodation, proliferation, and differentiated function as well as serving as three dimensional templates for some tissue/organ formation. As the knowledge in scaffolding design increases with the development of more tissue engineering research programs, there is increased need for a comprehensive studies focusing on scaffolding for tissue engineering.

The present work, divided in six chapters, is intended to explore the design, synthesis and applications of a specific bone tissue engineering scaffold based on collagen and hydroxyapatite. First chapter is dedicated to bone and bone scaffolding mainly related to collagen and calcium phosphates where hydroxyapatite is outstanding; chapter two shows the design and synthesis of hydroxyapatite nanoparticles and microspheres, while chapters three and four are good examples of how nanoHA microspheres as a biomaterial could be explored as scaffolds for osteoblasts and potential drug delivery systems for macromolecules such as antibiotics for the treatment of periodontitis; finally, the potential of a composite scaffold based on type I/III collagen and nanoHA was evaluated in a rat model, as reported in chapter VII.

# **CHAPTER I – BONE AND BIOMATERIALS BASED ON COLLAGEN AND HYDROXYAPATITE FOR BONE ENGINEERING**

In the last decades, the interest on new therapeutic strategies emerging from tissue engineering and gene therapy has increased in a vertiginous way, and likewise bone engineering has been performed daily with the hundreds of thousands of dental and orthopedic implants around the world, being bone the most implanted tissue after blood [1,2]. Millions of bone tissue replacements are required worldwide and for that reason many companies have had to develop new orthopedic materials. Therefore, there has been an evident research and development effort in the design of biomaterials used in substitution and bone repair. However, the current developments in the understanding of bone wound healing and the interface with artificial materials are still limited compared to what is required to engineer new materials that might provide scaffolds to grow new bone tissue in regenerative therapies [3-8].

Understanding the mechanisms of bone formation as well as the manner by which this extraordinary tissue repairs or regenerates is a good motivation for developing new and improved biomaterials aiming at mimicking nature.

Even though the mechanisms that regulate the formation and maintenance of the skeleton are not yet well understood, it must be clarified throughout this chapter that cell-signaling molecules and receptors play critical roles in these processes; due to their number, diversity and complexity; such as therapeutic regulation of bone growth and homeostasis. Bone is not just calcium and phosphate, its proteins and macromolecules are artificers with specific call in time and space!

A better understanding into the biomaterial development thought for bone regeneration consists in obtaining a conscious study of bone biology to identify “novel” solutions and possibly apply some to solve problems using synthetic materials. This subject will be discussed next.

# 1. BONE

## 1.1 CONCEPT OF BONE

Bone is a very hard and rigid organ of vertebrates due to a complex mineral substance, largely composed of calcium, phosphates and carbonates distributed throughout a soft organic matrix (Figure 1). The most remarkable fact about living bone is that it is living, that means blood circulation transports materials to and from bone (by arteries and veins, respectively), becoming it able to grow, change, remove, or suffer resorption, according to the stresses to which bone is being submitted. Besides, as a living tissue, bone is a highly specialized form of connective tissue characterized by large amounts of extra cellular matrix material (ECM), intracellular proteins and proteoglycans that hold tissue together, in the spaces between connective tissue cells. Although the skeleton is mostly constituted by bone tissue, tendons, ligaments and cartilage are also present, and all of them keep their own characteristics giving shape to the body (Table 1).

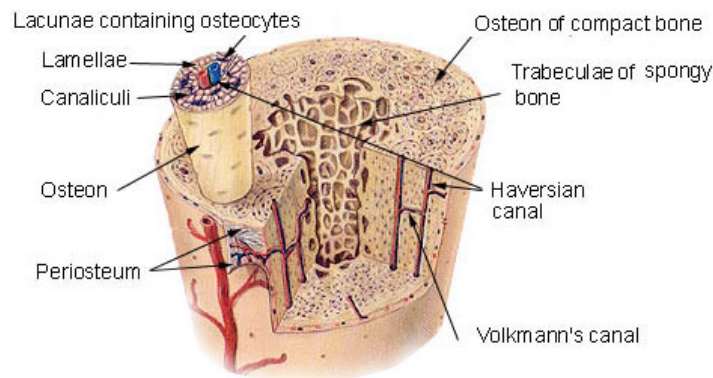


Figure 1. Bone composition: compact and spongy tissues.

Source ([www.wikipedia.org](http://www.wikipedia.org))

Table 1. Comparison between structures that compose skeleton [9]

| Structure       | Water<br>Composition | Proteoglycans<br>Composition | Collagen<br>Composition | Mineral<br>Composition |
|-----------------|----------------------|------------------------------|-------------------------|------------------------|
| Tendon/Ligament | Moderate             | Low                          | High                    | Low                    |
| Cartilage       | High                 | High                         | Low -<br>Moderate       | Low                    |
| Bone            | Low                  | Low                          | Moderate                | High                   |

In a comparison between the structures that conform the skeleton, it is interesting to note that high amounts of collagen make the tendon a tissue with good tensile strength; however, cartilage, is composed by proteoglycans that hold water and due to their presence, cartilage is stiff under compression. On the other hand, bone is more mineralized than tendon and cartilage and that induces bone to have higher compressive strength, while collagen helps bone to be tough.

Sometimes, bone is called “calcified cartilage”. However, in arthroidal joints cartilage has a unique superior quality of lubrication and shock absorption, and it has a very low dry friction coefficient ( $\approx 0.0026$ ), probably the lowest value of any known solid material [10].

## **1.2 BONE FUNCTIONS**

Provision of support, protection, locomotion, red blood cells production, and mineral homeostasis are some of the tasks performed by bone tissue.

### *1.2.1 Mechanical support*

Mechanical functions of bone are by far the most widely studied issue related to bone tissue. The mechanical nature of bone extends beyond basic description of strength and stiffness; it includes the developed mechanisms to avoid fatigue fracture, meaning fractures due to cyclic loading at physiologic load levels. It is interesting to highlight the fact that bone microstructure reflects the variety and diversity of mechanical requirements of each particular organism, for instance, a bovine femur can withstand considerable load and present rapid growth during lifetime, but in contrast, it is not prepared to decrease the potential for fatigue failure. On the other hand, canine femur are developed to prevent skeletal fatigue in bony structure, while not being adapted to massive weight or rapid growth [11].

### *1.2.2 Protection of vital structures*

At the gross anatomical level, two cortical plates compose bone with a trabecular zone in between, so-called spongy or trabecular and cancellous structures. This complex array allows for maximum absorption of energy and minimum trauma to the bone itself [11,12]. Cranial bone and ribs are examples of this kind of bone that protect vital organs such as the brain in the case of skull, or the heart and lungs for ribs.

### *1.2.3 Hematopoiesis*

Hematopoiesis is the process by which mammalian stem cells differentiate into several kinds of blood cell within the bone marrow [13]. In adults, hematopoiesis occurs in the spongy tissue at bone with large surface areas for the production of red blood cells, such as the iliac crest, vertebrae and proximal femur. These bones also have the responsibility for rapid bone turnover and play an important role in long-term control of calcium balance [14].

All lymphocytes are originated during this process from a common lymphoid progenitor before differentiating into their distinct lymphocyte types, which are Natural Killer cells (NK cells); and B and T cells [15].

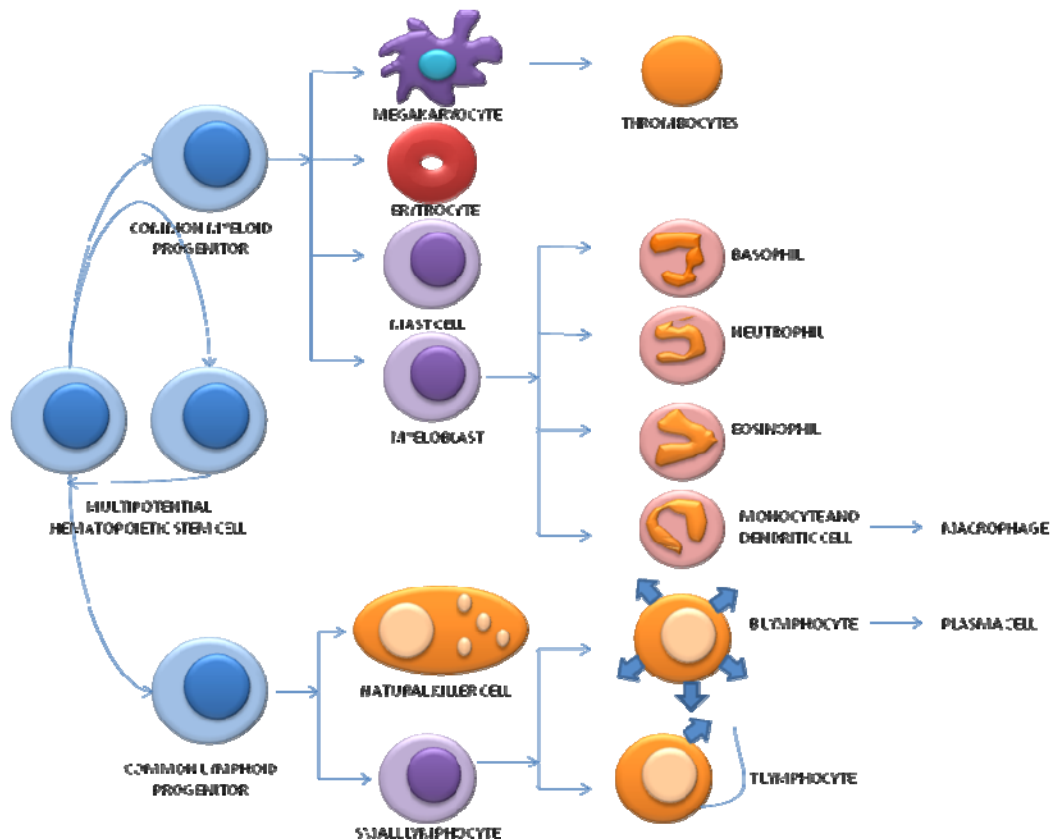


Figure 2. Hematopoiesis pathway

Hemocytoblast or multipotential hematopoietic stem cells convert into the common lymphoid progenitors, and these turn into lymphoblast from which prolymphocytes and lymphoid are derived. Prolymphocytes give rise to small lymphocytes, such as B and T lymphocytes, and natural killer (NK) lymphocytes [16]. B cells mature into B lymphocytes in the bone marrow, whereas T cells migrate and mature in the thymus. After maturation these lymphocytes enter the circulation and peripheral lymphoid organs (spleen, lymph nodes) where they survey for invading pathogens and/or tumor cells [13].

NK cells are part of the innate immune system, whereas T and B cells are part of the adaptive immune response. While NK cells defend the host from both tumors and cells infected by viruses; T and B cells recognize “non-self antigens” and respond to pathogens [16]. Even, their pathway of attack is different: NK cells release small cytoplasmic granules of proteins called *perforin* and *granzyme* that cause the target cell to die by apoptosis or necrosis; B cells respond to pathogens by producing large quantities of antibodies which then neutralize foreign objects like bacteria and viruses; and T cells, such as *helper T cells*, produce cytokines that control the

immune response, others such as *cytotoxic T cells*, produce toxic granules that induce the death of pathogen infected cells [13,15,16].

#### *1.2.4 Mineral homeostasis*

Bone, with 99% of the body's calcium, is not just the main storage for calcium but also for phosphorous ions, and many other ions that are needed for the adequate function of a variety of systems within the body, proper enzyme reactions, satisfactory blood clotting and transmission of nerve impulses [17]. Although some amount of calcium is found in trabecular bone, the largest volume comes from dense cortical bone.

#### *1.2.5 Locomotion and others*

Skeleton, mainly constituted by bone tissue, forms a system of levers to which the voluntary muscles are attached, allowing controlled and guided movement.

Bone also acts in the acid-balance of the body, buffering the blood against excessive pH changes, through the absorption or release of alkaline salts [11]. Also, bone tissue can store foreign elements like heavy metals, eliminating them from the blood for excretion and reducing their effect on other tissues. Besides, underwater hearing is by bone conduction [18,19] and localization of sound appears to depend on differences in amplitude detected by the conduction of sound to the inner ear through the bones of the skull.

### **1.3 BONE COMPOSITION**

Bones are composed by two types of tissues: compact and trabecular, and are covered by two membranes: periosteum and endosteum. Compact bone forms a rigid outer shell that resists deformation, while trabecular network provides strength with low mass due to the complex system of internal support by lamellae.

#### *1.3.1 Compact bone*

Compact bone, also called dense or cortical bone is a hard outer layer, which gives a smooth, white and solid appearance, and minimal gaps and spaces are found in it. 80% of total bone mass of an adult skeleton corresponds to dense bone. At microscopic level, cortical structure has a characteristic pattern of arrangement: lamellae and ground substance are arranged in concentric layers; Haversian channels found parallel to the long axis of the bone; Volkmann's channels that are interconnected with the Haversian channels and contain blood and lymph vessels and are innervated; lacunae are the cavities that occur at regular intervals in the concentrated layers; canaliculi interconnect several lacunae and Haversian channels; and the Haversian systems are the architecture formed by Haversian channels, lamellae, lacunae and canaliculi [11,12].

### *1.3.2 Trabecular bone*

Trabecular bone, also named as cancellous or spongy bone, is the filling of the inner part of bone. It is arranged as open cell porous networks that generate room for blood vessels and marrow. Presenting a ten fold increased surface area with respect to cortical bone, spongy bone holds the remaining 20% of total bone mass [11]. In bones with a substantial weight-bearing function it is the trabecular pattern that offers the maximum resistance to physical stress.

### *1.3.3 Periosteum*

Periosteum is a dense but thin fibrous membrane that covers the entire outer surface of a mature bone, not including the articulating joints, and serves as an attachment for tendons and muscles. The inner layer of periosteum, so-called osteogenic layer, contains the highly active cells that produce circumferential enlargement and remodeling of the growing of long bone. On the other hand, the outer layer is fibrous and comprises the entire periosteum [10]. Periosteum, attached to bone by strong collagenous fibers called Sharpey's fibers, is irrigated by blood vessels that nourish the surrounding bone and nerves, making it very sensitive to manipulation [17]. In a injury, cells originating from the inner periosteal layer become osteogenic [10].

### *1.3.4 Endosteum*

Endosteum, on the other hand, is the thin layer of vascular connective tissue lining the marrow cavity, and holds morphology and function similar to those of periosteum [14,17].

## **1.4 BONE CELLS**

Bone, a highly vascularised, innervated and mineralized conjunctive tissue, is composed of many different types of cells such as osteoclasts, osteoblasts, lining cells and osteocytes; that build structures based on lamellae of calcified matrix. The arrangement of these lamellae determines whether the bone is cortical or cancellous. Both cortical and trabecular bone contain specialized cells, organic matrix and mineral phase.

### *1.4.1 Osteogenesis*

Osteogenesis is dynamic process of laying down new bone material by osteoblasts. It occurs in two different ways: either by Intramembranous osteogenesis that is the direct laying down of bone into the primitive connective tissue (mesenchyme), or by endochondral osteogenesis that involves a cartilage precursor [20].

There are three main types of bone cells [21] (See Fig. 3, [22]): osteoblasts, responsible for bone formation; osteoclasts, responsible for bone resorption; and osteocytes, fully differentiated osteoblasts that have become embedded in matrix whose function is not clear [23]. Each type of cells will be discussed next.

#### *1.4.2 Osteoblasts*

Osteoblasts are small (20-30  $\mu\text{m}$ ), polyedric, and mono-nucleated cells originated from bone marrow, and they are the responsible of producing new bone named osteoid, the calcified matrix is mainly based on collagen type I and osteonectin, controlling calcium and mineral deposition [12], carrying out these osteoblast communications by protrusions or cell-cell contacts [24]. Active osteoblasts cover the bone surface as a sheath as a layer of plump cells, closely together along the surface of bone [24].

The osteoblast structure is grossly based on nucleus, with a single nucleoli; Golgi area, responsible for collagen synthesis; and rough endoplasmatic reticulum, parallel to cellular membrane that contains ribosomes; these two last acquire a prominent size when osteoblasts evolve towards osteocytes [24], due to their new role as osteoid builders. The whole structure of osteoblasts plays an active role in the synthesis of the proteins and polysaccharides of the bone matrix.

The half life time of human osteoblasts goes from 1 to 10 weeks, when they become less active, turning into the so-called bone-lining cells, beginning to elongate, becoming flat and staying parallel to the bone surface, decreasing their volume and increasing their base, and from that moment on, they are not involved in bone formation anymore, producing gaps between cells [24]. At the end of their life cycle, osteoblast may disappear by apoptosis mechanisms, or transform into both bone-lining cells or osteocytes [25].

Functions of osteoblasts into the osteodynamics are synthesis of collagenous and non-collagenous proteins of the organic matrix of bone; to direct the fibrils of the ECM (extracellular matrix), mediate the resorption process carried out by osteoclasts by means of specific cytokines [26]; and synthesis of growth factors [27].

#### *1.4.3 Osteoclasts*

Large and multi-nucleated cells that dissolve bone are named osteoclasts (100  $\mu\text{m}$ ) rich in mitochondrias and vacuoles, which are originated from bone marrow hematopoietic stem cells known as “Granulocyte-Macrophage Colony-Forming Units” (GM-CFU), precursors of macrophages and monocytes [28,29]. Osteoclasts have two special features in the membrane: a ruffled border, where resorption takes place, and a clear area rich in microfilaments, with integrins that serve as an anchor to the matrix. To this end, the osteoclasts move towards the area to be resorbed and then immediately adhere to the mineralized bone surface with the ruffled border and sealing the edges of the area with the integrins.

#### *1.4.4 Bone-lining cells*

Mature osteoblasts become bone-lining cells with flat appearance, and are thought to regulate the exchange of calcium and phosphorus ions into and out of the bone and respond to hormones by producing special proteins that activate osteoclasts.

#### 1.4.5 Osteocytes

Osteocytes derive from mature osteoblasts and can sense pressure or cracks in bone and may help to direct osteoclasts to the locations where they will dissolve bone [30]. Cortical or compact bone, containing osteocytes, is arranged concentrically around Haversian Channels. Cancellous or trabecular bone is formed by a network of bone lamellae, delimiting areolar cavities where the bone marrow is found [31].

Originated from osteoblasts, osteocytes, decrease about 30% when compared to osteoblast volume, at the expense of the cytoplasm so that the nucleus becomes the outstanding feature of the cell surrounded by giant rough endoplasmatic reticulum and Golgi area structures [24]. Osteocytes get smaller while filling the lacuna in which they are lying with bone matrix and elongate and orientate along axis parallel to collagen fibrils [12]. In histology, the study of osteocytes depends on the way of cutting, whether the cut is done longitudinally or transversally. In the first case, osteocytes can be observed flat and elongated and in the second they appear rounded or in triangular shapes [24].

#### 1.4.6 What are Stem Cells?

Stem cells, name proposed by the Russian histologist Alexander Maksimov in 1908, are non-specialized (undifferentiated) cells that have the extraordinary capacity to renew themselves for long periods, and develop into many different cell types in the body under certain physiologic or experimental conditions [32]. Each new cell resulting from the division of a stem cell has the potential to either remain a stem cell or become another type of cell but with a special function, i.e. brain cells, beating cells of the heart muscle, muscle cells, insulin-producing cells, bone cells, among others [33].

Research studies are conducted with two kinds of stem cells from animals and humans depending their source; these are embryonic stem cells, which are undifferentiated cells derived from embryos developed from eggs that have been fertilized *in vitro* and then donated for research purposes with informed consent of the donors; and adult or somatic stem cells, which are undifferentiated cells found in a differentiated tissue, or in the blood stream, that can renew themselves and differentiate (with certain limitations) to develop in all the specialized cell types of the tissue from which they are originated. However, scientists do not agree about whether or not adult stem cells may develop into all cell types.

A stem cell requires two properties, the first one is self-renewal capability that is the ability to go through numerous cellular cycles of division while remaining in the undifferentiated state; and the second is about potency that is the capacity to differentiate into specialized cell types.

In a rigorous sense, this requires stem cells to be either totipotent; being produced from the fusion of an egg and sperm cell that are able to construct a complete, viable, organism [33]; or pluripotent; descendants of totipotent cells; have the capacity to self-renew and be able to give

rise to any of the three somatic germ layers that comprise an organism (endoderm, mesoderm or ectoderm) [32].

Although multipotent; stem cells that can differentiate into those cells of a closely related family; or unipotent progenitor cells; stem cells that can produce only one cell type; are sometimes referred to as “stem cells” even when they are capable of differentiating into a limited number of tissue types.

Embryonic stem cells, the most studied pluripotent stem cells [34], and adult stem cells represent valuable sources of cells for applications in cell therapy, drug screening and tissue engineering. Fetal stem cells are primitive cell types found in the organs of fetuses [35]. The classification of fetal stem cells is often grouped within adult stem cells. However, a more clear distinction between the two cell types appears to be necessary.

Many efforts have been done to maintain their self-renewal and differentiation capabilities that are critical when expanding stem cells in culture.

The process of stem cells differentiation is conducted by internal signals that mean that a cell's genes are interspersed across long strand of DNA and carry coded instructions for all the structures and functions of a cell. On the other hand, external signals for cell differentiation include chemicals secreted by other cells, physical contact with neighboring cells, and certain molecules in the microenvironment (extracellular matrix).

Certainly, biomedical research concerning cell-based therapies, treatments where stem cells are induced to differentiate into a specific cell type required to repair damaged or destroyed cells or tissues, is focused in establishing precisely how stem cells remain unspecialized and self renewing for many years; and in recognizing the signals that cause stem cells to become specialized cells. However, several questions remain requiring answers such as: why can embryonic stem cells proliferate for a year or more under experimental conditions without differentiating in contrast to adult stem cells; and what are the factors in living organisms that normally regulate stem cell proliferation and self-renewal. These questions focus nowadays the attention of many scientists.

#### *1.4.7 Mesenchymal Stem Cells*

Mesenchymal stem cells or MSCs are multipotent stem cells that can differentiate into a variety of cell types. Cell types that MSCs have been shown to differentiate into in vitro or in vivo include osteoblasts, chondrocytes, myocytes, and adipocytes [36].

MSCs are characterized morphologically by a small cell body with a few cell processes that are long and thin. Their cell body contains a large, round nucleus with a prominent nucleolus which is surrounded by finely dispersed chromatin particles, giving the nucleus a clear appearance [37]. The remaining cell body contains a small amount of Golgi apparatus, rough endoplasmic reticulum, mitochondria, and polyribosomes. The cells, which are long and thin, are widely

dispersed and the adjacent extracellular matrix – ECM is populated by a few reticular fibrils but is devoid of the other types of collagen [38].

## 1.5 ANGIOGENESIS

Angiogenesis is an important physiological process involving the growth and development of new blood vessels both in health and in disease, from pre-existing vessels as well as in wound healing, where various angiogenic proteins including several growth factors stimulate it [39].

Endothelial progenitor cells are bone marrow-derived cells that circulate in the blood and have the ability to differentiate into endothelial cells, the cell that make-up the lining of blood vessels. Endothelial cells are selective filters that regulate the passage of gases, fluid and various molecules across their cell membranes. Different organs have different types of endothelium: some leaky and some very tightly bound [39].

Vascular endothelial cells, which divide only about once every three years in average, form the walls of blood vessels; nevertheless angiogenesis can stimulate them to divide and regulate by both activator and inhibitor molecules when it is required.

Normal angiogenesis occurs in the human body at specific times in development and growth. A process called *vasculogenesis* creates the primary network of vascular endothelial cells that will become major blood vessels. Afterwards, angiogenesis remodels this network into the small new blood vessels or capillaries that complete the body's circulation system [39].

In addition, angiogenesis is active in females a few days each month during the menstrual cycle, as new blood vessels form in the lining of the uterus to mature the egg during ovulation, and during pregnancy to establish the circulation between mother and fetus called placenta. As expected, angiogenesis is necessary for the repair or regeneration of tissue during wound healing [40].

The body controls angiogenesis by producing a precise balance of growth and inhibitory factors in healthy tissues. When this balance is disturbed, the result is either too much or too little angiogenesis. For instance, in response to hypoxia, or low O<sub>2</sub> pressure, mammalian cells increase the expression of a multitude of genes to stimulate cellular processes blood vessels growth [41].

Abnormal blood vessel growth, either excessive or insufficient, is now recognized as a “common denominator” underlying many deadly and debilitating conditions, including cancer, skin diseases, age-related blindness, diabetic ulcers, cardiovascular disease, stroke, and many others [42]. The list of diseases that have angiogenesis as an underlying mechanism grows longer every year.

How do new blood vessels grow? The cascade of events that leads to blood vessels growth starts with the release of angiogenic growth factors (proteins) that diffuse into the nearby tissues, and bind to specific receptors placed on the endothelial cells (EC) or nearby pre-existing blood

vessels. After this, EC become activated and signals are sent from the cell's surface to the nucleus, where new molecules, such as enzymes, are produced to dissolve tiny holes in the basement membrane surrounding all existing blood vessels. Afterwards, endothelial cells proliferate and migrate towards the diseased tissue; and integrins, specialized molecules of adhesion, serve as grappling hooks to help pull the sprouting new blood vessel sprout forward. Extra enzymes, such as matrix metalloproteinases – MMP, are produced to dissolve the tissue in front of the sprouting vessel tip to accommodate it. As the vessel extends, the tissue is remodeled around the vessel rolling up to form a blood vessel tube. Interconnection between blood vessels permits blood circulation, and specialized muscle cells provide structural support to the network and finally, blood flows then begins [40,42].

Some years ago, the controversy over whether blood stem cells were created, or born, in the endothelium or originated from another cell type in a nearby location, came to the end when scientists proved definitively that blood stem cells are made during mid-gestational embryonic development by endothelial cells.

Although normal tissues have an automatic mechanism to attract an increased blood supply when they require it, many tumors achieve rapid growth by switching on enhanced production of angiogenic signals. The new blood vessels supply the tumor with nutrients and oxygen, and they may also provide an easier escape route for metastatic cells [43]. Therefore, angiogenesis became one of the most studied biological processes by oncology scientists using in vitro techniques that require the addition of endothelial cells to the engineered skin. Endothelial cells may organize into vascular structures in culture with the aid of biomaterial scaffolds and coculture (i.e. fibroblasts and human umbilical vein endothelial cells HUVEC) with accessory cells, and vascular smooth muscle cells in a collagen matrix [44,45].

#### *1.5.1 Growth Factors*

Growth factors are proteins (see Table 2), which act as signaling molecules between cells, capable of stimulating cellular growth [46], proliferation, differentiation and maturation; and regulating a variety of cellular processes [47].

Table 2. Growth factors - Overview

| ABBR.                                                                                                                                                                                                                                | NAME                                                                                                                                                                                        | FUNCTION                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |                                                                                                                                                                                                                                                                        |                            |                 |                                                                                                                                                                                                                                                                        |                 |                                                                       |                  |                                                                                                                                                    |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------|-----------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------|-----------------------------------------------------------------------|------------------|----------------------------------------------------------------------------------------------------------------------------------------------------|--------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|-------------------------------------------------------------------------------------------------------------------------------|-------|---|---|-------|---|---|-------|---|---|-------|---------------------------|---|-------|---|----------------------------|--------|---|---|
| BMP                                                                                                                                                                                                                                  | BONE<br>MORPHOGENETIC<br>PROTEINS                                                                                                                                                           | Group of twenty growth factors and cytokines known for their ability to induce the formation of bone and cartilage, interacting with specific receptors on the cell surface: bone morphogenetic protein receptors – BMPRs. BMPs have an important role during embryonic development on the embryonic patterning and early skeletal formation [47].                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |                                                                                                                                                                                                                                                                        |                            |                 |                                                                                                                                                                                                                                                                        |                 |                                                                       |                  |                                                                                                                                                    |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
|                                                                                                                                                                                                                                      |                                                                                                                                                                                             | <table><tr><td>BMP</td><td colspan="2">Involved in development of</td></tr><tr><td></td><td>CARTILAGE</td><td>BONE</td></tr><tr><td>BMP-1</td><td>✓</td><td>✗</td></tr><tr><td>BMP-2</td><td>✓</td><td>Osteoblast differentiation</td></tr><tr><td>BMP-3</td><td>✗</td><td>✓</td></tr><tr><td>BMP-4</td><td>✗</td><td>✓</td></tr><tr><td>BMP-5</td><td>✓</td><td>✗</td></tr><tr><td>BMP-6</td><td>Joint integrity in adults</td><td>✗</td></tr><tr><td>BMP-7</td><td>✗</td><td>Osteoblast differentiation</td></tr><tr><td>BMP-8a</td><td>✓</td><td>✓</td></tr></table>                                                                                                                                                                                                                                                                                                                                                                                                                               | BMP                                                                                                                                                                                                                                                                    | Involved in development of |                 |                                                                                                                                                                                                                                                                        | CARTILAGE       | BONE                                                                  | BMP-1            | ✓                                                                                                                                                  | ✗      | BMP-2                                                                                                                                                                                       | ✓    | Osteoblast differentiation                                                                                                    | BMP-3 | ✗ | ✓ | BMP-4 | ✗ | ✓ | BMP-5 | ✓ | ✗ | BMP-6 | Joint integrity in adults | ✗ | BMP-7 | ✗ | Osteoblast differentiation | BMP-8a | ✓ | ✓ |
|                                                                                                                                                                                                                                      |                                                                                                                                                                                             | BMP                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   | Involved in development of                                                                                                                                                                                                                                             |                            |                 |                                                                                                                                                                                                                                                                        |                 |                                                                       |                  |                                                                                                                                                    |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
|                                                                                                                                                                                                                                      |                                                                                                                                                                                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | CARTILAGE                                                                                                                                                                                                                                                              | BONE                       |                 |                                                                                                                                                                                                                                                                        |                 |                                                                       |                  |                                                                                                                                                    |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
|                                                                                                                                                                                                                                      |                                                                                                                                                                                             | BMP-1                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | ✓                                                                                                                                                                                                                                                                      | ✗                          |                 |                                                                                                                                                                                                                                                                        |                 |                                                                       |                  |                                                                                                                                                    |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
|                                                                                                                                                                                                                                      |                                                                                                                                                                                             | BMP-2                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | ✓                                                                                                                                                                                                                                                                      | Osteoblast differentiation |                 |                                                                                                                                                                                                                                                                        |                 |                                                                       |                  |                                                                                                                                                    |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
|                                                                                                                                                                                                                                      |                                                                                                                                                                                             | BMP-3                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | ✗                                                                                                                                                                                                                                                                      | ✓                          |                 |                                                                                                                                                                                                                                                                        |                 |                                                                       |                  |                                                                                                                                                    |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
|                                                                                                                                                                                                                                      |                                                                                                                                                                                             | BMP-4                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | ✗                                                                                                                                                                                                                                                                      | ✓                          |                 |                                                                                                                                                                                                                                                                        |                 |                                                                       |                  |                                                                                                                                                    |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
|                                                                                                                                                                                                                                      |                                                                                                                                                                                             | BMP-5                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | ✓                                                                                                                                                                                                                                                                      | ✗                          |                 |                                                                                                                                                                                                                                                                        |                 |                                                                       |                  |                                                                                                                                                    |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
|                                                                                                                                                                                                                                      |                                                                                                                                                                                             | BMP-6                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | Joint integrity in adults                                                                                                                                                                                                                                              | ✗                          |                 |                                                                                                                                                                                                                                                                        |                 |                                                                       |                  |                                                                                                                                                    |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
| BMP-7                                                                                                                                                                                                                                | ✗                                                                                                                                                                                           | Osteoblast differentiation                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                                                                                                                                                                                                                                                                        |                            |                 |                                                                                                                                                                                                                                                                        |                 |                                                                       |                  |                                                                                                                                                    |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
| BMP-8a                                                                                                                                                                                                                               | ✓                                                                                                                                                                                           | ✓                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |                                                                                                                                                                                                                                                                        |                            |                 |                                                                                                                                                                                                                                                                        |                 |                                                                       |                  |                                                                                                                                                    |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
| FGF                                                                                                                                                                                                                                  | FIBROBLAST<br>GROWTH FACTOR                                                                                                                                                                 | Heparin-binding proteins involved in angiogenesis, wound healing, and embryonic development that interact with cell-surface associated heparin sulfate proteoglycans. FGFs play an important role in the processes of proliferation and differentiation of a considerable variety of cells and tissues [48,49].                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |                                                                                                                                                                                                                                                                        |                            |                 |                                                                                                                                                                                                                                                                        |                 |                                                                       |                  |                                                                                                                                                    |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
|                                                                                                                                                                                                                                      |                                                                                                                                                                                             | <table><tr><td>FGF Families</td><td>Function</td></tr><tr><td>FGF-1 to FGF-10</td><td>Bind fibroblast growth factor receptors FGFRs</td></tr><tr><td>FGF-11 to FGF14</td><td>Named iFGF, are involved in intracellular processes unrelated to FGFs</td></tr><tr><td>FGF-16 to FGF-23</td><td>Have systemic effects and they are not well characterized</td></tr></table>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              | FGF Families                                                                                                                                                                                                                                                           | Function                   | FGF-1 to FGF-10 | Bind fibroblast growth factor receptors FGFRs                                                                                                                                                                                                                          | FGF-11 to FGF14 | Named iFGF, are involved in intracellular processes unrelated to FGFs | FGF-16 to FGF-23 | Have systemic effects and they are not well characterized                                                                                          |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
|                                                                                                                                                                                                                                      |                                                                                                                                                                                             | FGF Families                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          | Function                                                                                                                                                                                                                                                               |                            |                 |                                                                                                                                                                                                                                                                        |                 |                                                                       |                  |                                                                                                                                                    |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
|                                                                                                                                                                                                                                      |                                                                                                                                                                                             | FGF-1 to FGF-10                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | Bind fibroblast growth factor receptors FGFRs                                                                                                                                                                                                                          |                            |                 |                                                                                                                                                                                                                                                                        |                 |                                                                       |                  |                                                                                                                                                    |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
|                                                                                                                                                                                                                                      |                                                                                                                                                                                             | FGF-11 to FGF14                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | Named iFGF, are involved in intracellular processes unrelated to FGFs                                                                                                                                                                                                  |                            |                 |                                                                                                                                                                                                                                                                        |                 |                                                                       |                  |                                                                                                                                                    |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
| FGF-16 to FGF-23                                                                                                                                                                                                                     | Have systemic effects and they are not well characterized                                                                                                                                   |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |                                                                                                                                                                                                                                                                        |                            |                 |                                                                                                                                                                                                                                                                        |                 |                                                                       |                  |                                                                                                                                                    |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
| VEGF                                                                                                                                                                                                                                 | VASCULAR<br>ENDOTHELIAL<br>GROWTH FACTOR                                                                                                                                                    | <table><tr><td>VEGF type</td><td>Function</td></tr><tr><td>VEGF-A</td><td>It has been mostly studied on cells of the vascular endothelium, and it has shown to stimulate endothelial cell mitogenesis and cell migration.<br/>It is also vasodilator and increases microvascular permeability and increases the interior of a vessel named lumen.</td></tr><tr><td>VEGF-B</td><td>Embryonic angiogenesis</td></tr><tr><td>VEGF-C</td><td>It is involved in angiogenesis, lymphangiogenesis and endothelial cell growth and survival, and can also affect the permeability of blood vessels.</td></tr><tr><td>VEGF-D</td><td>This protein is structurally and functionally similar to vascular endothelial growth factor VEGF-C. It is active for the development of lymphatic vasculature surrounding lung bronchioles.</td></tr><tr><td>PlGF</td><td>Placenta growth factor is important for vasculogenesis, angiogenesis during ischemia, inflammation, wound healing and cancer.</td></tr></table> | VEGF type                                                                                                                                                                                                                                                              | Function                   | VEGF-A          | It has been mostly studied on cells of the vascular endothelium, and it has shown to stimulate endothelial cell mitogenesis and cell migration.<br>It is also vasodilator and increases microvascular permeability and increases the interior of a vessel named lumen. | VEGF-B          | Embryonic angiogenesis                                                | VEGF-C           | It is involved in angiogenesis, lymphangiogenesis and endothelial cell growth and survival, and can also affect the permeability of blood vessels. | VEGF-D | This protein is structurally and functionally similar to vascular endothelial growth factor VEGF-C. It is active for the development of lymphatic vasculature surrounding lung bronchioles. | PlGF | Placenta growth factor is important for vasculogenesis, angiogenesis during ischemia, inflammation, wound healing and cancer. |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
|                                                                                                                                                                                                                                      |                                                                                                                                                                                             | VEGF type                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | Function                                                                                                                                                                                                                                                               |                            |                 |                                                                                                                                                                                                                                                                        |                 |                                                                       |                  |                                                                                                                                                    |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
|                                                                                                                                                                                                                                      |                                                                                                                                                                                             | VEGF-A                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | It has been mostly studied on cells of the vascular endothelium, and it has shown to stimulate endothelial cell mitogenesis and cell migration.<br>It is also vasodilator and increases microvascular permeability and increases the interior of a vessel named lumen. |                            |                 |                                                                                                                                                                                                                                                                        |                 |                                                                       |                  |                                                                                                                                                    |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
|                                                                                                                                                                                                                                      |                                                                                                                                                                                             | VEGF-B                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | Embryonic angiogenesis                                                                                                                                                                                                                                                 |                            |                 |                                                                                                                                                                                                                                                                        |                 |                                                                       |                  |                                                                                                                                                    |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
|                                                                                                                                                                                                                                      |                                                                                                                                                                                             | VEGF-C                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | It is involved in angiogenesis, lymphangiogenesis and endothelial cell growth and survival, and can also affect the permeability of blood vessels.                                                                                                                     |                            |                 |                                                                                                                                                                                                                                                                        |                 |                                                                       |                  |                                                                                                                                                    |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
| VEGF-D                                                                                                                                                                                                                               | This protein is structurally and functionally similar to vascular endothelial growth factor VEGF-C. It is active for the development of lymphatic vasculature surrounding lung bronchioles. |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |                                                                                                                                                                                                                                                                        |                            |                 |                                                                                                                                                                                                                                                                        |                 |                                                                       |                  |                                                                                                                                                    |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
| PlGF                                                                                                                                                                                                                                 | Placenta growth factor is important for vasculogenesis, angiogenesis during ischemia, inflammation, wound healing and cancer.                                                               |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |                                                                                                                                                                                                                                                                        |                            |                 |                                                                                                                                                                                                                                                                        |                 |                                                                       |                  |                                                                                                                                                    |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
| They are more specific of platelet-derived growth factor family of cystine growth factors and are important signaling proteins involved in both vasculogenesis and angiogenesis.                                                     |                                                                                                                                                                                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |                                                                                                                                                                                                                                                                        |                            |                 |                                                                                                                                                                                                                                                                        |                 |                                                                       |                  |                                                                                                                                                    |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |
| They stimulate cellular responses by binding to tyrosine kinase receptors – VEGFRs, on the cell surface, causing them to dimerize and become activated though trans-phosphorylation, although at different sites, times and extents. |                                                                                                                                                                                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |                                                                                                                                                                                                                                                                        |                            |                 |                                                                                                                                                                                                                                                                        |                 |                                                                       |                  |                                                                                                                                                    |        |                                                                                                                                                                                             |      |                                                                                                                               |       |   |   |       |   |   |       |   |   |       |                           |   |       |   |                            |        |   |   |

Table 2. Growth factors - Overview

| ABBR. | NAME                              | FUNCTION                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
|-------|-----------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| PDGF  | PLATELET-DERIVED<br>GROWTH FACTOR | <p>PDGF, composed of two polypeptide chains that may exist as a homodimer (-AA, -BB) or heterodimer (-AB), enhance wound repair [50], support angiogenesis [51,52], and stimulate cell proliferation in the fetal rat calvarial system and in cultures of osteoblast-like cells derived from adult human bone explants [53].</p> <p>The role of PDGF in osteoblast differentiation may be to increase the number of cells that can progress into osteoblastic lineage and express the osteoblast phenotype [54].</p> <p>PDGF expression at fracture sites in addition to its mitogenic effects indicates a role for PDGF in wound healing and fracture repair [52].</p> <p>PDGF and EGF are broad-specificity factors that can stimulate many types of cells to divide, such as fibroblasts, smooth muscle cells and neuroglial cells [55].</p>                                                                                                                         |
| TGF   | TRANSFORMING<br>GROWTH FACTOR     | <p>TGF is used to describe two classes of polypeptide growth factors: TGF-<math>\alpha</math> and TGF-<math>\beta</math>. Mainly found in bone, platelets, and cartilage TGF-<math>\beta</math> triggers growth, differentiation, and extracellular matrix synthesis [56].</p> <p>In addition to mitogens that stimulate cell division, there are factors, such as some members of the TGF-<math>\beta</math> family, that act on some cell to stimulate cell proliferation and others to inhibit it, or that stimulate at one concentration and inhibit at another [57].</p> <p>The effect of TGF over osteoclast differentiation, appears to depend on the mixture of cell types and cytokines that are present in the microenvironment in which osteoclast differentiation occurs [58]. Also, TGF-<math>\beta</math> causes cell retraction, thus leading to greater bone surface exposure, increasing osteoclast precursor attraction to the bone surface [59].</p> |

### *1.5.2 Hormones*

The bones are constantly remodeling, but breakdown and formation are equal. The kidney filters the blood, including a large amount of calcium, but most of this is taken back into the body by the kidney cells. When calcium and/or phosphorus are in short supply, the regulating hormones take them out of the bone to serve vital functions in other systems of the body. Too many withdrawals can weaken the bone. The regulatory hormones also play critical roles in determining how much bone is formed at different phases of skeletal growth [60] and how well bone strength and mass is maintained throughout life. For example, sex hormones and the growth hormone system described below are increased during puberty, a time of rapidly increased skeletal growth. Finally, it is important to remember that the effects of hormones and mechanical forces on the skeleton are closely linked. For example, the ability of bone to respond to mechanical loading is impaired in animals lacking the receptor for estrogen [61].

Three calcium-regulating hormones play an important role in producing healthy bone: 1) parathyroid hormone or PTH, which maintains the level of calcium and stimulates both resorption and formation of bone; 2) calcitriol, the hormone derived from vitamin D, which stimulates the intestines to absorb enough calcium and phosphorus and also affects bone directly; and 3) calcitonin, which inhibits bone breakdown and may protect against excessively high levels of calcium in the blood [62-65].

#### *1.5.2.1 Parathyroid hormone or PTH*

Four small glands adjacent to the thyroid gland produce PTH. These glands precisely control the level of calcium in the blood. They are sensitive to small changes in calcium concentration so that when calcium concentration decreases even slightly the secretion of PTH increases. PTH acts on the kidney to conserve calcium and to stimulate calcitriol production, which increases intestinal absorption of calcium. PTH also acts on the bone to increase movement of calcium from bone to blood. Excessive production of PTH, usually due to a small tumor of the parathyroid glands, is called hyperparathyroidism and can lead to bone loss. PTH stimulates bone formation as well as resorption. When small amounts are injected intermittently, bone formation predominates and the bones get stronger [66]. In recent years a second hormone related to PTH was identified called parathyroid hormone-related protein (PTHrP), which regulates cartilage and bone development in the fetus, but it can be over-produced by individuals who have certain types of cancer. PTHrP then acts like PTH, causing excessive bone breakdown and abnormally high blood calcium levels, called hypercalcemia of malignancy [67].

#### *1.5.2.2 Calcitriol and Vitamin D*

Calcitriol is the hormone produced from vitamin D [68]. Calcitriol, also called 1,25 dihydroxy vitamin D (1,25(OH)<sub>2</sub>D), is formed from vitamin D by enzymes in the liver and kidney. Calcitriol acts on many different tissues, but its most important action is to increase intestinal absorption of calcium and phosphorus, thus supplying minerals for the skeleton. Vitamin D

should not technically be called a vitamin, since it is not an essential food element and can be made in the skin through the action of ultra violet light from the sun on cholesterol. Many people need vitamin D in their diet because they do not derive adequate levels from exposure to the sun. This need occurred as people began to live indoors, wear clothes, and move further north. In northern latitudes the sun's rays are filtered in the winter and thus are not strong enough to make sufficient vitamin D in the skin. Vitamin D deficiency leads to a disease of defective mineralization, called rickets in children and osteomalacia in adults. These conditions can result in bone pain, bowing and deformities of the legs, and fractures. Treatment with vitamin D can restore calcium supplies and reduce bone loss [69].

In adolescence elevated serum 1,25(OH)<sub>2</sub>D levels have been found during growth spurts [70-72] suggesting that increased concentrations of growth hormone raise 1,25(OH)<sub>2</sub>D production. 1,25(OH)<sub>2</sub>D, in turn, increases the intestinal absorption of calcium and a significant positive correlation between serum 1,25(OH)<sub>2</sub>D and bone mass accumulation has been reported in pubertal girls [73]. In addition, in a 14-year follow-up study dietary vitamin D intake was positively associated with peak bone mass in adolescent females [74]. Researchers [75] found that in normal adolescents, girls with earlier menarche or regular menses had higher bone mass than those with later menarche or irregular menses. Also, estradiol has been found to be an important determinant of bone mineral gain in pubertal girls [76,77], and in males pubertal growth spurts with an increase in testosterone concentration has been shown to associate with skeletal growth and mineralization [78]. Thus, several factors affect the growing skeleton.

The main nutrients affecting bone metabolism are calcium, vitamin D, phosphate, sodium and potassium. All these nutrients are needed for bone growth and the mineralization process, but calcium is a threshold nutrient. Several studies have shown that an adequate intake of calcium contributes to the development of peak bone mass [79,80]. Also, increased calcium intake reduces bone loss in postmenopausal women [81]. Vitamin D increases intestinal calcium and phosphate absorption and thus is necessary for calcium homeostasis [82]. High sodium and protein intakes increase calcium excretion [83,84]. Also fiber, phytate, oxalate and caffeine may have negative effects on calcium absorption and bone metabolism [85].

#### *1.5.2.3 Calcitonin*

Calcitonin is a third calcium-regulating hormone produced by cells of the thyroid gland, although by different cells than those that produce thyroid hormones [86]. Calcitonin can block bone breakdown by inactivating osteoclasts, but this effect may be relatively transient in adult humans. Calcitonin may be more important for maintaining bone development and normal blood calcium levels in early life. Excesses or deficiencies of calcitonin in adults do not cause problems in maintaining blood calcium concentration or the strength of the bone. However, calcitonin can be used as a drug for treating bone disease [69].

#### *1.5.2.4 Sex hormones*

Along with calcium-regulating hormones, sex hormones are also extremely important in regulating the growth of the skeleton and maintaining the mass and strength of bone. The female

hormone estrogen and the male hormone testosterone both have effects on bone in men and women [87].

The **estrogen** produced in children and early in puberty can increase bone growth. The high concentration that occurs at the end of puberty has a special effect—that is, to stop further growth in height by closing the cartilage plates at the ends of long bone that previously had allowed the bones to grow in length. Estrogen acts on both osteoclasts and osteoblasts to inhibit bone breakdown at all stages in life. Estrogen may also stimulate bone formation. The marked decrease in estrogen at menopause is associated with rapid bone loss. Hormone therapy was widely used to prevent this, but this practice is now controversial because of the risks of increased breast cancer, strokes, blood clots, and cardiovascular disease with hormone therapy [69].

**Testosterone** is important for skeletal growth both because of its direct effects on bone and its ability to stimulate muscle growth, which puts greater stress on the bone and thus increases bone formation. Testosterone is also a source of estrogen in the body; it is converted into estrogen in fat cells. This estrogen is important for the bones of men as well as women. In fact, older men have higher levels of circulating estrogen than do postmenopausal women [69].

#### *1.5.2.5 Other important hormones*

Growth hormone from the pituitary gland is also an important regulator of skeletal growth. It acts by stimulating the production of another hormone called insulin-like growth factor-1 (IGF-1), which is produced in large amounts in the liver and released into circulation. IGF-1 is also produced locally in other tissues, particularly in bone, also under the control of growth hormone. The growth hormone may also directly affect the bone—that is, not through IGF-1 [88]. Growth hormone is essential for growth and it accelerates skeletal growth at puberty. Decreased production of growth hormone and IGF-1 with age may be responsible for the inability of older individuals to form bone rapidly or to replace bone lost by resorption [89]. The growth hormone/IGF-1 system stimulates both the bone-resorbing and bone-forming cells, but the dominant effect is on bone formation, thus resulting in an increase in bone mass. Thyroid hormones increase the energy production of all body cells, including bone cells. They increase the rates of both bone formation and resorption. Deficiency of thyroid hormone can impair growth in children, while excessive amounts of thyroid hormone can cause too much bone breakdown and weaken the skeleton [90].

The pituitary hormone that controls the thyroid gland, thyrotropin or TSH, may also have direct effects on bone [91]. Cortisol, the major hormone of the adrenal gland, is a critical regulator of metabolism and is important to the body's ability to respond to stress and injury. It has complex effects on the skeleton [92]. Small amounts are necessary for normal bone development, but large amounts block bone growth. Synthetic forms of cortisol, called glucocorticoids, are used to treat many diseases such as asthma and arthritis. They can cause bone loss due both to decreased bone formation and to increased bone breakdown, both of which lead to a high risk of fracture [93]. There are other circulating hormones that affect the skeleton as well. Insulin is important for bone growth, and the response to other factors that stimulate bone growth is impaired in individuals with

insulin deficiency [94,95]. A recently discovered hormone from fat cells, leptin, has also been shown to have effects on bone [96,97].

### 1.5.3 Chemotaxis

The direction of cell movement can be affected by the concentrating gradient of certain growth factors, a response called chemotaxis. It is very important for processes such as normal development, inflammation, angiogenesis, and wound healing in which directed migration of specific cell types is essential. It has been found that when a wound is caused in the human body, large amounts of PDGF, EGF, and TGF- $\beta$  are secreted at different times by cells around the wound to coordinate the influx of neutrophils, macrophages, fibroblasts, smooth muscle cells, and endothelial cells for fast and complete healing of the wound [98]. Lack of these chemotactic growth factors may impair the healing process, while overproduction can cause excessive repair or scarring [99]. A chemotactic response is typically a function of both the absolute concentration of the attractant and the steepness of its concentration gradient. Several hypotheses have been proposed to explain the underlying mechanism of these phenomena [100-103].

## 1.6 MOLECULAR STRUCTURE OF BONE

### 1.6.1 Organic matrix

The organic matrix called osteoid corresponds to one third of bone in weight and is formed mainly by proteins where collagen represents 90% (See Figure 3, [12]) 95% of which is collagen type I, 5% collagen type V and smaller amounts of type III and XII.

The more common molecular species of type I collagen is a heterotrimer composed of two identical  $\alpha 1(I)$  chains and an  $\alpha 2(I)$  chain. These molecules are assembled intracellularly through specific interactions between the precursors (pro- $\alpha$ ) chains. The pro- $\alpha$  chains of type I collagen differ from their respective metabolic derivatives ( $\alpha$  chains) by the presence of additional sequences in both the N- and C-terminal ends [104].

Since the axial length of a residue in the collagen folds is 0.286 nm, the triplet sequences in  $\alpha 1(I)$  and  $\alpha 2(I)$  allow the formation of an uninterrupted helical domain of about 300 nm. These three left-handed helices are twisted together into a right-handed coiled coil, a triple helix or “super helix”, a cooperative quaternary structure stabilized by numerous hydrogen bonds [105-107].

In the collagen molecule the sequence Arg-Gly-Asp (RGD) is found, which is recognized by the surface integrins of bone cells [108]. RGD contains hydroxylysine and hydroxyproline aminoacids, being the latter a specific marker for all collagen phenotypes present in urine in direct relationship with the bone re-absorption ratio [109].

Albeit, collagen has not calcium affinity and due to this, other proteins (such as Osteonectin, called SPARC as well) are involved in the mineral deposit [110-112].

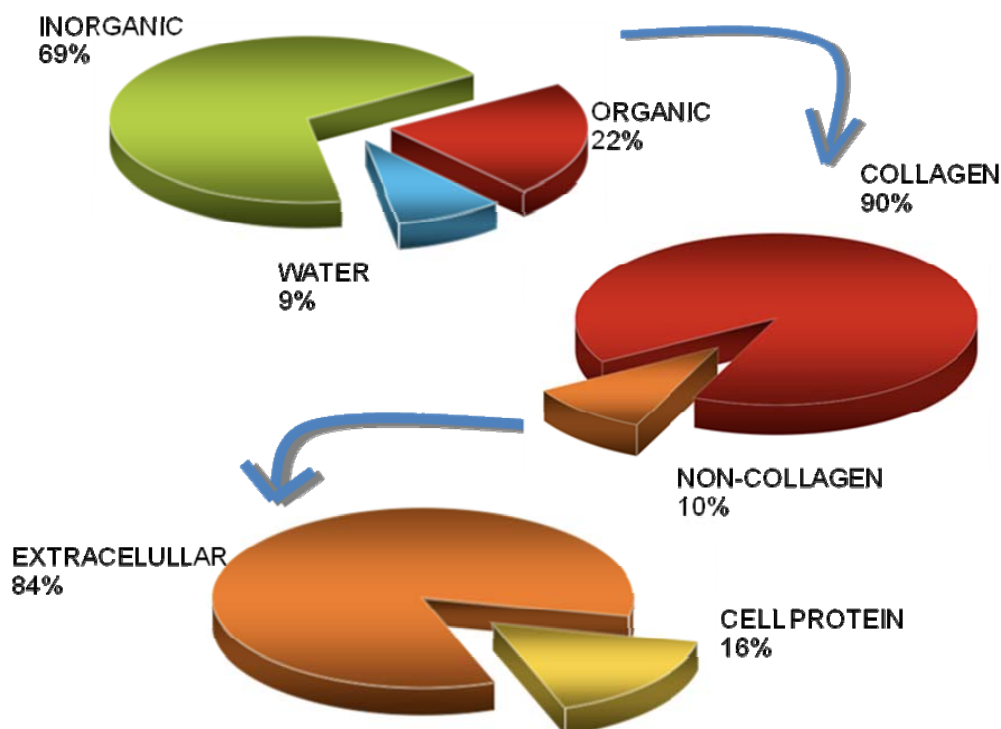


Figure 3. Distribution by weight of the constituents of whole cortical bone [12].

#### 1.6.1.1 Glycoproteins

Most of the non-collagenous proteins that are not proteoglycans are glycoproteins. They are modified by phosphorylation and sulfation by addition of N- and O- linked oligosaccharides depending on the type of tissue [27]. The glycoproteins group represents 25% of non-collagenous proteins and Osteonectin (ON), Alkaline phosphatase (ALP) and RDG proteins are part of this group [113,114].

Osteonectin (ON), so-called SPARC or BM40, with a molecular weight of ~43kD, represents around of 10% of non-collagenous protein, exhibits high affinity for collagen type I, calcium, and hydroxyapatite, due to its two high affinity calcium-binding sites (EF-hands) and twelve low calcium-affinity sites [27,113-117], fact that its very unusual. ON binds to collagen, mediates for hydroxyapatite deposition, and influences the cell proliferation [27]. It seems that ON is involved in the cell adhesion regulation to the matrix, becoming important in the normal mineralization process [115].

Immunocytochemical studies have localized this bone protein into cytoplasm of endosteal osteoprogenitor cells, osteoblasts, newly buried osteocytes and mineralized bone [118]. It has been identified by Immunohistochemistry in Osteoblast (odontoblast and ameloblast) and at points of association between proteoglycans and collagen fibers [114] suggesting that its major role is to confer stability on the ECM. By extension, ON is presumed to exert some control over the orientation and growth of mineral crystals in osteoid [118].

Alkaline Phosphatase, a dimeric protein with ~80kD of molecular weight, is found in the surface of osteoblast and its known that it releases inorganic phosphate and phosphoric acids required for mineralization [119,120], reduces the hydroxyapatite inhibitors such as pyrophosphates and acts as a calcium-binding protein [27].

On the other hand, RGD-containing proteins, named SIBLINGS (Small Integrin-Binding Ligand, N-linked glycoproteins) are fundamentally five [27,121]: Osteopontin (OP), sialoproteins, fibronectin, thrombospondin and vitronectin. They are glycoproteins with a fundamental role in the remodeling and regeneration bone mechanisms due to the presence of Arg-Gly-Asp (RGD) sequence that is a cell attachment sequence ligand for a class of cell-surface receptor named integrins [27], also RGD sequence is recognized by osteoclasts ( $\alpha v \beta 3$ ) among others [122].

#### *1.6.1.2 Proteoglycans*

Proteoglycans are big molecules that differ from glycosaminoglycans because these have long carbohydrate chains composed of repeating sulfated disaccharide subunits of varying compositions. Proteoglycans have a core protein with one or more covalently glycosaminoglycan (GAG) chain(s) [27]. This kind of proteins found in bone constitute the 10% of bone non-collagenous proteins, which are divided in four groups: hyaluronans and chondroitin-sulphate (big molecules involved in the early stages of bone morphogenesis), biglycans and decorin (smaller molecules that play an important role in the next steps of bone formation) [21].

Other proteins such as  $\gamma$ -carboxyglutamic acid proteins, that include Osteocalcin (OC) represent 15% of non-collagenous proteins of bone and link calcium and need vitamin K for its synthesis. OC is a small protein synthesized by osteoblasts and plaquets involving the presence of vitamins D and K.

#### *1.6.2 Mineral phase and water*

The mineral bone component represents 65% in weight and is formed of non-stoichiometric calcium phosphate and calcium carbonate (10:6:1) in small crystals of hydroxyapatite of nanometric scale ( $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ), and Mg, K and F are found in small concentrations [12,30]. Some proteins with adhesive properties favor mineralization while proteoglycans, Mg, ATP and pyrophosphate inhibit it [121]. In Table 3, the different species found in general bone are shown [12], however, bone mineral represents a complete array of the intermediates in the transition of  $\text{Ca}^{2+}$  and Pi (inorganic phosphorous) to solid phase of hydroxyapatite, for instance,  $\text{Ca}_4\text{H}(\text{PO}_4)_3$ , is present in intermediate stages of maturation. Following this trend, depending on the growth stage of bone; solid species may vary their concentrations in the mineral phase.

Water is the third major component of the bone family of materials. The importance of water for the mechanical functioning of bone cannot be underestimated. The values of several mechanical characteristics of dry bone are different from those of wet bone [123]. Water is located within the fibrils, in the gaps, and between triple-helical molecules. It is also present between fibrils and between fibers [124].

Table 3. Calcium, phosphate and carbonate species present in bone. Modified from [12].

| Formula                                             | Name                          | Ca/P ratio |
|-----------------------------------------------------|-------------------------------|------------|
| $\text{Ca}(\text{CO}_3)$                            | Calcium carbonate             | —          |
| $\text{Ca}(\text{HPO}_4) \cdot 2\text{H}_2\text{O}$ | Dicalcium phosphate carbonate | 1.0        |
| $\text{Ca}_4\text{H}(\text{PO}_4)_3$                | Octacalcium phosphate         | 1.33       |
| $\text{Ca}_{9x}(\text{PO}_4)_6x$                    | Amorphous calcium phosphate   | 1.3-1.5    |
| $\text{Ca}_3(\text{PO}_4)_2$                        | Tricalcium phosphate          | 1.50       |
| $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$        | Hydroxyapatite                | 1.67       |

## 1.7 BONE FORMATION

The embryonic skeletal development occurs during the fetal stage through two major pathways: intramembranous, in the bones of craniofacial structures, and endochondral ossification, that occurs in the long bones of the limbs.

It is doubtless that mesenchymal stem cells play a paramount role in bone formation. Mesenchymal stem cells appear to have the potential to undergo chondrogenesis; therefore, in developing intramembranous bone, there must be a set of conditions that could either enhance osteogenesis or inhibit chondrogenesis. Such knowledge should also facilitate designing protocols to enhance bone formation in endochondral bones, especially during fracture repairing because diaphyseal bone growth proceeds via the intramembranous pathway [125].

### 1.7.1 Intramembranous ossification

Intramembranous ossification involves direct differentiation of mesenchymal stem cells into osteoblasts, replacing sheet-like connective tissue membranes with bony tissue. Bones formed in this manner are called intramembranous bones. They include certain flat bones of the skull and some of the irregular bones [11,12]. The future bones are first formed as connective tissue membranes. Osteoblasts migrate to the membranes and deposit bony matrix around themselves [12]. When the osteoblasts are surrounded by matrix they are called osteocytes. Bone is formed from mesenchyme tissue along steps such as: development of ossification center, calcification, formation of trabeculae, and least and not last development of periosteum [14].

### 1.7.2 Endochondral Ossification

This mechanism involves the replacement of hyaline cartilage with bony tissue, as a result of condensation and differentiation of mesenchymal stem cells into cartilage, future skeletal components. When cartilage matures, it undergoes hypertrophy, afterwards it mineralizes and finally, it is invaded by blood vessels and osteoprogenitor cells that differentiate into osteoblasts to form the osteoid matrix [125].

When hydroxyapatite crystals are formed and become densely packed within the matrix, the tissue is called bone [126]. These bones are called endochondral bones representing a high percentage of bones of the skeleton. In this process, the future bones are first formed as hyaline cartilage models. During the third month after conception, the perichondrium (membrane of fibrous connective tissue

that invests cartilages except at joints) that surrounds the hyaline cartilage “models” becomes infiltrated with blood vessels and osteoblasts and changes into a periosteum [14]. Simultaneously, osteoblasts form a collar of compact bone around the diaphysis and cartilage in the center of the diaphysis begins to disintegrate. Afterwards, osteoblasts penetrate the disintegrating cartilage to replace it with spongy bone, forming in this way the primary ossification center that is continuous from this center toward the remainder bone. Consecutively, spongy bone is formed in the diaphysis, and osteoclasts break down the newly formed bone to open up the medullary cavity [10]. Initial bony matrix, mostly woven bone, is removed by osteoclasts and replaced by lamellar bone, with highly organized matrix supported by Haversian canal systems which it is a mechanically superior and predominant form in healthy and adult bone. From here onward and during development, blood vessels play an important role because that is the way to exchange nutrients and act as a source of precursor cell for osteoclasts, as well as an osteoblasts destiny [27,121].

Even, after birth, cartilage in the epiphyses continues to grow, increasing in length and the secondary ossification center form in the epiphyses [27]. When the secondary ossification is concluded, the hyaline cartilage is totally replaced by bone except in two areas. A region of hyaline cartilage remains over the surface of the epiphysis as the articular cartilage and another area of cartilage remains between the epiphysis and diaphysis [10].

Once bone cells are entrapped in the mineralized extracellular matrix, they are considered mature osteocytes and cannot further contribute to bone growth unless it is feed from the surrounding matrix as bone is remodeled.

## **1.8 FRACTURE HEALING**

Fracture healing is a perfect orchestration of proliferative physiological process, in which the body facilitates bone repair, protecting and subsequently healing the damaged area by means of chondroblasts and osteoblast originating from periosteum, main source of these precursor cells, and bone marrow (when present), endosteum, blood vessels and fibroblasts as secondary source. The healing pattern of bone defect is size-dependant [127], consequently, usual margins of 2-3 weeks are given to repair the majority of upper bodily fractures while, more than 4 weeks are given for lower bodily injuries [128].

A fracture is technically an abrupt break of the calcified structure of a bone caused by an extrinsic or intrinsic mechanical force. It is a very common and debilitating injury in the sense that it disables the individual to carry out his normal activities for some period of time. The “economics” of bone fractures are aggravated by the knowledge that a significant number of fractures do not heal properly, even after skilled surgical intervention [12].

The impact of fracture is the moment when the bone absorbs enough energy for a bone tissue failure to be introduced. The energy level required to introduce the bone failure is related to the volume of the bone and the rate of loading. The fractures formed can be classified based on the location and the pattern. Peri-articular fractures are located at the metaphysis or epiphysis of the bone [129]. Those fractures are known as being complex to stabilize due to restricted space

available for fixation at bone ends. Based on pattern, fractures can be classified into greenstick, spiral, comminuted, transverse, compound and vertebral compression [12] (See Fig 4).

Although immobilization and surgery facilitate fracture healing, bone ultimately heals through physiological processes divided in three major phases, which will be discussed next,

#### *1.8.1 Reactive Healing*

The phase of inflammation begins with bleeding from the damaged bone ends and from the associated soft tissue and a clot soon forms between the fragments stopping any further bleeding [130,131], the extravascular blood cells, known as fracture hematoma, which form the blood clot finally degenerate and die [38]. Factors involved in blood clotting lead directly to the phase of acute inflammation. Macrophages are attracted to the fracture during this acute phase but do not begin to predominate until the third day [132]. The soft parts in the region show the usual changes of acute inflammation with vasodilatation and the exudation of plasma and leucocytes [128].

The first evidence of increased cell division is to be found within about eight hours of the injury reaching a maximum in some twenty-four hours. This activity is first seen in the periosteum and tissues immediately around it extending throughout the whole length of the injured bone. However, within a few days this activity decreases to be confined to the area immediately adjacent to the fracture [133].

#### *1.8.2 Reparative Healing*

Days after fracture, cells of the periosteum replicate and transform into chondroblasts and form a slimy mass of firm consistency of a simple structure where nerves and blood vessels are absent, known as hyaline cartilage [134].

This phase called “natural” healing or the external bridging callus, which was initiated with the primary callus and consists in the formation of callus localized outside of the bone joining and immobilizing the moving fragments remaining after fracture [135]. The periosteal cells distal to the fracture gap develop into osteoblast [133] and form woven bone [27,121]. The conversion of the soft tissue is the next stage, the largely chondroid tissue becomes woven bone by endochondral ossification, described previously. Even fibroblast within the granulation tissue also develop into chondroblasts and form hyaline cartilage [130]. These two new tissues grow in size until they unite with their counterparts from other pieces of the fracture. This process forms the *fracture callus* [134].

The success is highly dependent on the blood supply from surrounding tissue, as oxygen supply to the fracture site is one of the primary factors influencing successful union of the bone.

At the end of this event, the fracture is considered healed verified both clinically and radiographically [129].

### *1.8.3 Remodeling*

Once satisfactory bridging is achieved, bone remodeling, the final stage, is initiated with the action of osteoblasts converting woven bone to the lamellar bone and by means of osteoclasts, unwanted bone is removed. Finally both the medullar cavity and the bone geometry and original strength are restored [135].

The unique properties of this phase of healing are relative independence from mechanical influences and ability to replace fibrous tissues with new bone tissues [135].

In the case of extreme mechanical rigidity at the fracture site, primary cortical healing takes place. It is the slowest healing process of all four. During this type of healing, bone union is achieved by direct osteonal penetration. Normally, this healing process is supported by the medullar callus activity [12].

## **1.9 BONE DISEASES**

The body systems that control the growth can be interrupted in several ways that result in various bone diseases and disorders; that can take place at or before birth, such as genetic abnormalities and developmental defects, as well as diseases such as osteoporosis and Paget's disease of bone that harm the skeleton later in advanced age. Also, there are several other disorders that indirectly affect bone by interfering with mineral metabolism [69].

### *1.9.1 Osteoporosis*

Osteoporosis is a disease characterized by low bone mass and structural deterioration of bone structure that causes bone fragility and increases the risk of fracture. Hip, spine and wrist are the susceptible bones to fracture in this condition. Osteoporosis is a result of normal ageing, however can emerge as a result of inequitable development of peak bone mass or excessive bone loss during mature age [136].

There are three principal mechanisms by which osteoporosis advances are: an inappropriate peak bone mass (insufficient mass and strength during growth), the second one is the abnormal bone resorption, and finally inadequate bone remodeling [61].

There are different categories of osteoporosis: a) primary osteoporosis, which is not caused by some other specific disorder [137]; b) idiopathic primary osteoporosis affects previous healthy children between the ages of 8 and 14 [138]; c) age-related osteoporosis, being the most common that begins early in life [139]; and d) secondary disorder is referred to bone loss caused by specific diseases (i.e. genetic disorder such as Gaucher's disease, hypogonadal states such as anorexia nervosa, endocrine disorders such as diabeters mellitus type I, gastrointestinal disease such as primary biliary cirrhosis, hematologic disorders such as hemophilia, rheumatic and auto-immune disease such as lupus, and others such as alcoholism and multiple sclerosis among others) [69] or medications (i.e. anticoagulants, anticonvulsants, glucocorticoids, lithium, metrotrexate, etc) [69,140].

#### *1.9.1.1 The problem*

Osteoporosis is a major global health problem that is increasing dramatically as the population ages. The World Health Organization (WHO) calculates that 70 million people worldwide have osteoporosis [141]. Hip fractures are the most intense consequence of osteoporosis and are related to lengthy hospital admissions, problems in developing daily activities, providing care at home, and a high rate of mortal cases. The occurrence of hip fracture is 1.5 million per year in the worldwide, a statistics estimated to grow to 2.6 million by 2025 and to 4.5 million by 2050 [141]. Osteoporotic fractures result in more than 500.000 hospitalizations, 800.000 emergency room visits, 2.6 million physician's office visits, and the placement of nearly 180.000 individuals in nursing homes [142] just in the United States every year. Total medical costs are estimated in approximately \$40.000 for each hip fracture [143]. One in five persons who sustain hip fracture end up in a nursing home, and 20% of them die before a year has passed, being of great importance the impact on the personal lives of the patients and their relatives. Even if they were ambulatory before their fracture occurred, two-thirds of the individuals who sustain hip fracture never return to their pre-fracture level of function, and many lose their ability to walk [144].

The WHO has declared 2002–2011 as the Decade of the Bone and Joint, uniting nations throughout the world in the commitment of energy and resources to accelerate progress in bone health and prevention of fractures.

#### *1.9.1.2 Symptoms and diagnosis*

Even osteoporosis itself has no specific symptoms; the increased risk of bone fractures is its most important concern. Osteoporotic fractures are those that occur in situations where healthy people would not normally break a bone, being the typical fragility fractures: vertebral column, ribs, hip and wrist.

Increasing awareness of osteoporosis and the development of treatment of proven efficacy is likely to increase the demand for the care of patients with this condition. This in turn will require more facilities for the diagnosis and assessment of osteoporosis, and particularly for the measurement of bone mineral.

The internationally accepted definition of osteoporosis is that it is a systemic skeletal disease characterized by low bone mass and micro-architectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture [145,146]. This view of osteoporosis includes the concept that bone mass is a paramount factor in the risk of fracture, but that other skeletal abnormalities contribute to skeletal fragility, while some non-skeletal factors also have effect on fracture risk [147].

In clinical practice, determinants such as bone mineral density (BMD), bone quality that is manifestation of the architecture (geometry, micro-architecture, trabecular thickness, trabecular connectivity, cortical thickness and cortical porosity) and matrix mineralization properties, are not generally measurable and consequently cannot help the clinician predict fracture risk [148].

Among the methods of measuring bone mass or density are: single and dual X-ray absorptiometry (SXA, DXA) that measures the mineral content of the whole skeleton, as well as of specific sites, including those most vulnerable to fracture [149]; Quantitative ultrasound (QUS) that has recently been used to assess skeletal status in osteoporosis with the advantage in that this not involves ionizing radiation and may provide information on the structural organization of bone in addition to bone mass [150]; Quantitative computed tomography (QCT) has been applied both to the appendicular skeleton and to spine but not yet to the proximal femur [150]; even Magnetic resonance imaging (MRI) initially appeared unsuitable for assessing bone, it provides some resolution of the internal structure of cancellous bone, however MRI is expensive and complex [149]; and finally osteoporosis can often be diagnosed by visual inspection of plain radiographs, albeit with low sensitivity [151].

### *1.9.1.3 Clinical Management*

**Pharmacological management.** Bisphosphonates therapy should be considered first-line therapy for the treatment of osteoporosis, in conjunction with lifestyle modifications and appropriate doses of calcium and vitamin D [152]. Bisphosphonates are an important option for the prevention of fractures in postmenopausal women. However, the complex instructions for the administration of oral bisphosphonates are inconvenient or unsuitable for many patients, and adherence to long-term therapy is poor [153]. The introduction of oral regimens for administration once weekly and, recently, once monthly has been associated with improved tolerability for patients, although adherence remains suboptimal [154]. The demonstration that once-yearly intravenous infusions of zoledronic acid produced a sustained reduction in bone turnover and increased bone mineral density raised the prospect that even less frequent administration and the hope of better adherence might be realized [155].

**Non-pharmacological management.** There is a persuasive body of evidence that nutritional factors play important roles in the development and maintenance of bone strength. Calcium and vitamin D have been the most extensively studied nutritional factors and appear to be important in bone health throughout life. Other dietary factors, energy, protein, micronutrients, and phytoestrogens all have significant roles to play in reducing osteoporotic risk. Dietary recommendations encourage a varied diet with adequate intakes of low-fat dairy products and fruits and vegetables [156]. On the other hand, exercise, as prevention of osteoporosis should begin at an early age. It has been found that the pre-pubertal years are the most crucial years to affect periosteal expansion. Promoting exercise and high-impact activities in children will instill in them the importance of maintaining bone strength. For the adult male and premenopausal woman at risk for osteoporosis, high-impact exercise varied with resistance and endurance training can improve BMD and muscle strength and prevent the decline of balance and flexibility that may occur with the natural aging process [157]. And finally fall prevention and maintaining independence and quality of life are important in the osteoporosis prevention and maintenance.

**Surgical management of fractures.** The major problems associated with fusion surgery to stabilize the spine fall into two main categories. First, this typically aged population is often in frail health, which means that any significant undertaking involving prolonged anesthesia, blood loss, and open surgery can lead to a high rate of mortality in these patients, who may already have

significantly compromised cardiovascular and pulmonary reserves. In addition, from a mechanical standpoint, obtaining surgical fixation in the osteoporotic spine with traditional screws and rods and cages is tenuous at best. The underlying bone is so osteoporotic that it will not be able to support the stress of surgical implants, thus leading to failure of fixation and surgical complications [158].

Recently, minimally invasive and very effective treatment options have emerged for treating these fractures. Vertebroplasty and kyphoplasty have surfaced as the “gold standard” options for acutely painful compression fractures [159]. The purpose behind vertebroplasty technique is to immediately increase the strength of the fractured vertebral body via intraosseous injection of polymethylmethacrylate (PMMA) that is a form of glue fixation for hip and knee implants, showing excellent track record for secure fixation with relative few adverse effects on local bone. On the other hand, kyphoplasty involves the use of an inflatable bone tamp or balloon technology to reach fracture reduction. Using a percutaneous cannula into the vertebral body, a nylon balloon is inserted and slowly inflated. The result is that the collapsed vertebral body is inflated restoring to pre-fracture level. To complete the process, the balloon is removed and PMMA is injected under very low pressure and filled the space created by the balloon [158].

### *1.9.2 Other bone diseases*

#### *1.9.2.1 Rickets and osteomalacia*

Severe vitamin D deficiency in children can cause rickets and the equivalent disease is called osteomalacia in adults, a disease in which bone is not mineralized properly [160]. Rickets is not only a marked weakness of bone and fractures but also bowing of the long bones, especially legs, and a characteristic deformity due to overgrowth of cartilage at the ends of the bones [69]. Thus, in osteomalacia longitudinal growth has stopped and deficient bone mineralization does not cause skeletal deformity but can lead to fractures, particularly of weight-bearing bones such as the pelvis, hip, and feet. Even when there is no fracture, many patients with rickets and osteomalacia suffer from bone pain and can experience severe muscle weakness [69]. Both diseases can generally be prevented by ensuring adequate levels of vitamin D, however they can have devastating consequences for affected individuals [161,162].

Rickets and osteomalacia are typically caused by any of a variety of environmental abnormalities. While rare, the disorder can also be inherited [163] as a result of mutations in the gene producing the enzyme that converts 25-hydroxy vitamin D to the active form, 1,25-dihydroxy vitamin D, or in the gene responsible for the vitamin D receptor. Osteomalacia can also be caused by disorders that cause marked loss of phosphorus from the body. This can occur as a congenital disorder or can be acquired in patients who have tumors that produce a protein that affects phosphorus transport in the kidney. Since vitamin D is formed in the skin by sunlight, the most common cause is reduced sun exposure. This is particularly important in northern latitudes where the winter sun does not have the power to form vitamin D in the skin [69].

#### *1.9.2.2 Paget's disease of bone (Osteitis deformans)*

Paget's disease of bone is a progressive, often crippling disorder of bone remodeling as a result of a disorganized structure due to excess numbers of overactive osteoclasts and osteoblast simultaneously. Those pagetic osteoclasts responsible for site-specific increases in bone turnover have more nuclei than normal osteoclasts [69,164].

Paget's disease commonly involves the spine, pelvis, legs, or skull but not in the entire skeleton, however, an early diagnosis can minimize this bone disease [69]. This bone disease causes bones to grow larger and weaker than normal, also might break easily, it can lead to other health problems such as arthritis and hearing loss.

More men than women have the disease and it is most common in older people. Many people do not know they have Paget's disease because their symptoms are mild. For others, symptoms can include: pain, enlarged bones, broken bones, and damaged cartilage in joints [165].

#### *1.9.2.3 Osteogenesis imperfecta*

A large number of genetic and developmental disorders affect the skeleton, among the more common and more important of these is a group of inherited disorders referred to as osteogenesis imperfecta (OI).

Osteogenesis imperfecta (OI) is a genetic disorder in which bones break easily. Sometimes the bones break for no known reason. OI can also cause weak muscles, brittle teeth, a curved spine and hearing loss. The cause is a gene defect that affects how you make collagen, a protein that helps make bones strong. Usually it is inherited the faulty gene from a parent and sometimes, it is due to a mutation, a random gene change [69].

OI can range from mild to severe and symptoms vary from person to person, from children who are stillborn due to multiple fractures in the womb and the inability to breathe, to children who suffer few fractures, to mild cases where fractures do not occur until later in life, much like in patients with osteoporosis [69]. A person may have just a few or as many as several hundred fractures in a lifetime. Treatments include exercise, pain medicine, physical therapy, wheelchairs, braces and surgery [166].

#### *1.9.2.4 Bone Cancer*

Bone cancer is a malignant tumor of the bone that destroys normal bone tissue [167]. Not all bone tumors are malignant. In fact, benign bone tumors are more common than malignant ones. Both malignant and benign bone tumors may grow and compress healthy bone tissue, but benign tumors do not spread, do not destroy bone tissue, and are rarely a threat to life.

Malignant tumors that begin in bone tissue are called primary bone cancer. Cancer that metastasizes to the bones from other parts of the body, such as the breast, lung, or prostate, is called metastatic

cancer, and is named for the organ or tissue in which it began. Primary bone cancer is far less common than cancer that spreads to the bones

Osteosarcoma, which arises from osteoid tissue in the bone, occurs most often in the knee and upper arm [167]. Chondrosarcoma, which begins in cartilaginous tissue. Chondrosarcoma occurs most often in the pelvis, upper leg, and shoulder. Sometimes a chondrosarcoma contains cancerous bone cells. In that case, it is classified as an osteosarcoma.

The Ewing Sarcoma Family of Tumors (ESFTs) that usually occur in bone, may also arise in soft tissue (muscle, fat, fibrous tissue, blood vessels, or other supporting tissue). Scientists think that ESFTs arise from elements of primitive nerve tissue in the bone or soft tissue [168].

#### *1.9.2.5 Oral health and bone disease*

Oral bone comprises both trabecular and cortical bone and formation and resorption throughout the life span. When oral bone loss exceeds gain, it can cause a loss of tooth-anchoring support or it can diminish the remaining ridge in those areas where partial or complete tooth loss has occurred. Oral bone loss is still a significant concern among adult populations among the world and it increases with age for both sexes. Estrogen deficiency and osteoporosis are causes associated with oral bone loss and attendant tooth loss [69].

On the other hand, Periodontitis is a concerned oral disease because promotes, in its more severe form, massive alveolar bone loss [169,170]. This disease is usually treated means by systemic administration of antibiotics in high dosages for long periods of time [171]. In the treatment of such infections, where vascularity is compromised, obtaining effective local antibiotic concentrations by parenteral administration is difficult [172], been the delivery of a active antimicrobial agent directly to the site of infection the most viable alternative treatment [173-180]. Several have reported the use of hydroxyapatite (HA) as a drug carrier because of its adequate mechanical properties and composition similarity to mineral bone and suggest that it may be used as a bone substitute [178,181-184].

Combining the sustained antibiotic release with the osteoconductive properties, resorbability of the porous HA microspheres, and potential use as injectable bone filling material, these systems provide a forth fold beneficial effect. These microspheres may therefore be considered as potentially good alternative systems to act as carriers for antibiotics and to enhance bone regeneration while treating periodontitis [185].

#### *1.9.2.6 Osteonecrosis*

Osteonecrosis is a disease caused by reduced blood flow to bones in the joints. With too little blood, the bone starts to die and may break down. Osteonecrosis is also known as: avascular necrosis, aseptic necrosis or ischemic necrosis [186].

Osteonecrosis is most often found in the hips, knees, shoulders, and ankles, even in one or several bones simultaneously. In people with healthy bones, new bone is always replacing old bone. This

process keeps bones strong and also happens when children grow or if a bone is injured. In osteonecrosis, bone breaks down faster than the body can make enough strong, new bone. The disease worsens and the bones in the joints break down whether a adequate treatment is not carried out [69].

Osteonecrosis can occur in people of any age, especially in their thirties, forties, and fifties; and is more common in people with illnesses such as: cancer or lupus. It can be caused when the blood flow to the bone decreases, but why this happens is not always clear. Some known causes of osteonecrosis are: steroid medications, alcohol use, injury, and increased pressure inside the bone. Some risk factors for osteonecrosis are: radiation treatment, chemotherapy, kidney and other organ transplants [186].

## 2. SCAFFOLDING FOR BONE ENGINEERING

Transplantation provides hope for many patients with tissue loss or organ failure, but inherent limitations such as donor shortage [187] and the required immune suppression therapy seriously hinder the potential benefits that such an approach can provide for many patients. The problem of current transplantation therapy has stimulated research for alternative solutions. An emerging interdisciplinary and multidisciplinary field aims to recreate biologically functional tissues and organs. This field called either tissue engineering or regenerative medicine, appeared almost 30 years ago and was created to represent a new concept that focuses on regeneration of neotissues from cells with the support of biomaterials and growth factors [188]. Man-made materials, not necessarily originally designed for body part replacements, are being used as implants and prostheses. That foreign materials' lack of biological function imposes serious health risks in patients [189].

Most of large-sized tissues and organs with distinct three-dimensional form will require support for their formation from cells. The support is called scaffold, template, or artificial extracellular matrix (ECM). The major function of scaffold is similar to that of the natural ECM that assists proliferation, differentiation, and biosynthesis of cells [188] in the neo-tissue/organ formation.

The regenerative medicine can be classified based on the use of scaffolds, thus, cell therapy without any use of scaffold and tissue engineering that needs scaffold as a support of tissue regeneration [188].

To fulfill the functions of a scaffold in tissue engineering, the scaffold should meet a number of requirements. First, it should have interconnected macropores, so that numerous cells can be seeded, migrate into the inside, increase the cell number and should be supplied by sufficient amounts of nutrients. Macropores make both vascular formation and waste transport possible. This is important for the survival of cells inside the scaffold. An optimal pore size is in the range between 100 and 500  $\mu\text{m}$ . Furthermore, scaffold should have an optimal porosity with adequate surface area and mechanical strength. The absorption kinetics of scaffold is also critical and depends on the tissue to be regenerated. If a scaffold is used for bone engineering [190], degradation of the scaffold biomaterial should be relative slow, as it has to maintain the mechanical strength until tissue regeneration is almost completed [188,191]. That is the case of inorganic scaffolds that have been used in addition to polymeric scaffolds, specifically for the bone tissue engineering and employ biomaterials such as HA and  $\beta$ -TCP.

In this chapter bioceramics, as inorganic biomaterials, and collagen, as a natural polymer, will be studied next to delineate the new concept of a composite based on collagen and HA in the field of bone engineering.

## 2.1 BIOCERAMICS

Bioceramics, as a subset of Biomaterials, range in biocompatibility from the ceramic oxides such as  $\text{Al}_2\text{O}_3$ ,  $\text{MgO}$ ,  $\text{SiO}_2$ , and  $\text{ZrO}_2$ , that are inert in the body, to the resorbable materials, which within the last 100 years, innovative techniques for processing ceramics and their composites can also be used to augment or replace various parts of the body, especially bone tissue [192].

Calcium phosphates bioceramics have been used in medicine and oral health since 1980's, according to reviews carried out by several authors [193-198]. Applications of bioceramics based on calcium phosphates can include from dental implants, percutaneous implants, periodontal treatments, alveolar augmentation, orthopedics, maxillofacial surgery, until spine surgery, among others; depending on what sort of application is required, different calcium phosphates are used.

Their relative inertness to the body fluids, high compressive strength, and aesthetically pleasing appearance led to the use of ceramics in dentistry as dental crowns. Bioceramics are also being used as reinforcing components of composite implant materials and for tensile loading applications, such as artificial tendons and ligaments, due to bioceramics high specific strength as fibers and their biocompatibility [199].

The implantable bioceramics must meet or exceed these desired properties: a) non-toxic, b) non-carcinogenic, c) non-allergic, d) non-inflammatory, e) biocompatible, and f) biofunctional for its lifetime in the host [192].

The concept of using synthetic resorbable ceramics as bone substitutes was introduced in 1969. The name of resorbable ceramics implies degradation upon implantation in the host, with the resorbable materials being replaced by endogenous tissues. Examples of resorbable ceramics are aluminum calcium phosphate, coralline, plaster of Paris (di-hydrated calcium sulfate), hydroxyapatite and tricalcium phosphate [192], with these two deserving a deeper explanation.

### 2.1.1 Calcium phosphates (CaP)

Calcium phosphates is the name given to a wide family of minerals containing calcium ions ( $\text{Ca}^{+2}$ ) together with orthophosphate ( $\text{PO}_4^{-3}$ ), metaphosphate or pyrophosphate ( $\text{P}_2\text{O}_7^{-4}$ ) ions and hydrogen ( $\text{H}^+$ ) or hydroxyl ions ( $\text{OH}^-$ ) [200].

The calcium phosphates and apatites in general have been of notably interest to biologists, industrial chemists and scientists, for many years because calcium phosphates form the mineral component of bones and teeth, and the more acid calcium phosphates are probably also involved in mineralization processes; being many of them originated in pathological calcifications. Calcium phosphates are widely used as biocompatible materials for bone replacement or for bone prostheses [200]. Calcium phosphates are broadly used in the production of phosphoric acid and fertilizers; They are also used

in the food industry as a raising agent in the cheese production; as well as in a variety of dental products.

On the other hand, apatites are a group of phosphate minerals, usually referred as hydroxyapatite, fluorapatite and chlorapatite, presenting high concentrations of  $\text{OH}^-$ ,  $\text{F}^-$ ,  $\text{Cl}^-$  ions, respectively, in the crystal. But also, the occurrence of carbonate group in apatites is very important because carbonate increases their chemical reactivity, particularly by increasing the solubility product and the rate of dissolution in acids, and by reducing the thermal stability [200].

In Table 4 the most commonly used apatitic and non-apatitic calcium phosphates are shown.

Table 4. Apatitic and Non-apatitic calcium phosphates. [200,201].

| Name                                                                     | Molecular Type                                                   | Ca/P Ratio |
|--------------------------------------------------------------------------|------------------------------------------------------------------|------------|
| <b>Non-apatitic calcium phosphates</b>                                   |                                                                  |            |
| Monohydrate calcium phosphate – MCPH                                     | $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$    | 0.50       |
| Monocalcium phosphate – MCP                                              | $\text{Ca}(\text{H}_2\text{PO}_4)_2$                             | 0.50       |
| Dicalcium phosphate di-hydrate – DCPD                                    | $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$                       | 1.00       |
| Dicalcium phosphate anhydrous – DCPA                                     | $\text{CaHPO}_4$                                                 | 1.00       |
| $\alpha$ - and $\beta$ - Tricalcium phosphate – $\alpha$ - $\beta$ - TCP | $\beta$ - $\text{Ca}_3(\text{PO}_4)_2$                           | 1.50       |
| Octacalcium phosphate – OCP                                              | $\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}$ | 1.33       |
| <b>Apatites</b>                                                          |                                                                  |            |
| Hydroxyapatite – HA                                                      | $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$                     | 1.67       |
| Carbonated apatite – CHA                                                 | $\text{Ca}_{10}(\text{PO}_4)_6\text{CO}_3$                       | 1.67       |
| Fluoroapatite – FHA                                                      | $\text{Ca}_5(\text{PO}_4)_3\text{F}$                             | 1.67       |
| Chlorapatite- ClHA                                                       | $\text{Ca}_5(\text{PO}_4)_3\text{Cl}$                            | 1.67       |

Calcium phosphates as biomaterials are available as powders, granules or blocks, However, these forms are of limited value when cavities are not easily accessible or when it would be preferable to perform percutaneous surgery that has been used much less frequently than open surgery [202].

#### 2.1.1.1 Dicalcium phosphate dehydrate(DCPD)

This phase is known as brushite (B), but also as calcium monohydrogen phosphate dihydrate, dibasic calcium phosphate dihydrate, and calcium hydrogen orthophosphate 2-hydrate. The mineral was discovered in 1865 in phosphatic guano from Avis Island in the Caribbean, and named after the American mineralogist G.J. Brush [203].

It has the chemical formula,  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ , characterized by a Ca/P ratio of unity. DCPD (B) is normally found in callus, bone, dental calculus [204], and kidney stones [205]. An important characteristic of DCPD is its use as a precursor for HA synthesis [206] because it can occur as an intermediate in apatitic mineralisation processes.

In addition, brushite is a common CaP phase, stable under weakly acidic environments [205]. However, its stability is normally questionable since it tends to lose its bonded molecular water to form monetite even upon storage on the shelf [207].

DCPD has a potential use for gene delivery with the precaution of remaining stable under physiological conditions, because the binding and condensation of DNA, which is so critical for efficient delivery of genes, are two processes that need to occur simultaneously during the formation of the carrier [207].

#### *2.1.1.2 Dicalcium phosphate anhydrous (DCPA)*

This ceramic is known as anhydrous dicalcium phosphate, dicalcium phosphate, calcium hydrogen orthophosphate, or monetite has the formula  $\text{CaHPO}_4$ . The mineral monetite was first described in 1882 in rock-phosphate deposits from the islands of Mnetta and Mona in the West Indies. DCPA does not appear to occur in dental calculus or other pathological calcifications, nor has it been found in normal calcifications. However DCPA has been reported in XRD studies of fracture callus and possibly in bone. Since it is less soluble than DCPD under all conditions of normal temperature and pressure, it might be expected to be of more frequent occurrence. However, its slow rate of crystal growth relative to DCPD might explain why DCPD forms instead of DCPA, even though it is less stable [203].

#### *2.1.1.3 Tricalcium phosphate (TCP)*

One of the calcium phosphates widely used is tricalcium phosphate (TCP) with formula  $\text{Ca}_3(\text{PO}_4)_2$ , it has a molar ratio Ca/P=1.5 and it is also known as calcium orthophosphate, tertiary calcium phosphate, tribasic calcium phosphate or “bone ash” because it is one of the main combustion products of bone. TCP is usually more soluble than synthetic HA and, upon implantation, it allows for good bone in-growth, due to its interconnected porosity of over 100 $\mu\text{m}$  pores diameter [208], and eventually it is replaced by endogenous bone [192]. There are alpha and beta crystal forms, being the first the stable form at high temperatures. A multicrystalline porous form of  $\beta$ -tricalcium phosphate has been widely used clinically as an artificial bone filler in the fields of dental – correcting periodontal defects and augmenting bony contours [209]– and orthopedic surgery [210], and its biocompatibility has been confirmed in experimental and clinical trials since the 1970s [211-214].

#### *2.1.1.4 Hydroxyapatite*

Also called hydroxylapatite (HA) is a naturally occurring mineral in a hexagonal crystal system, based on calcium phosphates with the formula:  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$  but usually written as the half of the molecule denoting that the crystal unit cell comprises two entities, in other words, that there is

the hydroxyl endmember of the complex apatite group. HA is a basic calcium phosphate and has a molar ratio Ca/P=1.67. On the HA surface, positive or negative charged ions can be found on the surface of HA, which means that the OH<sup>-</sup> ion can be replaced by fluoride, chloride, magnesium or carbonate [200].

Among calcium phosphates, HA has the lowest solubility in water at physiologic pH, indicating that it is almost impossible having Ca<sup>+2</sup> and PO<sub>4</sub><sup>-3</sup> ions dissolved in water at neutral pH. This fact explains how calcium ions in the body are in solid state and deposited in the osseous tissues. However, HA is soluble in acid solutions such as an infection or in the osteoclasts borders in biological conditions.

HA is the most stable calcium phosphate salt at normal temperatures and pH between 4 and 12 [201]. It is a compound of great interest in catalysis, the industry of fertilizers and pharmaceutical products, protein chromatography applications, water treatment processes, preparation of biocompatible materials, and mostly because it is the main inorganic component in calcified hard tissues such as bone and teeth of vertebrates [215-219]. Also HA forms functional irregularities resulting in cartilage arthritis, formation of renal, bladder and bile stones, and calcification of transplanted cardiac valves [220-223]. In this scenario, HA is considered as a model compound to study biomineralization phenomena [224-226].

HA is commonly used as a filler to replace amputated bone or as coating to promote osteointegration into prosthetic implants, also, many modern implants such as hip replacements and dental implants are supported by strong evidence, because HA is an inorganic component of the human bone and has a high bioactivity and biocompatibility [227]. A comparison of mechanical properties between human bone and typical bioceramics is shown in Table 5 [228]. HA has a flexural strength comparable to that of human bone. However, the elastic modulus and compressive strength of HA are higher than those of human bone, while fracture toughness is significantly higher for human bone than for HA [228].

Table 5. Comparison of mechanical features of human bone and bioceramics [228].

| Materials                      | Elastic Modulus (GPa) | Compressive Strength (MPa) | Flexural Strength (MPa) | Fracture Toughness (MPa.m <sup>1/2</sup> ) |
|--------------------------------|-----------------------|----------------------------|-------------------------|--------------------------------------------|
| Human bone                     | 7-30                  | 100-300                    | 50-150                  | 2-6                                        |
| HA                             | 86-110                | 500-1000                   | 115-200                 | 1                                          |
| TCP                            | 30-90                 | 400-700                    | 130-160                 | 1                                          |
| Al <sub>2</sub> O <sub>3</sub> | 300-400               | 4500                       | 550                     | 3-5                                        |

#### 2.1.1.5 Hydroxyapatite in nanometric scale

Natural crystals of bone and teeth exhibit nanostructures with needle-like or rod-like shapes well arranged within the polymeric matrix [229]. The mutual interaction between the natural crystal and the surrounding physiological environment is not yet well understood; however, the natural crystals do have a more dynamic response to environment compared to synthetic materials. One of the reasons may be the poor crystalline and nanostructured features of the natural crystals that are

formed in the natural environment [229]. Both structural characteristics give valuable dynamics to the crystals in terms of enhanced contact areas and degradation (or re-deposition) properly of the environment, which is essentially a structurally induced chemical and biological response [229].

Lately, nanoHA (10-100 nm) has received much attention owing to its superior functional properties over its microscale counterpart, particularly surface reactivity and ultra-fine structure, which are the most imperative properties for tissue-graft interaction upon implantation. During the past few years, significant research effort has been devoted to nanostructure processing of HA and its composites in order to obtain ultra-fine structures with physical, mechanical, chemical, and biological properties substantially different from their microscale counterparts and, simultaneously, analogous to natural bone mineral. Nanostructure processing improves the HA sinterability and enhances the mechanical reliability by reducing flaw sizes. The high volume fraction of grain boundaries in nanocrystalline ceramic compacts also provides for increased ductility and super-plasticity for low-temperature net-shape forming [230].

It has also been proven that the nanoHA, compared to conventional micro HA, helps osteoblast adhesion, differentiation and proliferation, osteointegration, and deposition of calcium-containing minerals on its surface, which conducts to enhanced composition of new bone tissue within a short period [231].

#### *2.1.1.6 Nanohydroxyapatite Synthesis Review*

Hydroxyapatite (HA) is the most stable calcium phosphate salt at normal temperatures and pH between 4 and 12. It is a compound of great interest in fields such as catalysis, the industry of fertilizers, pharmaceutical products, protein chromatography applications, water treatment processes, preparation of biocompatible materials, and mostly because it is main inorganic component in calcified hard tissues. For this reason there is a strong interest for synthetic, extra pure, well defined HA crystals for use in detailed physicochemical in vitro and in vivo studies, biomedical applications, and implantation surgery.

In literature, several methods to prepare HA nanocrystals have been reported (see Table 6). Essentially, there are two main nanoHA synthesis processes: wet method and solid state. Basically from wet method the most of processing synthesis are derived from, such as chemical precipitation, sol-gel, emulsion, biomimetics, among others. The synthesis of nanoHA from supersaturated aqueous solutions is advantageous due to low cost and simplicity, however, most of the synthetic routes followed until now led to the formation of nonstoichiometric products [232].

Difficulties encountered in preparing synthetic nanoHA crystals from aqueous solutions are mainly caused by the high chemical affinity of the material to some ions such as  $K^{2+}$ ,  $Na^{2+}$ ,  $NO_3^-$  and  $Cl^-$  [232]. Additionally, partial nanoHA crystal grains can be seen to aggregate into the so-called “secondary particle” as a result of the spontaneous congregation and the growth of nanometer sized particles to reduce the free enthalpy of the system [233], this phenomena can be overthrow using reagents such as surfactants with the aim of lower the surface tension of the liquid preparation, allowing easier spreading and avoiding crystal growth.

The complex nature of the calcium phosphates system and the role of kinetic (parameters that depending on the experimental conditions) prevail over the thermodynamics. The formation of synthetic nanoHA crystals from highly supersaturated solutions proceeds via intermediate precursor phases, which have a transient existence, such as  $\beta$ -TCP [234].

It was shown that the presence of chloride ions and fast precipitation of the crystals during preparation affected the crystal size and therefore the total surface area of the crystals. The effect is more pronounced when aging is absent. From the synthesis of nanoHA crystals from aqueous solutions it was concluded that aging and precipitation kinetics are critical for the purity of the product and its crystallographic features [235], and besides, the supersaturation level of the reactants, especially the concentration of  $\text{Ca}^{2+}$  ions, played a predominant role on the precipitate morphology for these classical acid-base reactions.

The use of extra pure reactants, which give no residual ions after reaction, the low precipitation, and finally, the long aging period, improved the quality and the characteristic of the crystals. During aging, the undergo a process that includes dissolution and re-crystallization. In this stage, the smaller crystals disappear in favor of the larger ones, which grow further. As a consequence the total number of crystals is reduced and the surface roughness of the crystals is reduced. Both phenomena result in a decrease of the specific surface area [201]. This is in accordance with the Oswald ripening law, which says that the number of smaller particles continues to shrink, while larger particles continue to grow in size [236].

Another outstanding variable, proved in the current study, is the rate of reactant addition, which affects the rate of HA nanocrystals nucleation. A slow addition rate ensures the absence of continuous nucleation.

Furthermore, an increase in the concentrations of starting raw materials decreased the crystallite size and the primary particle size of hydroxyapatite produced, and promoted the formation of needle-shaped apatite particles. The specific surface area of the starting  $\text{Ca}(\text{OH})_2$  powder had no influence on the crystallite size and specific surface area of the produced apatite particles [228].

In the case of ion substitution in the HA network it is suggested that in order to substitute a smaller Mg ion for a larger Ca ion, some additional structural changes may be needed to prevent destabilization of the structure and subsequent phase decomposition (formation of TCP besides HA) during heat treatment: the co-substitution of a second ionic species and more specifically  $\text{CO}_3^{2-}$  group is a simple way of achieving this [237].

Table 6. Nanohydroxyapatite processing methods - Review.

| SYNTHESIS METHOD       | CRYSTAL SIZE (NM) | CA AND P SOURCES                                                          |                                                                                                      | CONDITIONS                                                                                      | REF           |
|------------------------|-------------------|---------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|---------------|
| Chemical Precipitation | 10-100            | $\text{CaCl}_2$<br>$\text{Ca}(\text{NO}_3)_2$<br>$\text{Ca}(\text{OH})_2$ | $\text{P}(\text{OC}_2\text{H}_5)_3$<br>$\text{NH}_4\text{H}_2\text{PO}_4$<br>$\text{H}_3\text{PO}_4$ | Nanocrystal size, low crystallinity, homogeneous, reaction condition: RT-200°C Ageing until 24h | [235,238-240] |

| SYNTHESIS METHOD              | CRYSTAL SIZE (NM) | CA AND P SOURCES                                                                              |                                                                                                                                                           | CONDITIONS                                                                                                                                                                                                                                                                                                                                 | REF           |
|-------------------------------|-------------------|-----------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------|
| Hydrothermal                  | 15-65             | Ca(NO <sub>3</sub> ) <sub>2</sub><br>CaCl <sub>2</sub><br>DCPD<br>DCPA<br>Ca(OH) <sub>2</sub> | NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub><br>H <sub>3</sub> PO <sub>4</sub><br>(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>                      | Highly crystalline and phase pure material<br>50-400°C / 1-24 MPa<br>reaction in few seconds until 12h<br>Ageing until 8h                                                                                                                                                                                                                  | [241-251]     |
| Mechanochemical (Solid State) | 5-150             | Ca(OH) <sub>2</sub><br>CaCO <sub>3</sub><br>CaHPO <sub>4</sub> ·2H <sub>2</sub> O             | H <sub>3</sub> PO <sub>4</sub><br>Ca <sub>2</sub> P <sub>2</sub> O <sub>7</sub><br>CaCO <sub>3</sub><br>(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>  | Nanorods and nanofibers.<br>CO <sub>3</sub> <sup>2-</sup> and Mg <sup>2+</sup> substituted HA.<br>Ca deficient from stoichiometric HA<br>RT-1100°C / 1-6h of milling<br>High crystalline HA.<br>Need subsequent heat treatment.                                                                                                            | [252-259]     |
| Biomimetics                   | 50                | Ca(OH) <sub>2</sub><br>Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O<br>Egg shell      | (NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>                                                                                                          | Use of simulated body fluid (SBF) with different compositions.<br>Low crystallinity.<br>Use different substrates: glass, bioglass, silica, titanium, titania gels, etc.<br>Incubating substrates in SBF 37°C / 2 weeks / pH 7.4<br>Aging 30°C / 1 week                                                                                     | [260-262]     |
| Emulsion                      | 15-60             | CaCl <sub>2</sub><br>Ca(OH) <sub>2</sub><br>Ca(NO <sub>3</sub> ) <sub>2</sub>                 | Na <sub>2</sub> HPO <sub>4</sub><br>KH <sub>2</sub> PO <sub>4</sub><br>(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub><br>H <sub>3</sub> PO <sub>4</sub> | Use of oil and aqueous phases.<br>Use of surfactant ionic/nonionic template systems: DDBA <sup>1</sup> , C <sub>12</sub> E <sub>5</sub> , triton, n-hexanol, cyclohexane, Span 83 <sup>2</sup> , VA-10 <sup>3</sup> , NP-5 <sup>4</sup> , NP-9 <sup>5</sup> , NP-12 <sup>6</sup> , Dodecane, PEGDE <sup>7</sup> , citric acid<br>RT - 85°C | [263-269]     |
| Sol-gel                       | 10-60             | Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O                                          | P(OCH <sub>2</sub> CH <sub>3</sub> ) <sub>3</sub><br>(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub><br>(CH <sub>3</sub> O) <sub>3</sub> PO              | This method usually results in a fine-grain micro-structure containing a mixture of nm-submicron crystals, better accepted by the host tissue.                                                                                                                                                                                             | [233,270-272] |

<sup>1</sup> Didodecyldimethylammonium bromide; <sup>2</sup> Sorbitan Sesquioleate; <sup>3</sup> 2-methyl-2-ethyl heptanoic acid; <sup>4</sup> poly(oxyethylene)<sub>5</sub> nonyl phenol ether; <sup>5</sup> poly(oxyethylene)<sub>9</sub> nonyl phenol ether; <sup>6</sup> poly(oxyethylene)<sub>12</sub> nonyl phenol ether; <sup>7</sup> Pentaethylene glycol dedecyl ether.

Biomimicry is an ancient concept recently returning to scientific thought, that examines nature, its models, systems, processes, and elements – and takes inspiration from them to solve human problems sustainably. In engineering, the term “Biomimetic” is used for the process of understanding and applying biological principles to human design. In nanoHA synthesis, the term

biomimetics refers to the processing that emulates the nanoHA crystals formation and precipitation in the biological environment using, in most cases, synthetic body fluid – SBF.

SBF is prepared according to the chemical analysis of human body fluid, with ion concentrations nearly equal to those of the inorganic constituents of human blood plasma, and firstly presented by Tadashi Kokubo and co-workers [273] obtaining a coated surface with poorly crystallized calcium-deficient and carbonate-containing apatite, which was similar to “bone apatite”. This method is based on the calcium ions, dissolved from the glass-ceramic samples immersed in SBF, increasing the degree of supersaturation of the surrounding body fluid with respect to the apatite, and the hydrated silica that forms on the surfaces of the glass-ceramics providing favorable sites for further apatite nucleation. Once apatite is nucleated, it may be able to grow spontaneously in the body environment [274].

However, SBF simulates just the inorganic part of human blood plasma and does not contain proteins, glucose, vitamins, hormones, etc. The amount of some ions in human blood plasma can vary depending on the person’s sex, age and nutrition. The interest in using SBF for in vitro tests as well as for preparation of biomimetic apatite coatings has greatly increased within the last 5 years [275-277]. Thus, several SBF solutions such as revised (r-SBF) and modified (m-SBF) with ionic concentrations closer or equal to that of human blood plasma have been prepared [278]. However, the preparation of SBF has to be carried out under well-controlled conditions to avoid coagulation and precipitation that could influence the in vitro test results.

Other important method is the sol-gel synthesis of nanoHA ceramics that has recently attracted much attention. The sol-gel method offers mixing at molecular-level of the calcium and phosphorus precursors, which is able of improving chemical homogeneity of the resulting nanoHA to an important extent, in comparison with traditional methods such as solid state. The flexibility of the sol-gel method opens a great opportunity to form thin film coatings in a simple process for biomedical applications [279]. In this processing, it appears that the temperature that is required to form the apatitic structure depends largely on the chemical nature of the precursors, in addition to ageing time, longer than 24h, is critical for the solution system to stabilize such that a monophasic nanoHA can be produced. However, until now, there is no adequate explanation for the influence of the chemical nature of the precursors on phase formation [280].

## **2.2 COLLAGEN**

The most abundant proteins in the extracellular matrix are members of the collagen family. Thus, collagens are the major structural element of all connective tissues and are also found in the interstitial tissue of virtually all parenchymal organs, where the ultimate and classic functional role of collagens is serving to maintain the physical integrity of the different tissues and organs, exhibiting considerable morphological and architectural variability as assorted as the relative massive striated fibers in tendons and ligaments, the thin and often non-striated fibrils observed in portions of hyaline cartilage, the delicate reticular networks prevalent in several connective tissues, and the apparently amorphous aggregates that occur in a diverse of thin membranous sheets commonly recognized as basement membranes. Likewise, in certain occasions collagen can be

supporting elements such as amorphous or crystalline deposits of calcium phosphate salts, proteoglycan aggregates, and specific macromolecular glycoproteins such as fibronectin.

The better understanding of expression and function of collagen in general contributes to a better understanding of diseases that are based on molecular defects of collagen genes such as chondrodysplasias, osteogenesis imperfecta, Alport syndrome, Ehler's Danlos Syndrome, or epidermolysis bullosa; and besides, collagen degradation and disturbed metabolism are important in the course of osteoarthritis and osteoporosis [281,282].

Table 7. Various collagen types and their distribution in the body. Adapted from [283].

| Collagen Type                       |       | Tissue distribution                                                              |
|-------------------------------------|-------|----------------------------------------------------------------------------------|
| Fibril-forming collagens            | I     | Bone, dermis, tendon, ligaments cornea                                           |
|                                     | II    | Cartilage, vitreous body, nucleus pulposus                                       |
|                                     | III   | Skin, vessel wall, reticular fibres of most tissues (lungs, liver, spleen, etc.) |
|                                     | V     | Lung, cornea, bone fetal membranes; together with type I collagen                |
|                                     | XI    | Cartilage, vitreous body                                                         |
| Basement membrane collagen          |       | Basement membranes                                                               |
|                                     | IV    |                                                                                  |
|                                     | VI    | Widespread: dermis, cartilage, placenta, lungs, vessel wall, intervertebral disc |
| Anchoring fibrils                   | VII   | Skin, dermal- epidermal junctions; oral mucosa, cervix                           |
| Hexagonal network-forming collagens | VIII  | Endothelial cells, Descemet's membrane                                           |
|                                     | X     | Hypertrophic cartilage                                                           |
|                                     | IX    | Cartilage, vitreous humor, cornea                                                |
|                                     | XII   | Perichondrium, ligaments, tendon                                                 |
| FACIT collagens <sup>1</sup>        | XIV   | Dermis, tendon, vessel wall, placenta, lungs, liver                              |
|                                     | XIX   | Human rhabdomyosarcoma                                                           |
|                                     | XX    | Corneal epithelium, embryonic skin, cartilage, tendon                            |
|                                     | XXI   | Blood vessel wall                                                                |
| Multiplexins <sup>2</sup>           | XV    | Fibroblasts, smooth muscle, kidney, pancreas                                     |
|                                     | XVI   | Fibroblasts, amnion, keratinocytes                                               |
|                                     | XVIII | Lungs, liver                                                                     |

<sup>1</sup> FACIT collagen: Fibril Associated Collagens with interrupted triple helices.

<sup>2</sup> Multiplexins: Multiple triple-helix domains and interruptions

### 2.2.1 Structure and function

In the last decade the collagen family expanded dramatically (see Table 7) [283]. All members are characterized by containing domains with repetitions of the proline-rich tripeptide Gly-X-Y involved in the formation of trimeric collagen triplehelices. The functions of this heterogeneous family are not confined to providing structural components of the fibrillar backbone of the ECM, however, additional protein domains define a great variety of additional functional roles.

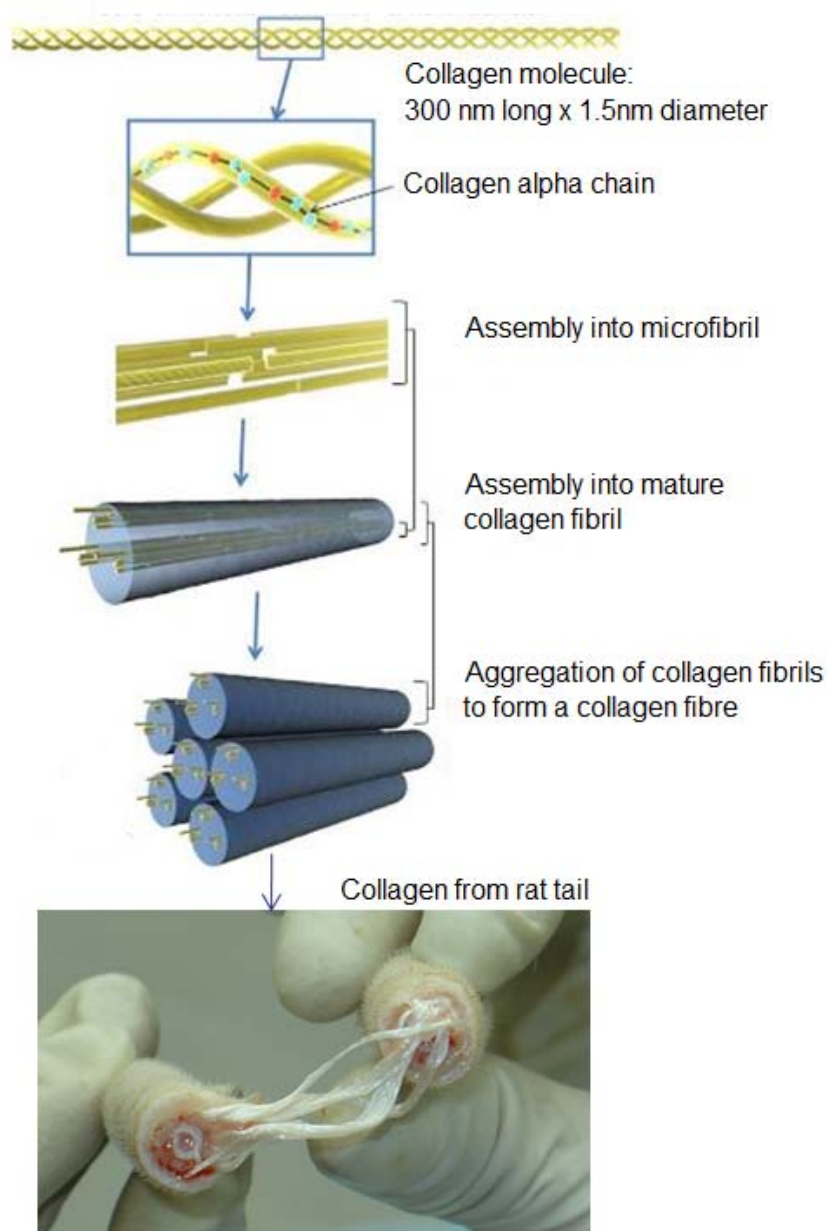


Figure 4. Collagen structure.  
Adapted from the source. Source: [www.orthoteers.com](http://www.orthoteers.com)

The knowledge about the molecular structure, biosynthesis, assembly and turnover of collagen is important to understand embryonic and fetal developmental processes as well as pathological processes linked with many human diseases.

Fibrillar collagens contain rigid, rod-like molecules with three subunits,  $\alpha$  chains, folded into a right-handed collagen triple helix. Each  $\alpha$  chain consists of about 1000 amino acid residues and is coiled into an extended, left-handed polyproline II helix, and it turns, twisted into a right-handed superhelix [284] (Figure 4).

Based on their structure and supramolecular organization, collagens can be grouped into fibril-forming collagens, fibril-associated collagens (FACIT), network-forming collagens, anchoring fibrils, transmembrane collagens, basement membrane collagens and others with unique functions (Table 7); and are characterized by considerable complexity and diversity in their structure, presence of additional non-helical domains, assembly, and function among others. The most abundant and widespread is the fibril-forming collagen family with about 80% of the total collagen in the body [283]. Types I and V collagens fibrils contribute to the structural backbone of bone and types II and XI collagens predominantly contribute to the fibrillar matrix of articular cartilage.

Despite the rather high structural diversity among the different collagen types, all members of the collagen family have a right-handed triple helix composed of three  $\alpha$ -chains, which might be formed by three identical chains (homotrimers) that is the case of collagens II, III, and others or by two or more different chains (heterotrimers) as in collagens types I, IV, V, among others [107].

Type I collagen, formed as a heterotrimer by two identical  $\alpha 1(I)$ -chains and one  $\alpha 2(I)$ -chain, is the most abundant and best studied collagen, forming more than 90% of the organic mass of bone and is the major collagen of tendons, skin, ligaments, cornea, and many interstitial connective tissues with the exception of very few tissues such as hyaline cartilage, brain, and vitreous body. In vivo, mostly incorporated into composite containing either type III collagen (in skin and reticular fibres) [285]. In most organs and notably in tendons and fascia, type I collagen defines considerable biomechanical properties concerning load bearing, tensile strength, and torsional stiffness in particular after calcification in bone [283].

On the other hand, type III collagen is a homotrimer of three  $\alpha 1(III)$ -chains and is widely distributed in collagen I and it is an important component of reticular fibres in the interstitial tissue of the lungs, liver, dermis, spleen, and vessels. This homotrimeric molecule also often contributes to mixed fibrils with type I collagen and is also abundant in elastic tissues [286].

Collagens serve within the body to a large extent for the maintenance of the structural integrity of tissues and organs; however, they also contribute to the entrapment, local storage, and delivery of growth factors, such as IGF-I and -II bound to the collagenous matrix of bone, and cytokines and therefore play important roles during organ development, wound healing and tissue repair [287,288]. Therefore, bone represents a major reservoir of these biomacromolecules within the body; thus, degradation of collagen network by osteoclasts during bone remodeling is thought to

release matrix-bound IGFs to induce new bone formation via stimulation of osteoblastic activity in a paracrine manner [283].

### 2.2.2 Collagen Porous scaffolds preparation

Every tissue and organ has its own three dimensional matrix organization in which cells align new ECM components according to the scaffolds provided. Therefore, scaffolds with a specific 3D structural design resembling the actual ECM of a particular tissue certainly have great potential in tissue engineering.

Collagens are the most ubiquitous and abundant components in mammals and provide strength and structural integrity to all organs, including skin, tendon, and bone; therefore, collagens –specially type I collagen – have been used as biomaterials in a variety of applications because of a number of useful properties such as low antigenicity and appropriate mechanical characteristics [289].

In the fabrication and structural design of collagen scaffolds exist some important parameters that is worth to be studied deeper: orientation of collagen fibers and fibrils that is of crucial importance for the functioning of organs and tissues and plays a decisive role in their biomechanical properties [289]; pore size and porosity [312].

Structure of the scaffolds can be controlled directly using the employed freezing process and the heat transfer processes associated with it [312]. Differences in heat transfer rates during the freezing process of the collagen suspension have a great effect on the microstructure of the fabricated scaffolds. If the cooling rate is fast enough to extract all the crystallization heat, crystallization starts simultaneously in the entire collagen suspension, resulting in the formation of many small ice crystals and thus small pores, in the contrary case, if the cooling rate is not rapid enough, ice crystals formation is slower, allowing the growth of larger ice crystals [301-302].

Table 8. Tensile strength performance of different tissues - Comparison

| Tissue                                           | Tensile strength (MPa) | References |
|--------------------------------------------------|------------------------|------------|
| Tendon                                           | 50 – 100               | [303, 304] |
| Skin                                             | ~20                    | [305]      |
| Lung                                             | 1-2                    | [306, 307] |
| Collagen scaffold (non-croslinked <sup>a</sup> ) | ~0.1                   | [308, 309] |
| Collagen scaffold (croslinked <sup>b</sup> )     | 0.7                    | [308, 309] |

a: Scaffolds frozen at -80°C

b: Cross-linking using 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide/N-hydroxysuccinimide

### 2.2.3 Collagen scaffolds Cross-linking

Chemical cross-linking also decreased the susceptibility of the scaffolds to enzymatic digestion *in vitro* and reduced the *in vivo* degradation rate after subcutaneous implantation in adult Wistar rats (non-crosslinked scaffolds for 2-3 weeks, cross-linked scaffolds 10-12 weeks) [309,310]

## 2.3 NANOCOMPOSITES

Bone is an amazing and true nanocomposite. It is a complex and a highly specialized form of connective tissue pertaining to the formation of the skeleton of the body. It is a good example of a dynamic tissue, since it has a unique capability of self-regenerating or self-remodeling to a certain extent throughout the life without leaving a scar [290].

A single-phase material – also called monolithic – does not always provide all the essential features required for bone growth, which attends to persist on research of an ideal bone graft. There is, therefore, a great need for engineering multi-phase materials – also called composite – with structure and composition similar to natural bone [291]. Recently, many advanced methods that conform nano-crystal-contained composites – called nanocomposites – at room temperature, have become available and they have a variety of biomedical applications [272,292,293]. Thus, nanocomposites, particularly HA and collagen based, have gained much recognition as bone grafts not only due to their composition and structural similarity with natural bone but also because of their unique functional properties such as larger surface area and superior mechanical strength than their single-phase constituents [294,295].

Nanocomposites could play a pivotal role in bone grafting as a new class of bone graft material, which uses a combination of several nanoscale bone graft materials and/or in conjunction with osteoinductive growth factors and osteogenic cellular components. Furthermore, the term nanocomposite can be defined as a heterogeneous combination of two or more materials in which at least one of those materials should be within a nanometer-scale. Nanomaterial, on the other hand, is considered as a new class of material as it possesses superior properties over its microscale counterpart. Nanocrystalline HA promotes osteoblast cell adhesion, differentiation, and proliferation, osteointegration and deposition of calcium containing minerals on its surface better than microcrystalline HA; hence, enhancing the formation of new bone tissue within a short period [231,296,297].

### 2.3.1 Synthesis of collagen porous scaffolds

Obtaining homogeneous collagen slurry is an important step in the initial preparation of scaffolds, as well as selecting a collagen type appropriate to each application. Collagen type I is widely used in bone tissue regeneration, sometimes mixed with type II, IV or V.

Collagen can be purchased or be obtained from bovine Achilles tendons which must be defatted, freed from adhering non-collagenous material, and frozen in liquid nitrogen. The frozen tendon pieces are pulverized using a universal cutting mill and sieved through a 0.5-mm sieve with continuous cooling using liquid nitrogen. Insoluble type I collagen (containing its telopeptides) is isolated from the pulverized material using salt solutions (0.1-M and 1.0-M) [289].

Generally, an acid suspension of collagen mixture is blended for 90 minutes at 4°C. A cooling system maintained the temperature of the suspension at 4°C throughout the entire mixing process to avoid fiber denaturization. Slurry is then degassed under vacuum (50 mTorr) for 60 minutes to remove the many the visible air bubbles created during mixing. After degassing, the slurry is prepared for freeze-drying as follows. First, the slurry is poured into plastic pans at room temperature and then placed into a freeze-dryer. The temperature of the freeze dryer was decreased at a constant rate from 20°C to -40°C over a period of 60 minutes. The temperature was held constant at -40°C for 60-120 minutes, and then sublimated at 100-200 mTorr, 0°C for 17 hours. This process produced the porous structure of the collagen scaffold. This process has been shown to produce scaffolds with uniform pore size, which is that of the ice crystal that originally occupied the pore space while frozen [311, 312].

Secondly, collagen scaffolds are stiffened using a physical crosslinking process called dehydrothermal treatment (DHT). This process took place inside a vacuum oven at 50 mTorr. The vacuum was maintained at 105°C for 24 hours to induce covalent crosslinks between the polypeptide chains of the collagen fibers. Completed scaffolds were placed in aluminum foil envelopes and stored in a dessicator at room temperature until next crosslinking [313] (Figure 5).



Figure 5. Collagen type I/III scaffolds following the above procedure. (Own work)

Scaffold pore size has been shown to influence cellular activity, thus, the influence of freezing temperature is an important variable important to be included into the study of scaffold synthesis. The optimal scaffold pore size that allows maximal entry of cells [314] as well as cell adhesion and matrix deposition has been shown to vary with different cell types [315, 316]. There is evidence that tissue synthesized in a scaffold with uniform pore architecture shows superior biomechanical properties compared to tissue synthesized in a scaffold with a non-uniform pore structure [317].

There is a main consideration in the assembly of a polymeric tissue engineering scaffold and it is relating to the curing method or the method by which the polymer is assembled into a bulk material known as cross-linking. Cross-linking involves the formation of covalent or ionic bonds between individual polymer chains. Typically either a radical or ion is needed to promote cross-linking a long with an initiator, such as heat, light, chemical accelerant, or time. The advantages of cross-linking polymers are that they often have significant mechanical properties. However, one major

disadvantage is the possible cytotoxicity issues with cross-linking systems. The multiple components used by cross-linking polymers as well as the necessary chemical reaction may lead to the use of cytotoxic components or the formation of cytotoxic reaction byproducts [318].

The interactions or mere proximity of proteins can be studied by the clever use of cross-linking agents. Examples of some common cross-linkers are the imidoester crosslinker dimethyl suberimidate, the NHS (N-hydroxysuccinimide) crosslinker and formaldehyde. Each of these cross-linkers induces nucleophilic attack of the amino group of lysine and subsequent covalent bonding via the crosslinker. EDC (also EDAC or EDCI, acronyms for 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) is a water soluble carbodiimide usually obtained as the hydrochloride. Common uses for this carbodiimide include peptides synthesis, protein cross-linking to nucleic acids and preparation of immunoconjugates. EDC is often used in combination with NHS to increase coupling efficiency or create a stable amine-reactive product cross-linker functions by converting carboxyls into amine-reactive isourea intermediates that bind to lysine residues or other available primary amines [319].

Scaffolds manufactured in this study were cross-linked via a dehydrothermal process after freeze-drying to stiffen the collagen network by introducing covalent cross-links between the polypeptide chains of the collagen fibers without denaturing the collagen into gelatin. Cross-linking was carried out in a vacuum oven at a temperature of 105°C under a vacuum of 50mTorr for 24 h [312, 320].

Table 9. Tensile elastic modulus of various porous scaffolds.

| Scaffold material <sup>a</sup>                         | E*(GPa)  |
|--------------------------------------------------------|----------|
| Cross-linked collagen I                                | 20 ± 2   |
| Cross-linked collagen I + hydroxyapatite nanoparticles | 200 ± 14 |
| Cross-linked collagen I + HA                           | 18 ± 2   |
| Cross-linked collagen I + chitosan nanofibers          | 86 ± 6   |

<sup>a</sup> Lyophilizate with 75% porosity, measured at 37°C, 70% relative humidity, cross-head speed 50 mm/min

### 2.3.2 Nanohydroxyapatite association to Collagen

Bone tissue is a natural composite material that contains collagen fibrils and HA nanocrystals in a hierarchical organization. On the smallest length scale, collagen triple helices spontaneously form nanoscale bundles, which act as a template for the crystallization of HA nanocrystals. The fabrication of artificial materials that mimic the structure and properties of natural bone is a significant challenge since it involves both organic and inorganic nanophases that have a specific spatial relation [298].

Nanocomposite bone graft made of nanoHA and collagen facilitates greater osteoconduction and related functions than conventional bone grafts [299]. This system exhibits some features of natural bone in composition and structure to a certain extent. Innovations in the processing of nanocomposites are raising the possibility of obtaining bone grafts with improved performance.

### *2.3.3 Bone Tissue Engineering using Col/HA composite scaffolds*

When bone is lost due to injury and/or illness, the defects are generally filled with natural bone because artificial bone materials have problems of bioaffinity. However, natural bone also has supply and infection problems. If an artificial material has the same biological properties as bone, it can replace natural bone for grafting. Thus, Kikuchi and coworkers [322] synthesized a hydroxyapatite and collagen composite by a simultaneous titration coprecipitation method. The composite obtained showed a self-organized nanostructure similar to bone assembled by the chemical interaction between HA and Col.

Major problems with the treatment of osteomyelitis are associated with poor antibiotic distribution at the site of infection due to limited blood circulation to the skeletal tissue. Improved treatment procedures have been used in drug delivery systems that include bioceramics and natural and synthetic polymers. Hydroxyapatite and collagen composite for sustained antibiotic release can be an efficient support in the treatment of osteomyelitis because most of the antibiotic release may be associated with composite bioresorption, permitting antibiotic release through the healing process [321].

There have been few investigations of its mechanical and biological benefits in direct comparison with a collagen equivalent. Thus, Song and coworkers [323] produced a biomedical membrane made of a nanocomposite that with improved mechanical, chemical and biological properties than pure collagen, very useful in the field of guided bone regeneration.

Wahl et al. [324] manufactured scaffolds from type I collagen with or without HA by critical point drying with mean pore sizes from 44-1135  $\mu\text{m}$  and microchannels are neatly incorporated via an indirect solid freeform fabrication (SFF) process, which could aid in reducing the different constraints commonly observed with other scaffolds.

And finally, just to cite few some examples, Zhang et al. [325] designed a hierarchical structure made by self-assembly of nano-fibrils of mineralized collagen resembling extracellular matrix. The collagen fibrils were formed by self-assembly of collagen triple helices. HA crystals grew on the surface of these fibrils in such a way that their c-axes were oriented along the longitudinal axes of the fibrils. The mineralized collagen fibrils aligned parallel to each other to form mineralized collagen fibers.

The present study was aimed to design and synthesize a nanocomposite based on nanohydroxyapatite and type I/III collagen as scaffold to be used in bone tissue engineering. One of the main concerns was the method to be used in order to bind, more than a physically way like the above examples, nanocrystals to the collagen net. Thus, SPARC (secreted protein acidic and rich in cyteine) was chose to play the role of chemical bridge between nanoHA and collagen fibrils, making of these synergism the only nanocomposite designed to used a collagen scaffolds with nanocrystals of hydroxyapatite and SPARC with several benefits such as: collagenous and porous scaffold that aids cellular growing and proliferation; increased specific area due to the HA

nanoparticles; and the presence of SPARC increases cellular activity and chemically binds collagen to nanoHA due to its affinity with calcium ions in its domains, fact never taken into account before by other group of researchers in bone tissue engineering.

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## CHAPTER II – NANOPARTICLES OF HYDROXYAPATITE: PREPARATION, CHARACTERISATION, AND CELLULAR APPROACH – OVERVIEW

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**Abstract.** Nanostructure syntheses have been applied to derive hydroxyapatite in nanometric scale for orthopaedic and dental implant applications due to its biocompatibility and similarity to natural apatite characteristics, becoming to a preferred material in comparison to hydroxyapatite in micro scale. This overview presents some of the most studied processes, of obtaining nanophased hydroxyapatite, enriched with own results and including additional and interesting information about cell-nanohydroxyapatite performance in vitro experiments.

### 1. Introduction

When bone is lost due to injury and/or illness, the defects are filled with natural or artificial bone grafts, although, infections and bioaffinity are collateral problems, respectively. Hence, research has recently been focused towards artificial materials, that might present biological properties similar to those of bone and that might replace natural bone by grating.

In recent years, synthetic hydroxyapatite (HA) has shown substantial interest and importance due to its chemical similarity with the natural calcium phosphate. Calcium orthophosphates are salts of the tribasic phosphoric acid,  $H_3PO_4$ , and this forms three groups of compounds that contain  $H_2PO_4^-$ ,  $HPO_4^{2-}$  or  $PO_4^{3-}$  ions [1]. The first group are compounds formed only under rather acidic conditions, thus, they are not found normally in biological systems. Second and third groups of compounds are found in the mineral constituents of bones and teeth, as well in some pathological calcifications.

Among apatites, Hydroxyapatite (HA),  $(Ca_{10}(PO)_6(OH)_2)$  is known to be the major inorganic constituent of bone, corresponding to almost 70% in dry weight, of bones hard tissues [2-7]. The term apatite was firstly coined by Werner in 1786, which is derived from the Greek word *apataw* [1] that means “to deceive” because frequently the minerals, belonging to this group, were misleading due to similar appearances with valuable stones.

Biological apatites, which comprise the mineral phases of calcified tissues (enamel, dentin, and bone), slightly differ from pure HA in stoichiometry, composition and crystallinity, and in other

physical and mechanical properties [8]. There are several reports dealing with the precipitation of HA, modifying stoichiometries from aqueous solutions in order to obtain different Ca/P ratios [9-15]; and since Jarcho and colleagues' work in 1976 until now, many studies about HA preparation have been reporting the synthesis of HA crystal in the nanometric, sub-micrometric and micrometric size ranges [16-20]. However, the various parameters that affect size, shape, and crystallinity of nanoscale HA (1-100 nm) still need further study.

## **2. Hydroxyapatite particles**

In general calcium phosphates are all white solids, unless doped with a coloured ion; most are sparingly soluble in water, and some, like HA, are quite insoluble, but all dissolve in acids [1]. HA has a relatively high thermal stability and phase purity even after high temperature sintering (1000-1300°C) [8].

HA has been proposed as substitute for defective bones or teeth. It is known to be biocompatible, bioactive, i.e. ability to form a direct chemical bond with surrounding tissues, osteoconductive, non-toxic, non-inflammatory and non-immunogenic agent [4, 21-22].

HA appears to form a direct chemical bond with hard tissues [23-24] and on implantation of HA particles of porous blocks in bone, new lamellar cancellous bone forms within 4 to 8 weeks [25-26]. HA ceramics have a flexural strength comparable to human bone. However, the elastic modulus and compressive strength of HA ceramics are higher than those of human bone, while the fracture toughness is significantly higher for human bone than for HA ceramics [27].

Nowadays, HA is widely applied as a coating artificial joint components used in orthopaedics and dental implants and hold a great potential as a biomaterial for bone implantation because of its bioactivity, dissolution range, and resorption properties close to those of natural bones [28]. According to the literature, conventional HA has been widely applied in the forms of powder, dense or porous blocks [4, 29].

Currently, significant research effort has been dedicated to developing inorganic nanosize crystalline materials because of their new possibilities deriving from size and their potential in biology, electronics, optics, transport, and information technology [4, 30-38]. It is believed that nanostructured calcium phosphate ceramics can improve the sintering kinetics due to a higher surface area and subsequently improve mechanical properties significantly [39]. Furthermore, most likely the nanoscale sizes of the particles will provide an increase in specific surface area, which in turn can cause an increase in the binding capacity of macromolecules such as DNA [40] and thus might dramatically affect the biological outcome.

Differences in shrinkage between the agglomerates are also responsible by the occurrence of small cracks in sintered HA [41]. Therefore, synthesis of agglomerate-free or softly agglomerated nanostructured HA may be an important step towards achieving improved mechanical properties in dense nanostructures [42].

Recently, HA prepared at nano level has been playing an increasingly significant role in various biomedical applications owing to its unique functional properties of high surface area to volume ratio and its ultrafine structure similar to biological apatite, which is having a great impact on cell-biomaterial interactions [43-44]. This type of HA has been considered mainly in the form of particulate for the treatment of periodontal osseous defects and alveolar ridge augmentation because of its easy fabrication, handling and close surface contact with the surrounding tissue [45].

The attractive advantages described above justify a further review of HA nanocrystals synthesis, characterisation and its behaviour in contact with cells.

### **3. NanoHA applications**

Needle-like HA particles are suitable for preparing filters for ion exchange between cations [37] or for protein adsorption [35-36, 46]. In particular, colloidal HA has been used as adsorbents for chromatography to separate proteins and catalysts for chemical reactions such as the Michael-type addition and methane oxidation [38, 47-48].

NanoHA preparation has a considerable academic and commercial interest for bone replacement and coatings on metallic joint prostheses. HA occurs in ammoniated superphosphates, and in calcareous soils as a reaction product of monocalcium phosphate monohydrate ( $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$ ) or brushite. HA is also used in column chromatography for protein separation [31-33, 49]. In addition, nanofiber-shaped HA is a promising candidate material for reinforcing ceramic or polymer matrices used in the biomedical applications [50].

Magnetic nanoparticles have been developed to such an extent that the functional properties have been specially tailored for biological applications such as cell separation, drug delivery, a contrast agent for magnetic resonance imaging (MRI), and a heat mediator for hyperthermia [51].

Obtaining non-agglomerated nanosized bioceramic particles of high-surface area is interesting for many applications including injectable/controlled setting bone cements, high strength porous/non-porous synthetic bone grafts, the reinforcing phase in nano-composites that attempt to mimic the complex structure and superior mechanical properties of bone [52]; and as potentially useful materials for a number of biomedical applications including scaffolds for tissue engineering and as carriers for non-viral gene delivery [40].

### **4. NanoHA preparation methods**

Considering the numerous applications of calcium phosphate compounds in biomedical fields, several nanoHA synthesis techniques and variations have been developed.

HA nanostructured can be produced from dry and wet reactions, being this one the most widely studied. Wet chemical reactions to form calcium phosphate materials can be generally divided in two kinds of process in terms of reagents concentrations: non-homogeneous and homogeneous processes [53]. A drop-wise addition of a donor reagent of phosphorous into a calcium hydroxide solution or salt slurry is involved in the non-homogeneous process; while, in the second one, calcium and phosphorous salts are solubilised in water under a strict pH control. In both cases, a long ageing time is required to let the precipitation reactions be concluded, in order to obtain uniformity of a single ion concentration during the precipitation phase [53].

Previous work mainly placed emphasis upon controlling the stoichiometry of the product, while, with the development of nanotechnologies, considerable effort is now focused on controlling the morphology and size [54-57] due to studies that have shown that many clinical capabilities of HA highly depend on its morphology and size [58].

#### **4.1 Mechanochemical Processing**

The principle on which mechano-chemical synthesis during the reduction of powders particles is

founded, relates to the energy provided by the impaction that mill balls exert on powder particles constrained towards the internal wall of the jar, 50 Kbar for conventional ball mills, and the temperature rise in the collision region may vary from few degrees to few hundred degrees [59]. This method takes advantage of the perturbation of surface-bonded species by pressure to enhance thermodynamic and kinetic reactions between solids [60]. During milling, the energy transmitted to crystalline particles results in a dislocation cell structure that develops into random nanosized particles increasing the milling time [61]. Spherical particles with 10-50 nm in size of carbonated-substituted HA were produced from phosphate di-hydrogen ammonium and calcium carbonate as precursors after 6h of milling [62].

## 4.2 Sol-gel Route

Sol-gel technique is considered as an elective method for the preparation of highly pure powder due to the possibility of a strict control of the process parameters. This particular synthesis process induces better chemical homogeneity and its product is characterized by nano-size dimensions of the primary particles, that improve the contact reaction and the stability at the artificial/natural bone interface [53]. In addition, sol-gel is characterized by being a low temperature process because there is a highly reactive powder without any degradation phenomena occurring during sintering. Nevertheless, hydrolysis of phosphates and expensive raw materials are the limitations to this technique [63].

In other studies [64] citric acid ( $C_6H_8O_7$ ) was added as a chelating agent to avoid agglomeration of HA particles in the presence of calcium nitrate and trimethyl phosphate as precursors in a sol-gel processing. Primary and secondary particles produced have 50 nm and 100-200 nm, respectively, where these last are aggregates of the first ones. Through the addition of citric acid, the substitution of organic macromolecule for hydrate molecule can lower the reaction speed making favourable the HA gel particles uniformly distributed by the organic constituent and difficult for them to aggregate and grow.

## 4.3 Biomimetic Process

The biomimetic process is inspired in the biomineralization of bone, which involves the deposition of apatitic crystals on a matrix rich in collagen fibrils, the structural macromolecules that create the scaffold within which the biological mineral is formed. Moreover, the biological matrix contains acidic macromolecules rich in negatively charged groups, mainly carboxylates but also sulfates and phosphates. These macromolecules are believed to control nucleation, polymorphism, growth, chemical composition, shape, dimensions, orientation, and texture of the crystals [65]. The presence of the acidic macromolecules usually inhibits the nucleation and growth of calcium salts in aqueous solution, whereas they promote calcification when adsorbed on a substrate [66]. Several synthetic macromolecules and polyelectrolytes are known to influence nucleation and growth of calcium salts, resembling the role that biological macromolecules play *in vivo*. Even after extensive investigations related to biomineralization, many intermediate stages are still unclear. This complex biological process means crystallization of apatite, formed by biological means, onto the surface of either already formed bone or biological macromolecules, being possible this process in presence of hundreds of biological compounds having some influence in the mineralization process [67].

For that purpose, synthetic body fluids (SBF) has been prepared by several authors [8, 67-74] similarly with the chemical analysis of human body fluid, with ion concentrations nearly equal to those of the inorganic constituents of human blood plasma; were firstly used by Kokubo *et al.* [70] to prove the similarity between *in vitro* and *in vivo* behaviour of certain glass-ceramic compositions (See Table 1).

Table 1. Ion concentrations of SBF solution and “human plasma”.

| Ion                            | Human plasma<br>(mM) | Kokubo et al.,<br>1990 [70]<br>(mM) | Bayraktar & Tas,<br>1999 [68]<br>(mM) | Bigi et al., 2000<br>[8]<br>(mM) |
|--------------------------------|----------------------|-------------------------------------|---------------------------------------|----------------------------------|
| Na <sup>+</sup>                | 142.0                | 142.0                               | 142.0                                 | 141.5                            |
| Cl <sup>-</sup>                | 147.8                | 125.0                               | 103.0                                 | 124.5                            |
| HCO <sub>3</sub> <sup>-</sup>  | 4.2                  | 27.0                                | 27.0                                  | 27.0                             |
| K <sup>+</sup>                 | 5.0                  | 5.0                                 | 5.0                                   | 5.0                              |
| Mg <sub>2</sub> <sup>+</sup>   | 1.5                  | 1.5                                 | 1.5                                   | 1.5                              |
| Ca <sub>2</sub> <sup>+</sup>   | 2.5                  | 2.5                                 | 2.5                                   | 2.5                              |
| HPO <sub>4</sub> <sup>2-</sup> | 1.0                  | 1.0                                 | 1.0                                   | 1.0                              |
| SO <sub>4</sub> <sup>2-</sup>  | 0.5                  | 0.5                                 | 0.5                                   | 0.5                              |

SBF is known as a meta-stable buffer [75], however, its preparation must be very careful because a small variance in either the preparation steps or the storage temperatures, has a radical effect on the phase purity and the kinetics of the HA precipitation processes.

Two routes were proposed [8] using di-ammonium hydrogen phosphate ((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>) and calcium nitrate solutions (Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O) as phosphate and calcium ions sources, respectively. The syntheses were carried out at 37°C, to avoid SBF degradation reaching HA powder with Ca/P ratio of 1.67. In addition, Mg- and CO<sub>3</sub><sup>2-</sup> substituted HA was performed obtaining particles of 30-40 nm in size [71] and it was found that pH is a primary variable in the mineralization process [67].

#### 4.4 Hydrothermal Method

There are not many studies on the hydrothermal synthesis of nanoHA. The source of calcium phosphates can be either organic or inorganic. The purity and mechanical property of the products from the organic source are comparatively inferior to those from the inorganic one, i.e. calcium phosphates extracted from bone are likely to retain traces of products are equally biocompatible [76]. In general, the high purity chemical reagents are much more expensive than bone ash, so it is interesting to prepare calcium phosphates compounds with minimal impurities from bone or its related substances. Low temperature (≤ 200°C) hydrothermal treatment is recommended [77] as an attractive inexpensive and environmentally friendly solution process with high purity products.

The organization mechanism of organic surfaces; that can control the nucleation of inorganic materials by geometric, electrostatic, and stereochemical complementary between incipient nuclei and functionalized substrates; has been reviewed by several authors [78].

#### 4.5 Emulsion Technique

Microemulsion is a technique based on the use of two immiscible liquids such as water that has a high dielectric constant being dispersed under agitation in an oil phase, having low dielectric constant, both phases stabilized by an amphiphilic surface-active agent or surfactant in order to control interfacial tension. Microdroplets of water, formed when the aqueous phase is dispersed surrounded by a monolayer of surfactant molecules in the hydrocarbon phase, are stabilized in a non-aqueous phase by a surfactant acting as a microreactor or nanoreactor in which reactions are conducted [39]. These self-assembling media of reverse micelles can be used in the synthesis of nanophase materials without excessive agglomeration, and with high surface area. Hence, nanoHA with spherical morphology can be produced by this method. It has been shown to be one of the few

techniques, able to produce a particle size in the range of nanometers with minimum agglomeration [79].

Nanocrystalline HA particles have been synthesized using a microemulsion technique [39] with Ca and P ions and NP-5 and NP-12 surfactant in cyclohexane organic solutions. It was shown that for HA nanopowders, their surface area, morphology and particle size were strongly affected by composition of the microemulsion system, pH, aging time, temperature and metal ions concentration. The results of nanoHA particles characterisation showed surface areas around 130 m<sup>2</sup>/g and particle sizes in the range of 30-50 nm with needlelike morphology, among others.

Sonoda and co-workers [80] have reported HA synthesis with small size and aspect ratio using pentaethyleneglycol dedecyl ether emulsion similar to a nanoemulsion. Lim et al. [81] have reported the synthesis of HA powders with a specific surface area between 42 and 80 m<sup>2</sup>/g and an average particle size between 0.53 and 1.15  $\mu$ m with agglomerated morphology; and formation of nanocrystalline HA in nonionic surfactant emulsion where the specific surface area varied from 7-75 m<sup>2</sup>/g depending on petroleum ether concentration. Lower average surface area (26 m<sup>2</sup>/g) with high particle size (200 nm), including a strongly agglomerated morphology of 45  $\mu$ m in size, was reported [82].

The reverse microemulsion technique [83] appears as a nanoHA technique where the particle length depends directly of hydrophile-lipophile balance (HLB) value, reaching particles from 21 up to 93 nm but without any uniform shape.

For HA nanofibers, the hydrothermal microemulsion [84], regarding 1D structures, appears as a variation of emulsion technique and led to a substantial increase in the aspect ratio, a fairly satisfying uniformity in diameter (50-120 nm), a well-crystallized product, and a shorter reaction time. The hydrothermal method has been proved to be an effective method to synthesize materials in nano dimensions. It was also was proved that, after the HA nucleation in CTAB reverse micelles, the surfactant head groups preferentially adsorb on surface planes parallel to the c axes of the nuclei, resulting in the formation and growth of anisometric particles. These tiny particles then aggregate in a directional manner though their c axes onto ultrahigh-aspect-ratio HA nanofibers [84].

#### **4.6 Chemical Precipitation**

The most commonly used technique for the formation of HA is the precipitation, which involves wet chemical reactions between the calcium and phosphate precursors under controlled temperature and pH conditions [85-89]. However, this method has some inherent disadvantages such as the difficult control of the pH value over 9 to avoid the formation of a Ca-deficient HA, which during sintering forms tricalcium phosphates [53]. Chemical synthesis of HA powders in neutral and /or slightly acidic aqueous media is known to be a more complicated and difficult task [90-91].

In our own experiments, during titration it has been observed that viscosity decreased as a function of H<sub>3</sub>PO<sub>4</sub> addition, thus, at pH 10.5 the white solution obtained is visibly less viscous in comparison with the starting point at pH 13. These findings are in agreement with reported elsewhere [92], where to a solution of HA different percentages of a polyacrylic acid dispersant (Dispex N40) were added, showing that for pH values between 9.5 and 11 the suspensions show a minimum in viscosity for the three concentrations of dispersant. These results indicated that the best condition for the stabilization of the HA suspensions is achieved for a pH around 10.5 [92].

Recently, as a variation of chemical precipitation, surfactant-based template systems are being

explored to synthesize nanosize materials and they are assumed to be very efficient templates for controlling particle size and shape [79]. Agglomeration can cause lower sintered density as well as crack-line voids during densification. High aspect ratio morphology in the starting powder can also cause poor packing, which results in exaggerated grain growth during sintering [39]. Consequently, to produce sintered monolithic HA parts with desired mechanical properties, one needs to use agglomerate-free low aspect ratio fine HA powders with high surface area.

Nanostructure processing of HA in the presence of yttria-stabilized zirconia by chemical precipitation and pressure-assisted sintering of these powders, has also been reported [93].

## **5. Materials and Methods**

### **5.1 NanoHA synthesis**

Aqueous solution of calcium hydroxide ( $\text{Ca}(\text{OH})_2$ ) and ortho-phosphoric acid ( $\text{H}_3\text{PO}_4$ , 85%), both of analytical grade, were used as starting reagents for the preparation of HA nanoparticles according to the following procedure: firstly, 100 ml of an aqueous suspension of  $\text{H}_3\text{PO}_4$  (0.6M) was very slowly added, drop by drop, to a 100 ml of an aqueous suspension of  $\text{Ca}(\text{OH})_2$  (1M) added with sodium dodecylsulfate (SDS) into the calcium solution while stirring vigorously for about 30 min at room temperature. Concentrated NaOH was added until a final pH of 10.5 was obtained. The white solution obtained was washed using de-ionized water and dried in oven at 80°C for 24 h.

### **5.2 NanoHA characterisation**

The characterisation methods listed below were performed without any further physical or thermal treatment of nanopowder.

#### *5.2.1 Physical Characterisation*

##### **Biodegradation study**

In order to investigate the degradation behavior of nanoHA synthesized was carried out a comparison of biodegradation of three types of nanoHA, one of them was the commercial nanoHA (nH-1) and the other two were prepared by different routes: chemical precipitation (describe above, nHA-2) and hydrothermal process (nHA-3). PBS and SBF were the solutions to which were exposed discs of nanoHA during 1, 3, 7 and 15 days at 120 rpm and 37°C.

##### **Inductively Coupled Plasma**

Three specimens of nanoHA describe above, were dissolved in conc. HCl, afterwards were diluted in a 2% of  $\text{HNO}_3$  solution to obtain concentrations in the range of 10 – 100 ppm for each element (Ca, P). Quantification was performed using an ICP-OES (Inductively Coupled Plasma – Optical Emission Spectrometer, Activa-S, Horiba Jobin Yvon, NJ). Atoms in the plasma emitted light to detect phosphorus and calcium at 213 nm and 317 nm, respectively. The intensity of the energy emitted at the chosen wavelength was proportional to the element concentration in the analyzed sample, and the values obtained were compared with standards in the range of concentration above mentioned.

##### **Scanning electron microscopy analysis – SEM**

The powder morphology was analyzed using digital imaging and scanning electron microscopy (SEM). Using a JEOL JFC-100 fine coat ion sputter device, samples were sputter coated with gold. Then they were studied with a JEOL JSM-6301F microscope at an accelerating voltage of 5 kV.

### **Transmission electron microscopy – TEM**

The size and morphology of the nanoHA powder was determined using a Transmission Electron Microscope (TEM, Zeiss model EM 902A) at an accelerating voltage of 80kv. A nanoHA solution was prepared by dispersing 0.5mg of nanoHA in 1mL of ethanol. This was placed in an ultrasonic bath overnight, to ensure thorough dispersion. A drop of the solution, agitated for 1min in vortex, was placed on a Formvar carbon-coated copper 400 grid and left for 1 min. Excess liquid was removed with filter paper and the grid was allowed to dry for 10 min. TEM images were acquired and observed with Axion Camera Zeiss and AxionCs 40AC Zeiss, v 4.2.0.0 software.

### *5.2.2 Chemical Characterisation*

#### **Infrared spectroscopy**

Chemical characterisation was performed using Fourier transformed infrared (FTIR) spectroscopy. For this purpose, HA was ground in an agate mortar and thoroughly mixed with spectroscopic grade KBr. 1-2 mg of HA powder sample was mixed with 200 mg of KBr powder. Pellets were analyzed using a PerkinElmer System 2000 spectrometer. FTIR was performed to determine the various functional groups in phosphates and carbonate substitution in the synthesized HA powder.

#### **X-ray Photoelectron Spectroscopy - XPS**

XPS analysis was performed using VG Escalab 200R facility equipped with hemispheric detector with 5 channeltrons (energy steps of: 2-200 eV) X rays source with doubleMg/Al anode; pre-chamber with both temperature and atmosphere controlled on gun in pre-chamber and in main chamber (ISS) Electron gun for Auger spectroscopy

#### **X-ray diffractometry – XRD**

X-ray diffraction characterisation of nanoHA powder was conducted using a Panalytical X'Pert PRO alpha-1 with a RTMS X'Celerator detector. It used Ni-filtered Cu Ka radiation over the 2 $\theta$  range of 10-90° at a scan rate of 2.4° /min and with a sampling interval of 0.002° at 40 mA and 45 kV.

### *5.2.3 In vitro performance*

MG-63 cells were cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air, in a-MEM (GIBCO 22561-021), 10% fetal bovine serum (FBS), 1% fungizone and 0.5% gentamicin. For the subculture, the cell monolayer was washed twice with phosphate buffered saline and incubated with trypsin-EDTA solution (0.05% trypsin, 0.25%EDTA) for 5 min at 37°C to detach the cells. Cells were re-suspended in culture medium for re-seeding. There were synthesized porous and uniform microspheres (500nm) based on nanoHA particles that were sterilized at 120°C for 20 min, placed on a 96 PS culture plate (14 mg of microspheres in each well, c.a 1cm<sup>2</sup> of surface area) and incubated with PBS overnight. Cells were then seeded on microspheres pre-conditioned with a-MEM with 10%FBS, 1% fungizone and 0.5% gentamicin. MG-63 cells were cultured (2×10<sup>4</sup> cells/cm<sup>2</sup>) up to 7 days under controlled conditions (cells seeded on 96 TCPS plates) and on the surface of both type of microspheres. The control cultures and seeded microspheres were evaluated throughout the incubation time (7 days) for cytotoxicity (Neutral Red) and observed by confocal microscopy (CSLM) and scanning electron microscopy (SEM). Medium was substituted by fresh one every other day.

### **Cytotoxicity tests (Neutral Red)**

Cytotoxicity of HA-1 and HA-2 microspheres was investigated on MG-63 osteoblast-like cells, which express a number of features characteristic of relatively immature osteoblasts (34) using Neutral Red assay. A 0.4% w/v solution of neutral red dye tested for cell cultures (Color Index 50040) in distilled water was prepared and stored at 4°C (stock solution). The working solution was prepared immediately before use by diluting the stock solution in complete medium to yield a final concentration of 50 µg/mL. Five replicates were performed for each type of microspheres. The negative control was microspheres in culture medium in PS 96 wells culture plates. The positive control was MG-63 in culture medium in 96 wells TCPS culture plates. After incubation for 1, 3, 5, and 7 days at 37°C, the supernatant was discarded, 0.150 mL of neutral red dye solution were added to the wells for 3 h at 37°C. The supernatant was removed, the wells washed twice with PBS and 0.1 mL of lysing solution (50% ethanol in 1% acetic acid) was added. The color intensity of each well was read at 540 nm with reference to 620nm, the optical densities of the replicates were averaged and the viability read in negative control was discounted.

### **Cell morphology (SEM and CLSM)**

For SEM, microspheres-cells constructs were washed twice with PBS and fixed with 1.5% glutaraldehyde in 0.14% cacodylate solution for 30min at room temperature. They were dehydrated by suspending in successive ethanol solutions (50%, 70%, 80%, 90%, and 100%) for 10min in each solution. After dehydration, the samples were transferred to hexamethyldisilazan and air-dried at room temperature overnight. Finally, the microspheres-cells constructs were sputter coated with gold and examined under a JEOL JSM-6301F microscope at accelerating voltage of 5kV.

For CSLM, samples were washed twice with PBS, fixed in 4% v/v formaldehyde (methanol-free; Polyscience) for 15 min, permeabilized with 0.1% v/v Triton X-100 for 5 min, and incubated in 10 mg/mL bovine serum albumin and 100 µg/mL RNase for 1h at room temperature. F-actin filaments were stained with Alexafluor-conjugated phalloidin (Molecular Probes) for 20 min at RT in dark, and nuclei were counterstained with 10 µg/mL propidium iodide (Sigma) for 15 min. Finally, samples were washed with PBS, mounted in Vectashield® and stored at 4°C in dark. CLSM images were acquired on a BioRad MRC 600 microscope.

## **6. Results**

### **Biodegradation**

The disintegration of nanoHA discs in both solutions (PBS and SBF), in crescent order after 15 days was nHA-3> nHA-2> nHA-1. All nanoHA samples increased their weight during the experience, that fact was evidenced in SEM images (data not showed) where it was observed deposition of crystals (NaCl) and new nanoHA formation. This fact suggests that synthesized nanoHA were more biodegradable than commercial sample, obtaining an interest feature for a biomaterial used in bone tissue engineering.

### **ICP**

ICP accounts deserve special attention the value found for the loss of mass (20.7% ) which indicates that there is a presence of volatiles substances, which may be OH, H<sub>2</sub>O or CO<sub>2</sub>. As the hypothesis of CO<sub>2</sub> will be unlikely, thus, this value should correspond to OH and H<sub>2</sub>O. However, as the stoichiometric amount of OH in apatites rarely exceeds 5%, it is suggested that the sample contains an excess of H<sub>2</sub>O, probably encapsulated in its structure. The value of Cl is found compatible with a chlorine-apatite. The values of CaO and P<sub>2</sub>O<sub>5</sub> are relatively low for a natural apatite. However, if the analysis is recalculated, disregarding the value of water, following values are obtained CaO = 55% and P<sub>2</sub>O<sub>5</sub> = 42%, values perfectly compatible with a stoichiometric apatite.

## SEM

Morphological results from SEM investigation are shown in Figure 1, where it is observed that the morphology of the HA nanocrystals presents a uniform needle-like shape.

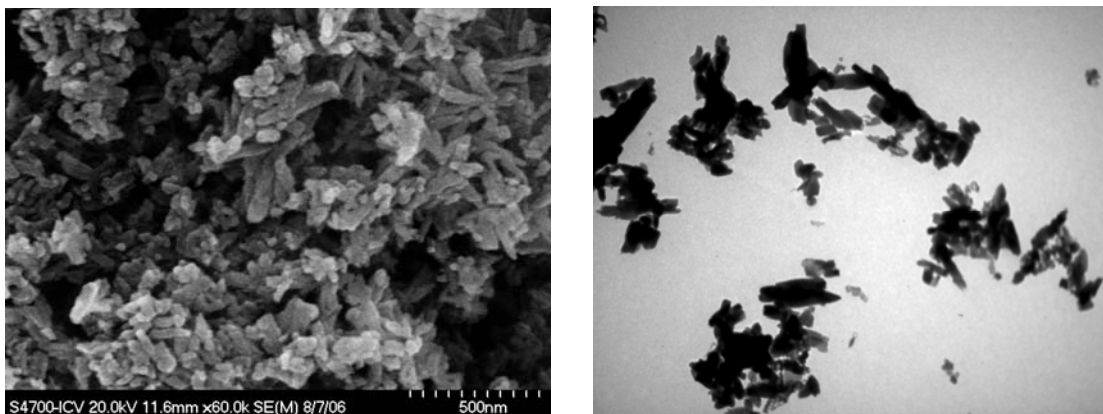


Figure 1. SEM (left) and TEM (right) micrographs. NanoHA particles have been studied using these methods to characterize morphologically these nanocrystals that are presented in agglomerates of few microns. Nanometric particles of HA exhibit in both micrographs bar and needle-like shapes with 80x20nm dimensions in mean. (SEM, scale bar=500nm; TEM, 12000x)

## TEM

Morphology of nanoHA particles using Transmission Electron Microscopy is observed in the Figure 1. Dispersed crystals of nanoHA, due probably to the surfactant use with bar and needle-like shapes, are observed in a high magnification.

## FTIR

FTIR results (data not showed) suggested the presence of bands characteristic of  $\text{PO}_4^{2-}$  at 471 ( $\nu_2$ ), 563 ( $\nu_4$ ), 961 ( $\nu_1$ ) and 1000  $\text{cm}^{-1}$  ( $\nu_3$ ) [94];  $\text{OH}^-$  vibrational due to water at 602  $\text{cm}^{-1}$  and occluded water at 1638 and 3431  $\text{cm}^{-1}$ . It is important to point out that bands at 1422  $\text{cm}^{-1}$  ( $\nu_3$ , in-plane stretching band) and 872  $\text{cm}^{-1}$  (out-of-plane stretching band) suggesting the presence of a carbonated nanoHA, which may have practical applications. It is well known that the presence of carbonate in calcium phosphate materials is an important factor contributing to their reactivity and to the *in vivo* integration of the implant [95]. The bands at 1545, 1450 and 880  $\text{cm}^{-1}$  were thought to be due to a small fraction of the total  $\text{CO}_3^{2-}$  ions replacing  $\text{OH}^-$  ions in the lattice [1].

## XPS

Results from XPS are presented in Table 1 and Figure 2. It is noteworthy that the atomic ratio of material P/Ca or as well more current expressed Ca/P = 1,71 near to the natural apatite with a calcium / phosphorous ratio of 1,67. Besides, Table 2 presents P2p and Ca 2p 3/2 values similar to natural apatite, as well.

Table 2. Binding energies (eV) of core electrons and surface atomic ratios of apatite

| Sample  | P 2p  | Ca 2p <sub>3/2</sub> | O 1s  | C 1s  | P/Ca<br>atomic | $\text{CO}_3^{2-}$ /Ca<br>atomic |
|---------|-------|----------------------|-------|-------|----------------|----------------------------------|
| Apatite | 133.2 | 347.2                | 531.0 | 289.7 | 0.585          | 0.106                            |

## XRD

A typical XRD profile of HA ceramic synthesized by calcium hydroxide and ortho-phosphoric acid to ceramic conversion has been shown in Figure 2. XRD analysis of the nanoHA powder indicated the presence of monophase non-crystalline compound.

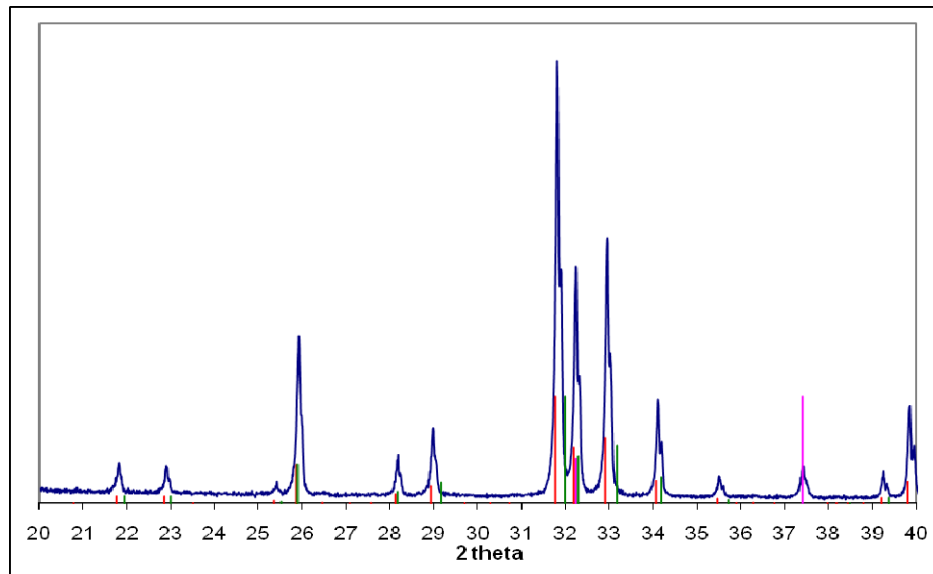


Figure 2. XRD spectra of nanoHA en powder. It is indicated that results (blue line) fit the most of peaks (vertical lines) that exhibits natural hydroxyapatite.

## Biological Assesment

In the present work, the cytotoxicity tests of nanoHA it was assessed by the synthesis of microspheres based on nanoHA particles [96]. Biological evaluation was performed using human MG-3 osteoblast-like cells line. Cells seeded on TCPS, used as a control, showed the same trend, but with a higher growth rate than microspheres. The control presented less number of viable cells in the first day probably due to the less available surface area compared to the microspheres. The nanoHA microspheres presented a significant number of viable until the end of the culture, and high growth rate as well.

SEM images of MG-63 osteoblast cells cultured for 7 days on the surface of nanoHA microspheres based on nanosized hydroxyapatite are presented in Figure 3. The surface of nanoHA microspheres is almost completely covered by layers of cuboidal cells which became denser along the entire time of culture, forming confluent monolayers. CLSM images in Figure 3 show nanoHA microspheres uniformly colonized by osteoblasts cells after 5 days of culture.

### *Statistical analysis*

Data are presented as mean  $\pm$  standard deviation ( $n=5$ ) and were analyzed using the Student t-test for independent samples. Differences between groups were considered statistically different when  $p < 0.05$ .

## 7. Discussion

In general, the nanoHA synthesis using a number of methods; including mechano-chemical, sol-gel, biomineralization, chemical precipitation, emulsion techniques, batch hydrothermal processes,

being all of these the most used methods to processing nanoHA; having several disadvantages such as requiring a very precise control over reaction conditions, expensive starting materials, large amounts of toxic organic solvents, or time consumption [97]. However, chemical precipitation is the most outstanding process due to its simplicity and good results without many sophisticated instruments, equipments or reagents.

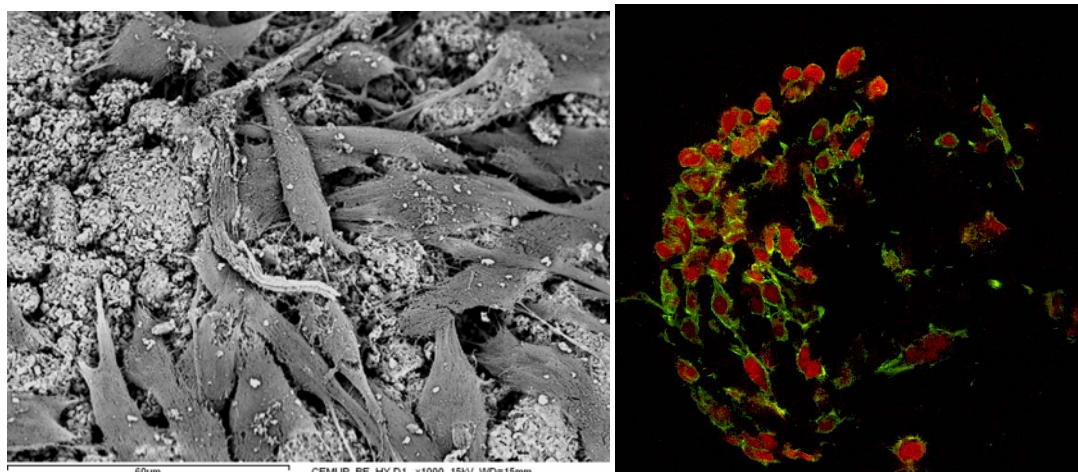


Figure 3. SEM (left) and Confocal (right) micrographs of seeded microspheres with MG-63 osteoblast cells after 7 days of culture. In the left, osteoblasts proliferation was observed on nanoHA microspheres means scanning electron spectroscopy using backscattered electron imaging. In the right, a confocal laser scanning microscopy image shows aligned osteoblasts proliferating on microsphere surface where nuclei is stained in red and cytoplasm stained in green.

NanoHA prepared by chemical precipitation was characterized using different method verified results about morphology, size, chemical composition and biological behavior. The use of different methods provides to researcher support for initial results and a better understanding about material from different points of views.

Synthesized nanoHA can be useful as nanocrystals, aggregates, powder, or as well conforming discs and microspheres, been a versatile material in bone engineering scaffolding with many action fields. So far as we know, any review or overview has revised extensively many preparations processes, characterization methods enriched with own data that exemplify the knowledge, as presented in this work.

## 8. Conclusions

Therefore it can be concluded that new nanoHA based materials are certainly among the most promising challenges in bioactive ceramics for the near future, and consequently, the research effort put in their development will continue to increase.

Being bone engineering one of the action fields where nanoHA play an important role, others such as pharmaceutical, cosmetics, catalysis and instrumentation, are industrial sectors benefited from the new technology applied in the nanoHA processing.

Therefore, it can be conclude that among bioactive ceramics, the hydroxyapatite and its processing into particles smaller than one micron, have poured on this well-known ceramic, interesting new

abilities in several areas. Specifically in biomedical engineering are brought new opportunities in scaffolding, drug delivery system and even DNA transportation.

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# ANEX I



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(12) **Patent Application Publication**  
**Brito Lopes et al.**

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(43) **Pub. Date: Oct. 22, 2009**

(54) **PRODUCTION METHOD FOR CALCIUM  
PHOSPHATE NANO-PARTICLES WITH HIGH  
PURITY AND THEIR USE**

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514/769

## ABSTRACT

The present invention provides a continuous process for producing calcium phosphate nanoparticles in a network mixer or static mixer reactor, fed by a calcium solution, a phosphorous solution and an alkaline solution and, optionally, one solvent or dispersing agent. The proposed process enables the micromixing control, which is essential to form nanometric structures, but it is also a determining factor in the crystals purity, crystallinity and morphology. The reactants distribution scheme at the inlet of the reactor and along the reactor, performed continuously or varying in time, is also a crucial factor to programme the pH of the reactant media along the reactor. The calcium phosphate nanoparticles suspension that exits the reactor can be submitted to further aging, ultra-sounds, separation, drying, sintering and milling processes. Some calcium phosphates are considered biomaterials, used as: food additives and nutritional supplements; bone graft for bone replacement, growth and repair; biocements and coating of metallic implant. Some of the most recent applications include their use in cosmetics, toothpaste and in esthetical treatments for diminishing wrinkles by stimulating conjunctive tissue formation.

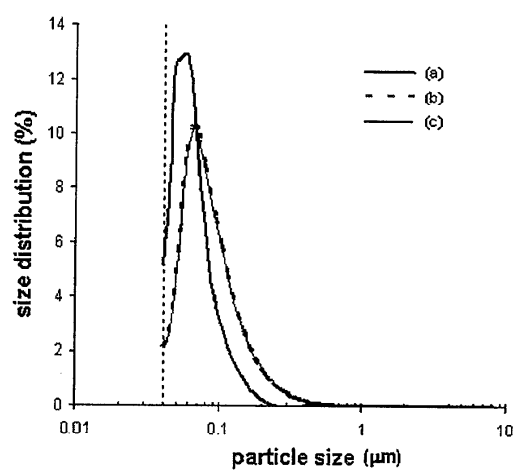


FIG. 1

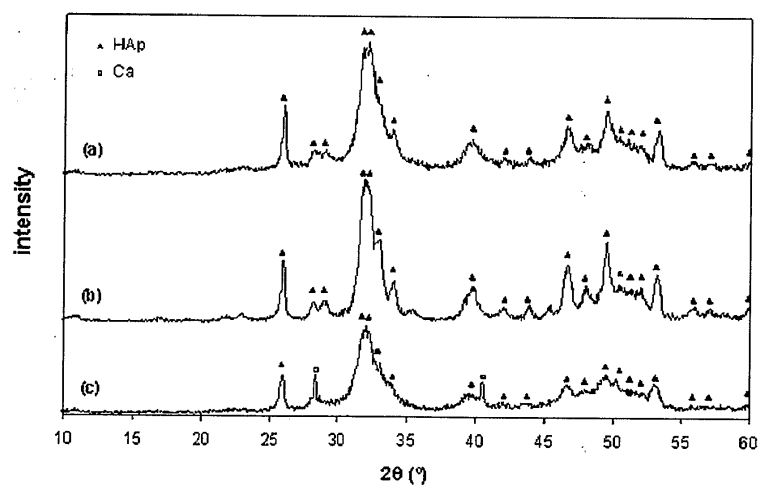


FIG. 2

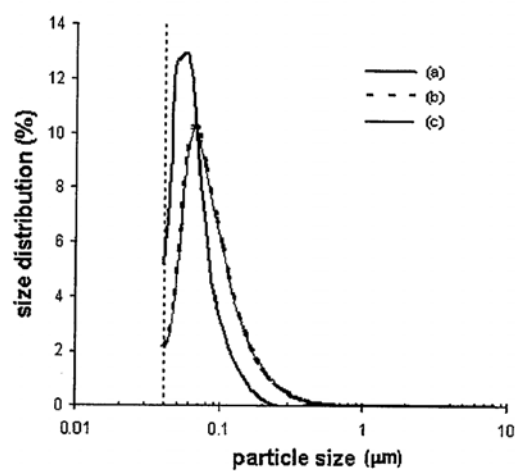


FIG. 1

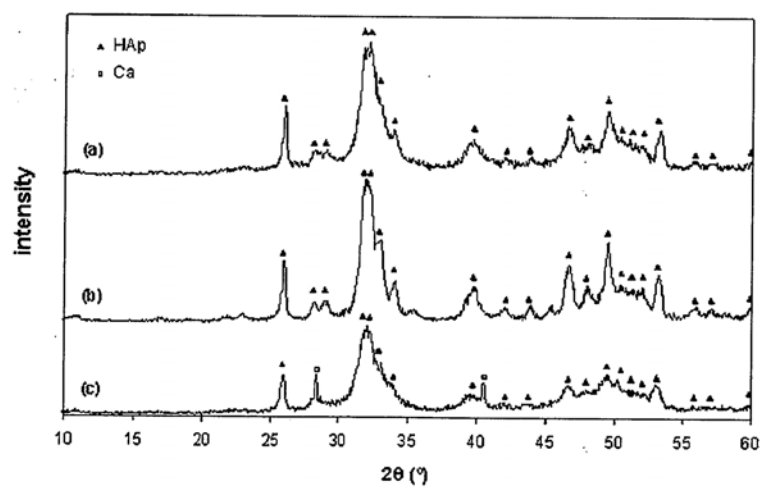


FIG. 2



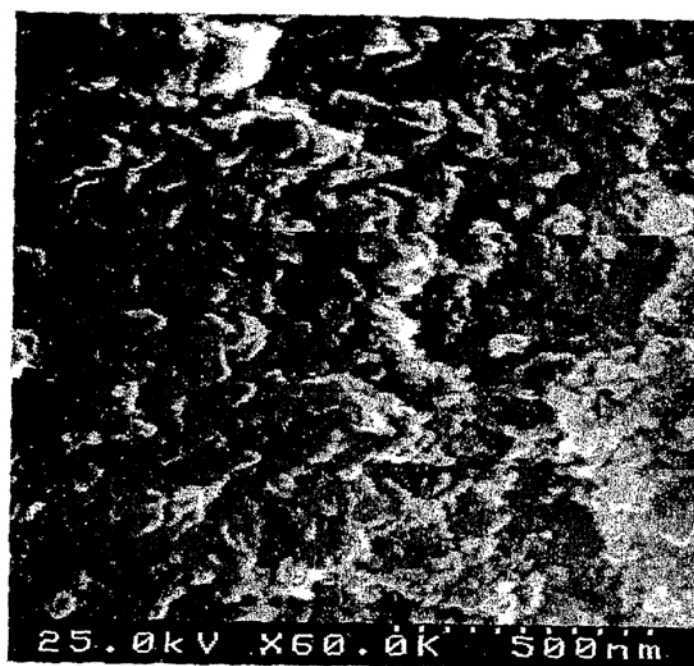


FIG. 5

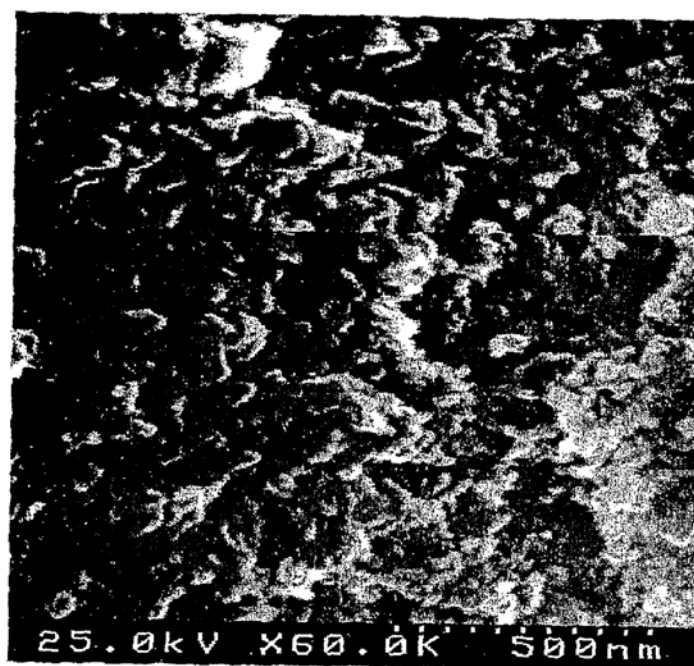


FIG. 5

# **PRODUCTION METHOD FOR CALCIUM PHOSPHATE NANO-PARTICLES WITH HIGH PURITY AND THEIR USE**

## **FIELD OF THE INVENTION**

**[0001]** The present invention provides a continuous process for producing calcium phosphate nanoparticles in a network mixer or static mixer reactor, fed by a calcium solution, a phosphorous solution and an alkaline solution and, optionally, one solvent or surfactant agent, being therefore related with the chemical industry synthesis domain.

## **SUMMARY OF THE INVENTION**

**[0002]** The present invention provides a continuous process for producing calcium phosphate nanoparticles in a network mixer or static mixer reactor, fed by a calcium solution, a phosphorous solution and an alkaline solution and, optionally, one solvent or surfactant agent.

**[0003]** The proposed process enables the micromixing control, which is essential to form nanometric structures, but it is also a determining factor in the crystals purity, crystallinity and morphology. The reactants distribution scheme at the inlet of the reactor and along the reactor, performed continuously or varying in time, is also a crucial factor to programme the pH of the reactant media along the reactor. The calcium phosphate nanoparticles suspension that exits the reactor can be submitted to further aging, ultra-sounds, separation, drying, sintering and milling processes.

## **BACKGROUND OF THE INVENTION**

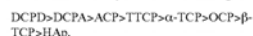
**[0004]** Calcium Phosphates (CP's) are inorganic compounds constituted by  $\text{Ca}^{2+}$  and  $\text{PO}_4^{3-}$  ions at different stoichiometric amounts, see Table 1; furthermore the substitution of some ions of the crystallographic structure by ions such as  $\text{F}^-$ ,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$  and  $\text{CO}_3^{2-}$  can provide different properties to the substituted calcium phosphates. Some calcium phosphates are considered as biomaterials and, therefore, are used in several food, biomedical and pharmaceutical applications; and more recently are also used in the cosmetic industry.

TABLE 1

| Calcium Phosphates: name, short-name and chemical formula |               |                                                                   |
|-----------------------------------------------------------|---------------|-------------------------------------------------------------------|
| Name                                                      | Short-name    | Chemical Formula                                                  |
| Monocalcium Phosphate Monohydrate                         | MCPM          | $\text{Ca}(\text{H}_2\text{PO}_4)_2 \times \text{H}_2\text{O}$    |
| Dicalcium Phosphate Dihydrate                             | DCPD          | $\text{CaHPO}_4 \times 2\text{H}_2\text{O}$                       |
| Dicalcium Phosphate Anhydrous                             | DCPA          | $\text{CaHPO}_4$                                                  |
| Octacalcium Phosphate                                     | OCP           | $\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \times 5\text{H}_2\text{O}$ |
| Amorphous Calcium Phosphate                               | ACP           | $\text{Ca}_3(\text{PO}_4)_2$                                      |
| $\alpha$ -Tricalcium Phosphate                            | $\alpha$ -TCP | $\alpha\text{-Ca}_3(\text{PO}_4)_2$                               |
| $\beta$ -Tricalcium Phosphate                             | $\beta$ -TCP  | $\beta\text{-Ca}_3(\text{PO}_4)_2$                                |
| Hydroxyapatite                                            | HAp           | $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$                      |
| Tetracalcium Phosphate                                    | TTCP          | $\text{Ca}_4(\text{PO}_4)_2\text{O}$                              |

**[0005]** The most important biocompatible calcium phosphates are the Hydroxyapatite (HAp) that is considered a bioactive ceramic, and the  $\beta$ -Tricalcium Phosphate ( $\beta$ -TCP) that is a bioresorbable ceramic. HAp is the main mineral component of the human bone, and its bioactivity makes HAp

thermodynamically stable in physiologic environments, promoting at the surface of the bone implant a strong biological and chemical reaction with the surrounding tissue.  $\beta$ -TCP, like most of all CP's (excluding HAp) is considered as a bioresorbable material due to its dissolution when exposed to physiological environments making possible the natural bone replacement. The reabsorbing rate is directly proportional to the CP solubility, which is also affected by the pH. Generally, CP's can be ordered by their decreasing solubility as follows:



**[0006]** In state-of-the-art, it can be found several methods for CP's production, mainly CP's can be prepared wet chemical reactions and by solid-state reactions. The wet chemical process have different routs as chemical precipitation, hydrothermal processing and hydrolyses of others CP's. Chemical precipitation is an advantageous process due to its simplicity and low implementation cost. It is a very versatile method that allows the control of product properties such as morphology, size and reactivity, and, therefore, it is a widely used method for the production of nano-particles. Chemical precipitation is normally implemented in stirred reactors, followed by filtering, washing and drying processes (Conn and Jessen, 'Process for Producing Hydroxyapatite' U.S. Pat. No. 4,32,477, 1982), (RUDIN et al., 'Method for Producing Nano-sized Crystalline Hydroxyapatite' WO0202461, 2002), (Ying, Ahn and Nakahira, 'Nanocrystalline apatites and composites, prostheses incorporating them, and method for their production' US6013591, 2000), (Ahn, 'Tricalcium phosphates, their composites, implants incorporating them, and method for their production' US2005031704, 2005). Additionally, the processes can include other stages such as micro-wave radiation treatments and/or aging (Murugan e Seeram, 'Production of Nano-Sized Hydroxyapatite Particles' US2005226939, 2005), addition of a solvent (sol-gel method) (Ren and Zhou, 'Method for synthesizing nano Hydroxyapatite micro powder containing carbonate radical' CN1587195, 2005), and wet chemically seeding stage immediately followed by drying with spray atomization (Luo, 'Methods of Synthesizing Hydroxyapatite Powders and Bulk Materials' US5858318, 1999).

**[0007]** However, the implementation of the wet chemical process in stirred tanks has disadvantages related to the calcium phosphate stoichiometry and the process reproducibility in terms of the some properties of the CP's. The stoichiometry, defined as the molar ratio between  $\text{Ca}^{2+}$  and  $\text{PO}_4^{3-}$  ions (Ca/P ratio), is a crucial parameter to produce the desired calcium phosphate, see Table 2. For the case of HAp production, the Ca/P ratio is even determining, since for values lower than 1.67, it can be formed a deficient Hydroxyapatite (DHAp), represented by the chemical formula  $\text{Ca}_{10-x}(\text{HPO}_4)_x(\text{PO}_4)_{6-x}(\text{OH})_{2-x}$ . The reproducibility is an issue for industrial-scale batch production, since different batches have commonly different properties, namely purity, crystal size and morphology, crystallinity degree, and particle size distribution.

**[0008]** The mentioned disadvantages are intrinsic to the stirred reactor technique, which through intensive-energy mixing provides a good macromixing quality due to the recirculation patterns inside the reactor. However, in many real applications, the recirculation patterns lead to the formation of stagnation zones and short-cuts that are hardly overcome. Nonetheless, assuming that these problems can be avoided, stirred reactors cannot ensure a good micromixing quality,

# **PRODUCTION METHOD FOR CALCIUM PHOSPHATE NANO-PARTICLES WITH HIGH PURITY AND THEIR USE**

## **FIELD OF THE INVENTION**

**[0001]** The present invention provides a continuous process for producing calcium phosphate nanoparticles in a network mixer or static mixer reactor, fed by a calcium solution, a phosphorous solution and an alkaline solution and, optionally, one solvent or surfactant agent, being therefore related with the chemical industry synthesis domain.

## **SUMMARY OF THE INVENTION**

**[0002]** The present invention provides a continuous process for producing calcium phosphate nanoparticles in a network mixer or static mixer reactor, fed by a calcium solution, a phosphorous solution and an alkaline solution and, optionally, one solvent or surfactant agent.

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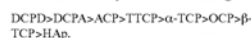
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**[0008]** The mentioned disadvantages are intrinsic to the stirred reactor technique, which through intensive-energy mixing provides a good macromixing quality due to the recirculation patterns inside the reactor. However, in many real applications, the recirculation patterns lead to the formation of stagnation zones and short-cuts that are hardly overcome. Nonetheless, assuming that these problems can be avoided, stirred reactors cannot ensure a good micromixing quality,

which is an essential factor for the CP's purity, crystal size and morphology, crystallinity degree, and particle size distribution. Micromixing defines the mixing phenomena at the molecular scale, which is the scale where the chemical reactions occur, and it is determining for the selectivity of the overall process and for the final product properties. Therefore, for the production of nanomaterials, the process should be controlled at that molecular scale, by ensuring a good micromixing quality.

TABLE 2

| Influence of Ca/P rates and Calcium Phosphates formation conditions and stability |      |                                                                                                                                                              |
|-----------------------------------------------------------------------------------|------|--------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Calcium Phosphate                                                                 | Ca/P | Stability and formation conditions                                                                                                                           |
| Formed at room temperature                                                        |      |                                                                                                                                                              |
| MCPM                                                                              | 0.5  | Formed at pH < 2 (approximately)                                                                                                                             |
| DCPD                                                                              | 1.0  | Formed at 2 < pH < 4, nucleation and fast growth up to pH = 6.5                                                                                              |
| DCPA                                                                              | 1.0  | Formed at higher temperatures than DCPD but slightly stable                                                                                                  |
| OCP                                                                               | 1.33 | Nucleation and fast growth for 6.5 < pH < 8, more stable than DCPD or ACP at the same range                                                                  |
| ACP                                                                               | 1.5  | Formed in an early stage of the reaction when precipitation occurs at high concentrations and for 4 < pH < 8, but is spontaneously converted in DCPD and OCP |
| HAp                                                                               | 1.67 | Precipitation occurs at pH > 8, and is the calcium phosphate most stable                                                                                     |
| Formed at high temperature                                                        |      |                                                                                                                                                              |
| $\alpha$ -TCP                                                                     | 1.5  | Formed after heating above 1180° C, followed by fast cooling. Less stable than DCPD or OCP                                                                   |
| $\beta$ -TCP                                                                      | 1.5  | Formed after heating up to 1189° C. More stable than DCPD or OCP at 6 < pH < 8                                                                               |
| TTCP                                                                              | 2.0  | Formed after heating above 1500° C. Unstable in acid solutions                                                                                               |

## DESCRIPTION OF THE INVENTION

[0009] The present invention provides a method for wet chemical production of calcium phosphate nanoparticles production by controlling the micromixing quality. Mixing is a crucial parameter of industrial processes and micromixing quality for precipitation processes is critical to define the crystals size and shape, as well as the particles size distribution. Ineffective mixing can lead to non-reproducible processes and low quality products, and therefore, it is often necessary to implement complex purification processes downstream the reactor, increasing the cost of the final product.

[0010] Motionless or static mixers have become standard equipment in the process industries since the 1970s, and are employed in a wide range of industries since they couple a better mixing efficiency with lower energy consumption. Static mixers are used inline in an once-through process or in a recycle loop where they supplement or even replace a conventional stirrer. In many continuous processes, static mixers are undeniably an attractive alternative to conventional agitation. Static mixers eliminate the need for mechanical stirrers and therefore have a number of benefits: small space requirement; low equipment cost; no power requirement except pumping; the flow of materials through them may be induced by gravity, pressure difference or by using the exist-

ing potential or kinetic energy; easy and quick installation; low set-up and operating costs; self-cleaning; reduced maintenance requirements.

[0011] The standard design of a static mixer is a series of identical, flow-modifying motionless inserts, called elements, built into a tubular housing. These motionless inserts use the pressure difference or the kinetic and potential energy of the processed materials, causing splitting, shifting, shearing, rotating, accelerating, decelerating and recombining of materials.

[0012] There are more than two hundred commercial static mixer models currently available. Commercial static mixers are available from a number of manufacturers and in different types, shapes and geometries. Commercial static mixers can be divided into five main families: open designs with helices (e.g. Kenics® static mixer); open design with plates (e.g. LPD®, Komax®, Inliner® and HEV® static mixers); corrugated plates (e.g. SMV® static mixer); multilayer designs (e.g. SMX®, SMXL® and SMRX® static mixers); and closed designs with channels (e.g. ISG® static mixer).

[0013] Alternatively, network mixers (Lopes, Laranjeira, Dias e Martins, 'Network Mixer and Related Mixing Process' WO2005077508, 2005), differ from static mixers as they are not composed by elements that are inserted inside a tubular housing, but promote mixing without mechanical stirring due to its interior geometry constituted by a series of interconnected channels and chambers. The unique available commercial version is the NETmix® technology (registered Portuguese brand), which enables the fluid micromixing control in an optimized and reproducible way, essential factor for the proper control of complex chemical reactions, like nanoparticles production. The NETmix® reactor consists in a network of interconnected chambers and channels creating zones of complete mixing and of complete segregation, which are carefully designed in order to program the mixing intensity and quality, either locally as well along the reactor. This is one of the main advantages present in the NETmix® reactor when compared with other static mixers where the mixing is difficult to control.

[0014] The present invention enables the micromixing quality control and ensures the process reproducibility for calcium phosphates nanoparticles produced by wet chemical precipitation implemented in a network mixer or static mixer reactor, fed by a calcium solution, a phosphorous solution, and an alkaline solution; and, optionally, a solvent or a surfactant agent. This process controls the mixing at the molecular level, ensuring a good micromixing quality, essential for the production of particles with nanometric structure and high purity, with controlled crystallinity, particle size and crystal size and morphology. This invention also allows programming the reactants injection scheme with a given distribution at the inlet and along the reactor, performed continuously or varying in time, which enables to programme the pH of the reactant media along the reactor in order to produce the CP's with the desired specifications.

## BRIEF FIGURE DESCRIPTION

[0015] FIG. 1: Particle size distribution curves of three hydroxyapatite samples produced in the NETmix® reactor.

[0016] FIG. 2: X-ray diffraction spectra of three hydroxyapatite samples produced in the NETmix® reactor.

[0017] FIG. 3: Scanning Electron Microscopy image of the example 1 hydroxyapatite sample.

[0018] FIG. 4: Scanning Electron Microscopy image of the example 2 hydroxyapatite sample.

[0019] FIG. 5: Scanning Electron Microscopy image of the example 3 hydroxyapatite sample.

#### DETAILED DESCRIPTION OF THE INVENTION

[0020] The most widely method used in CP's production is the wet chemical precipitation reaction, at temperatures below 100° C. The rigorous control of the micromixing is crucial for the production of CP's by chemical precipitation in order to get high purity and control the crystal size and morphology, particle size distribution and crystallinity. The processes conventionally can not control the micromixing quality and intensity, and, therefore, a rigorous process control of pH, temperature, stirring and reactants feed flow rate is vital to ensure the product purity. Some of the strategies normally applied to improve chemical homogeneity and stoichiometry of resulting CP's are to promote a slow precipitation and to use very diluted reactants solutions.

[0021] Because of the above mentioned reasons, the network mixer or static mixer reactors are promising technologies for the continuous production of CP's nanoparticles, or other substituted CP's nanoparticles by including in their crystalline structures other ions, such as F<sup>-</sup>, Na<sup>+</sup>, K<sup>+</sup>, Mg<sup>2+</sup> and CO<sub>3</sub><sup>2-</sup>. These reactors operate at low temperatures, making possible the production of MCPM, DCPD, DCPA, OCP, ACP and HAp. The production of  $\alpha$ -TCP,  $\beta$ -TCP and TTCP requires further thermal processing at high temperatures, as mentioned at Table 2.

[0022] The present invention provides a process that enables the micromixing quality control for the CP's wet chemically synthesis, and allows programming the reactants distribution scheme at the inlet and along the reactor, performed continuously or varying in time, which enables to programme the pH of the reactant media along the reactor in order to produce the CP's with the desired specifications. The reactor also has a thermostatzation system which enables an easy temperature control, according to the formulation specification.

[0023] The reactor used can be a static mixer or a network mixer with a feed system that allows different configurations of reactants injection schemes achieved by means of valves and flow distributors.

[0024] The available reactants are:

- [0025] 1. one source of Ca<sup>2+</sup> ions;
- [0026] 2. one source of PO<sub>4</sub><sup>3-</sup> ions;
- [0027] 3. one alkaline agent;
- [0028] 4. one solvent or surfactant agent;
- [0029] 5. water to adjust the concentrations of each of the above aqueous solutions.

[0030] Solutions with different concentrations and compositions are prepared by mixing different amounts of the reactants mentioned from 1 to 5. These solutions are specified accordingly to the injection scheme chosen to feed the reactor. The distribution of the reactants can be performed at the reactor inlet or along the reactor, continuously or time variably. The synthesized product can be collected as an aqueous suspension at the reactor's outlet or at any position along the reactor. The obtained suspension can be submitted to further separation processes (decantation, centrifugation, filtration or similar) to increase the solids content in the suspension, which is then washed in order to eliminate all the ions of the remaining solution, and finally can be submitted to a drying

process. After drying, the powder product can be milled and/or thermally treated (calcination, sintering).

[0031] Therefore, the production of calcium phosphates nanoparticles requires the understanding and the control of the micromixing quality and the appropriated reactants injection scheme with distribution at the reactor's inlet or along the reactor, continuously or time variably. This can be performed by feeding to a network mixer or static mixer reactor several streams containing Ca<sup>2+</sup> and PO<sub>4</sub><sup>3-</sup> ions, and an alkaline reactant, with possibility of adding one or more solvents and/or one or more surfactant or tensioactive agents.

[0032] Therefore, it is necessary to provide to the reactor the following streams:

- [0033] a) one Ca<sup>2+</sup> ions source;
- [0034] b) one PO<sub>4</sub><sup>3-</sup> ions source;
- [0035] c) one alkaline source to adjust the reaction pH;
- [0036] d) solvents and/or surfactant or tensioactive agents;
- [0037] e) water to adjust the concentrations of each of the above aqueous solutions;
- [0038] f) prepare all necessary solutions to feed the reactor with different concentration and compositions, obtained by combining in different proportions the reactants mentioned previously: a), b), c), d) and e);
- [0039] g) use a network mixer or static mixer reactor that ensures an efficient and homogeneous mixing, equipped with feed distributors at the reactor's inlet and/or along the reactor to allow different reactants injection schemes;
- [0040] h) the reactant solutions mentioned in f) can be fed at the so said reactor, through a distribution scheme at the inlet or along the reactor, in a continuous or time varying mode;
- [0041] i) thermostatzation of the reactants and/or the reactor in order to ensure a proper temperature for the reaction, where the rigorous choice of operating conditions for the steps defined at f), g), h) and
- [0042] i) enables the nanoparticles or microparticles production with nanometric crystalline or amorphous structures.

[0043] The Ca/P molar ratio should be comprised between the values of 0.5 ≤ Ca/P ≤ 2.0, so that calcium phosphates can be obtained in anhydrous or hydrated forms.

[0044] The said calcium phosphates can have some stoichiometric variations resulting from substitution of some ions of the crystallographic structure by other ions, as for example F<sup>-</sup>, Mg<sup>2+</sup>, Na<sup>+</sup>, CO<sub>3</sub><sup>2-</sup> and K<sup>+</sup>.

[0045] The said calcium phosphates exhibit nanometric structure (crystal size in the nanometric range) and can be provided as nanoparticles or microparticles.

[0046] The said calcium phosphates can have a controlled crystallinity degree, which can vary from amorphous to crystalline structure.

[0047] The said calcium phosphates can have a controlled morphology, which can vary from spherical to needle-like geometry.

[0048] The calcium phosphates suspension produced in the so said reactor can be further processed to concentration, separation, drying, thermal treatment and/or milling stages to obtain final products in the form of suspensions, slurries or dried powders, with a concentration range varying from 0.1% to 100% of any specific calcium phosphate or a mixture of different calcium phosphates.

**[0049]** The present invention provides a methodology to produce calcium phosphates nanoparticles with high purity, to be applied in several industrial fields, namely in food industry, as food additives and nutritional supplements, in biomaterials as bone graft for bone replacement, growth and repair, bioceramics and metallic prosthesis coatings. It can also be used as catalysts for water treatment and as absorbents in chromatographic columns. Recent applications include drug delivery, cosmetics, tooth paste and in esthetical treatments to diminishing wrinkles by stimulating conjunctive tissue formation.

#### Nanoparticles Characterization

**[0050]** X-Ray Diffractometer (Philips X'pert mod. MPD, Nederland) was used to determine the crystalline phases presented in the nanoparticles produced in the reactor with micromixing quality control. The diffractograms (FIG. 2) were obtained with Cu K $\alpha$  ( $\lambda=1.54056$  nm) produced at 40 kV and 50 mA and diffraction angles between  $3^\circ < 2\theta < 60^\circ$  with a step size of  $0.05^\circ$  2 $\theta$ /s. The crystallite size was estimated by the Scherrer formula

**[0051]** Laser Diffraction Particle Size Analyzers (Beckman Coulter LS 230, equipped with Polarization Intensity Differential Scattering, USA, Fraunhofer optical model, with shape factor=1.0, i.e., the height/diameter particle ratio is 1:1) was used to determine the particle size distribution curves (FIG. 1) and average particles diameters. The lower detection limit for particles size is of 40 nm.

**[0052]** BET Surface Area Analyser (Micromeritics Gemini II-2370, with sample degasification temperature of  $200^\circ\text{C}$ . in 12 h, 5 point analysis and equilibrium time of 50 s) was used to determine specific surface areas using BET method.

**[0053]** Scanning Electron Microscope (Hitachi S-4100, Japan, V<sub>acc</sub>=25 kV) was used to characterize the particles morphology. Samples were prepared by fixing the powder in double-side adhesive conductive carbon tape, and then coating it with carbon (FIGS. 3 to 5).

#### APPLICATION EXAMPLES

##### Example 1

Needle-Like Shaped Hydroxyapatite Nanoparticles Production.

**[0054]** Production of hydroxyapatite nanoparticles at  $25^\circ\text{C}$ . was performed in the commercial NETmix® reactor with 15 inlet ports to feed the reactants solutions and 15 outlets for product recovery. Preparation of 0.5M Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O solution, 0.3M NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> solution and 14 solutions of NUOH with different pH values (between 8 and 14). The calcium and phosphorous solutions were fed in one single reactor point (inlet 1). The ammonia solutions were fed on the inlets 2 to 15, with increasing pH order from port 2 to port 15. The hydroxyapatite suspension produced was analysed to determine the particle size distribution curve (FIG. 1.a), where the average particles diameter was  $d_p=63$  nm. Due to equipment limitations, it was not possible to obtain size distribution ranges lower than 40 nm. Therefore, the real average particle diameter is lower than the referred value ( $d_p < 63$  nm). The suspension obtained was centrifuged, washed, dried at  $80^\circ\text{C}$ . under vacuum and milled. The hydroxyapatite powder was analysed by XRD (FIG. 2.a) proving that the as prepared sample is fairly crystalline and that the average crystals size is  $d_c=5.9\pm 3.6$  nm, estimated by the Scherrer formula. The SEM

image shows that particle morphology is needle-like (FIG. 3) with a shape factor of 5:1. Therefore, particle average diameter is 5 times lower than that obtained by the Granulometer ( $d_p < 13$  nm).

##### Example 2

Spherical Shaped Hydroxyapatite Nanoparticles Production.

**[0055]** Production of hydroxyapatite nanoparticles at  $25^\circ\text{C}$ . was performed in the commercial NETmix® reactor with 15 inlet ports to feed the reactants solutions and 15 outlets for product recovery. Preparation of a Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O solution with pH adjusted to 11 by addition of a KOH solution and a NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> solution with pH adjusted to 12 by addition of a KOH solution. Calcium and phosphorous solutions were alternatively fed at the reactor's inlet: odd inlets were used for calcium solution and even inlets for phosphorous solution, guarantying global stoichiometry (Ca/P molar ratio) in any part of the reactor as 10:6. The produced hydroxyapatite suspension was analysed to determine the particle size distribution curve (FIG. 1.b), and the average particles diameter was of  $d_p=82$  nm. Due to equipment limitations described before, the real average particle diameter is lower than this value. The hydroxyapatite powder was analysed by XRD (FIG. 2.b) proving that the sample is highly crystalline and that the average crystals average size is  $d_c=4.9\pm 1.3$  nm. The SEM image shows particles with spherical morphologies (FIG. 4). Therefore, the shape factor used in the Laser Diffraction particle size analysis was very similar to the real value. This sample presented a specific surface BET area of  $80.3\text{ m}^2/\text{g}$ .

##### Example 3

**[0056]** Spherical Shaped Hydroxyapatite Nano Particles Production with Low Crystallinity.

**[0057]** Production of hydroxyapatite nanoparticles at  $25^\circ\text{C}$ . was performed in the commercial NETmix® reactor with 15 inlet ports to feed the reactants solutions and 15 outlets for product recovery. Preparation of a CaCl<sub>2</sub> aqueous solution containing 20% in volume of ethanol and pH=11 (by adding a KOH solution) and KH<sub>2</sub>PO<sub>4</sub> aqueous solution containing 20% in volume of ethanol and pH=12 (by adding a KOH solution). The calcium and phosphorous solutions were fed alternatively at the reactor's inlet: odd inlets were used for calcium solutions and even inlets for phosphorous solution, guarantying the global stoichiometry (Ca/P molar ratio) in any part of the reactor as 10:6. FIG. 1.c shows that hydroxyapatite particles size distribution curve is very similar to one of example 2. However, this sample has lower crystallinity than the previous one, as it can be confirmed by the XRD analysis (FIG. 2.c). The SEM image shows spherical shaped particles morphology (FIG. 5).

1. A method for the production of calcium phosphates particles with high purity comprised by controlling the micro-mixing quality and distribution injections schemes of reactants at the reactor's inlet or along it, continuously or variably in time.

2. The method according to claim 1, which comprises feeding to a network mixer or static mixer reactor different streams containing Ca<sup>2+</sup> ions, PO<sub>4</sub><sup>3-</sup> ions and one alkaline reactant, with possibility of adding one or more solvents and/or one or more surfactant or tensioactive agents.

3. The method according to claim 1, comprised by the following steps:

- a) provide one  $\text{Ca}^{2+}$  ions source;
- b) provide one  $\text{PO}_4^{3-}$  ions source;
- c) provide one alkaline source to adjust the reaction pH;
- d) provide solvents and/or surfactant or tensioactive agents;
- e) provide water to adjust the concentrations of each of the above aqueous solutions;
- f) prepare all necessary solutions to feed the reactor with different concentration and compositions, obtained by combining in different proportions the reactants mentioned previously: a), b), c), d) and e);
- g) use a network mixer or static mixer reactor that ensures an efficient and homogeneous mixing, equipped with feed distributors at the reactor's inlet and/or along the reactor to allow different reactants injection schemes;
- h) the reactant solutions mentioned in f) can be feed at the so said reactor, through a distribution scheme at the inlet of the reactor or along it, in a continuous or time varying mode;
- i) thermostatzation of the reactants and/or the reactor in order to ensure a proper temperature for the reaction, where the rigorous choice of operating conditions for the steps defined at f), g), h) and i) enables the nanoparticles or microparticles production with nanometric crystalline or amorphous structures.

4. The method according to claim 1, wherein the Ca/P molar ratio is in the range  $0.5 \leq \text{Ca/P} \leq 2$ , so that calcium phosphates can be obtained in anhydrous or hydrated forms.

5. The method according to claim 4, wherein one of the calcium phosphates forms produced is hydroxyapatite.

6. The method according to claim 1, comprised by the calcium phosphates be chemically modified by the substitution of some ions of the crystallographic structure by other ions, as for example  $\text{F}^-$ ,  $\text{Na}^+$ ,  $\text{Mg}^{2+}$  and  $\text{CO}_3^{2-}$ .

7. The method according to claim 1, comprised by the calcium phosphates suspension production in the so said reactor can be further processed to concentration, separation, drying, thermal treatment and/or milling stages to obtain final products in the form of suspensions, slurry or dried powder,

with a concentration range varying from 0.1% to 100% of any specific calcium phosphate or a mixture of different calcium phosphates.

8. Nanoparticles and/or microparticles of calcium phosphates having high purity and nanometric structure, comprised by being produced by the method in accordance with claim 1.

9. Nanoparticles and/or microparticles of calcium phosphates having high purity and nanometric structure, according to claim 8, wherein the crystallographic structure has a controlled crystallinity degree, which can vary from amorphous structure to crystalline structure.

10. Nanoparticles and/or microparticles of calcium phosphates having high purity and nanometric structure, according to claim 8, wherein the morphology is controlled, from spherical to needle-like geometry.

11. Use of the nanoparticles and/or microparticles according to claim 8, comprised by production of biomaterials used in biomedicine, preferably as bone graft for bone replacement, growth and repair, bioceramics and metallic prosthesis coatings.

12. Use of the nanoparticles and/or microparticles according to claim 8, comprised by their application in food industry, preferably as alimentary additives and nutritional supplements.

13. Use of the nanoparticles and/or microparticles according to claim 8, comprised by their application in pharmaceutical industry, preferably in drug delivery and controlled release.

14. Use of the nanoparticles and/or microparticles according to claim 8, comprised by their application in cosmetics industry, preferably in teeth related products and cosmetic products.

15. Use of the nanoparticles and/or microparticles according to claim 8, comprised by their application esthetical treatment to stimulating conjunctive tissue formation.

16. Use of the nanoparticles and/or microparticles according to claim 8, comprised by their application as catalysts for water treatment and as absorbents in chromatographic columns.

\* \* \* \* \*

## CHAPTER III - MICROSPHERES BASED ON HYDROXYAPATITE NANOPARTICLES AGGREGATES FOR BONE REGENERATION

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**Keywords:** microspheres, hydroxyapatite, bone regeneration, alginate, nanoparticles, chemical precipitation.

**Abstract.** This study concerns the preparation and characterization of microspheres associating alginate and two different types of hydroxyapatite (HA), which are intended to be used as drug delivery systems and bone regeneration matrices. Hydroxyapatite nanoparticles (HA-1 and HA-2) were prepared using a chemical precipitation synthesis based on  $H_3PO_4$ ,  $Ca(OH)_2$  and a surfactant, SDS (sodium dodecylsulphate), as starting reagents. These two powders of nanoHA and alginate were used to prepare two different types of microspheres. Both powders and microspheres were characterized using FTIR, TEM, SEM, mercury porosimetry analysis and X-ray diffraction. Results show that pure hydroxyapatite (HA) and mixtures of HA/ $\beta$ -TCP in the nanometer range were obtained from both HA syntheses. Microspheres with different characteristic were obtained from these two types of hydroxyapatite.

### 1. Introduction

Bone exhibits natural hydroxyapatite crystals with needle-like or rod-like shapes, well arranged within the polymeric matrix of collagen type I. These natural nanoparticles formed in physiological

environment have a more dynamic response when compared to synthetic material with larger particle size [1]. In order to prepare fine HA powders, many chemical processing routes have been employed, including hydrothermal reactions, sol-gel synthesis, pyrolysis of aerosols and micro-emulsion, biomimetic process, and chemical precipitation, which is the most used alternative. Nowadays, several improvements injectable bone substitutes are being developed as minimally invasive cell carriers for tissue regeneration both for bone and cartilage [2-4]. Desired characteristics of synthesized hydroxyapatite are fine and uniform particle size, in the nanometer range, phase homogeneity and minimized degree of particle agglomeration [5].

This work describes the preparation and characterization of novel nano-hydroxyapatite-alginate microspheres intended to be used as an injectable bone filling material or as enzyme delivery matrices. The purpose of this work is to characterize the morphology and chemical composition microspheres based on hydroxyapatite obtained by two different procedures.

## **2. Materials and Methods**

Aqueous solutions of calcium hydroxide ( $\text{Ca}(\text{OH})_2$ ) and ortho-phosphoric acid ( $\text{H}_3\text{PO}_4$ , 85%, both of analytical grade, were used as reactants for the preparation of two different types of HA nanoparticles. HA powder (HA-1) was synthesized according to a previously reported procedure [6]. Briefly, 1L of an aqueous suspension of  $\text{H}_3\text{PO}_4$  (0.6M) was slowly added drop by drop to a 1L of an aqueous suspension of  $\text{Ca}(\text{OH})_2$  (1M) while stirring vigorously for about 2h at room temperature. Concentrated NaOH was added until a final pH of 10.5 was obtained. The white solution obtained was washed using de-ionized water and dried in oven at 80°C for 24h. HA powder 2 (HA-2) was synthesized in the same way, with the difference of the addition of sodium dodecyl sulphate addition (10g) into the calcium solution. The alginate/nanohydroxyapatite microspheres preparation has been reported elsewhere [6].

Chemical characterization was performed using Fourier transformed infrared (FTIR) spectroscopy. For this purpose, hydroxyapatite and microspheres were reduced to powder and analyzed as KBr pellets using a PerkinElmer System 2000 spectrometer.

The size and morphology of the nanosized HA was determined using a Transmission Electron Microscope (TEM, Zeiss model EM 902A) at an accelerating voltage of 80kV, TEM images were acquired and observed with Axion Camera Zeiss and AxionCs 40AC Zeiss, v 4.2.0.0 software.

The microspheres morphology was analyzed by both digital imaging and Scanning Electron Microscopy (SEM). Using a JEOL JFC-100 fine coat ion sputter device, samples were sputter coated with gold. The HA microspheres were then studied with JEOL JSM-6301F microscope at an accelerating voltage of 5kV.

X-ray diffraction (XRD) characterization of all prepared samples was conducted using a Panalytical X'Pert PRO alpha-a with a RTMS X'Celerator detector. It used Ni-filtered Cu K $\alpha$  radiation over the  $2\theta$  range of 10-90° at a scan rate of 2.4°/min and with a sampling interval of 0.002° at 40mA and 45kV.

### 3. Results

Figure 1 shows the needle-like shaped morphology of the HA-1 and HA-2 nanoparticles after sintering process. SEM images of the microspheres are presented in Figure 2. After the sintering process their size is decreased due to alginate burn-off, even though their spherical shape is (Figures 2c and 2d). The pores shown in both samples (Figures 2e and 2f), seem to be present in larger amount in HA-1 than in HA-2 microspheres, albeit the interconnectivity is clear in HA-2 microsphere sample. Mercury porosimetry analyses confirmed higher average porous diameter and porosity in HA-2 than HA-1 (results reported elsewhere) [6]. XRD patterns of all samples are shown in Figure 3. HA-1 powder is represented by the pattern in Figure 3a and some undefined humps may be seen, due to the non-sintered state. Figure 3a and pattern c present many similarities even though after the sintering process they revealed the presence of different phases. The pattern in Figure 3b shows clearly the peaks corresponding to pure monoclinic HA, however, Figure 3d shows a mixture of monoclinic HA and  $\beta$ -TCP.

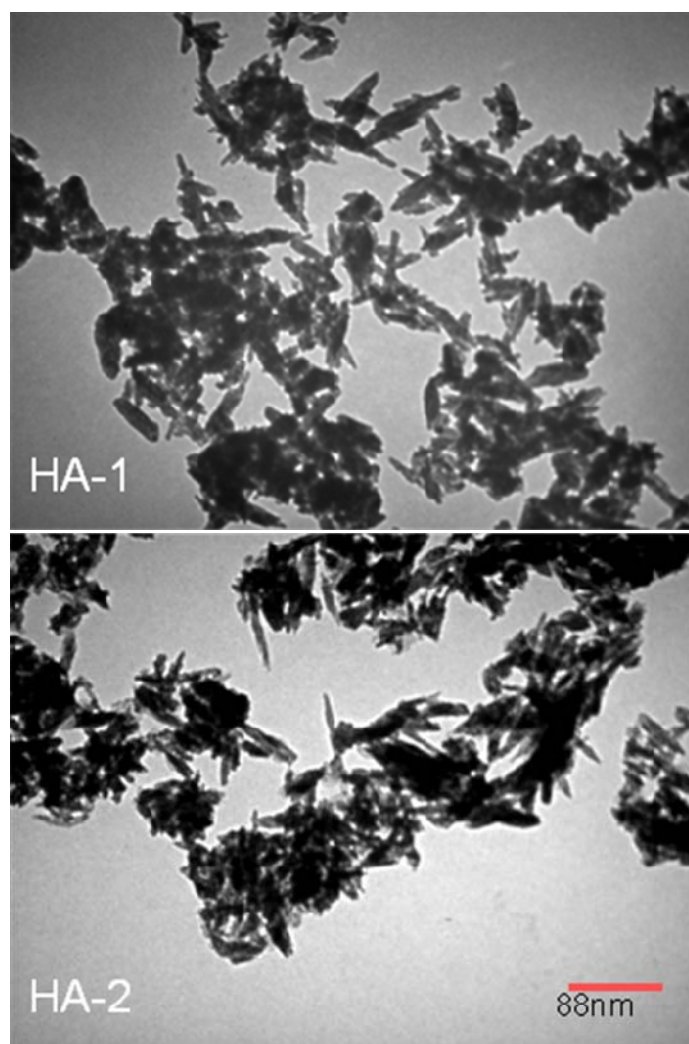


Figure 1. SEM images from HA-1 and HA-2 powders

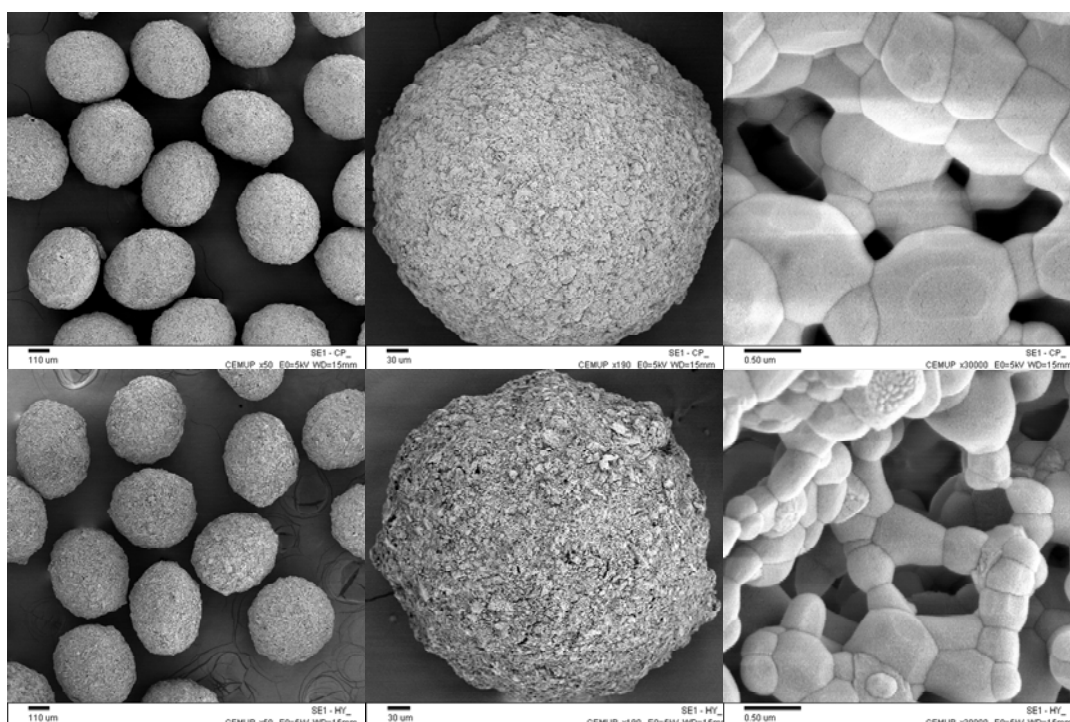


Figure 2. SEM images of HA-1 (up) and HA-2 (down) microspheres (a, b: x50; c,d: x190 and e,f: x30000).

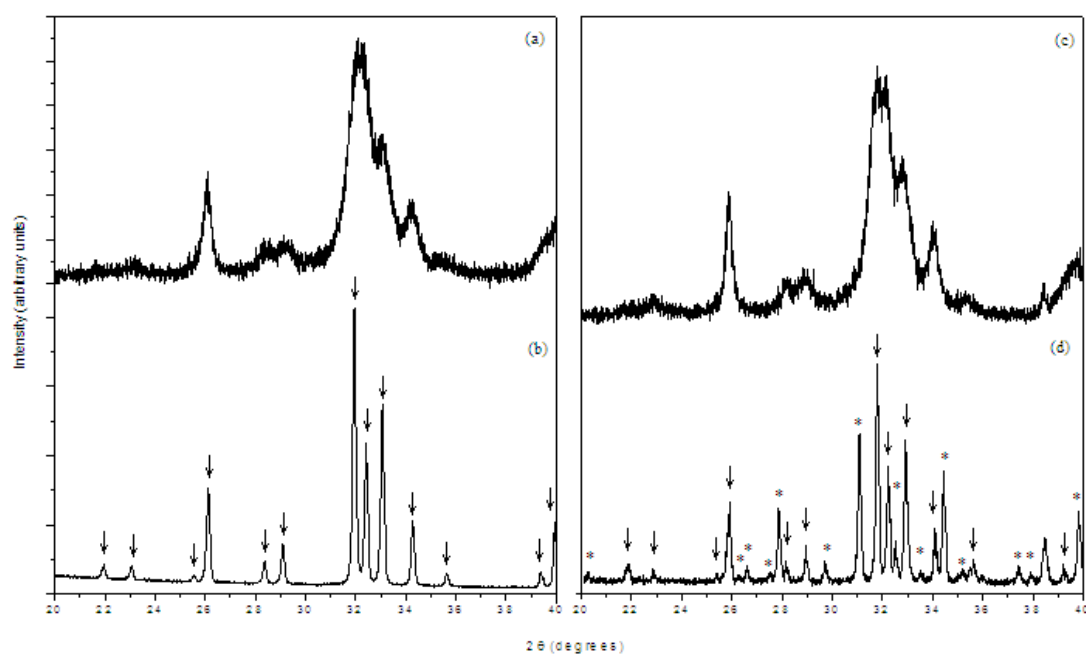


Figure 3. XRD spectra from HA powders and microspheres (a) HA-1 powder, (b) HA-1 microspheres, (c) HA-2 powder, (d) HA-2 microspheres, ↓: monoclinic HA, \*:β-TCP.

#### 4. Discussion

TEM and SEM images showed the orientation and size of the nanoparticles aggregates and also verified the traditional dimensions and morphology of the HA nanoparticles are maintained in the microsphere structure due to the fast sintering process, avoiding the complete particles melting. The characterisation analyses included FTIR, TEM, SEM, mercury porosimetry and XRD, FTIR analysis indicated that the characteristic spectra bands of both ceramic powders, HA-1 and HA-2, are maintained in the microspheres, suggesting that sodium alginate did not induce subsequent modifications in the ceramics structures. It is important to point out the multiple binding sites on the microsphere surface (Fig 2e,f) that could allow the binding to several macromolecules such as enzymes or antibiotics, among others. This feature may enhance their role as drug delivery system components to be used in biomedical applications.

#### 5. Conclusions

Preparation and characterisation of two different types of hydroxyapatite and alginate microspheres, intended to be used as drug delivery systems and bone regeneration matrices, were studied in the present work. Hydroxyapatite nanoparticles (HA-1 and HA-2) were prepared using a chemical precipitation synthesis based on  $\text{H}_3\text{PO}_4$ ,  $\text{Ca}(\text{OH})_2$  as starting reagents, with addition of the surfactant SDS (sodium dodecyl sulphate) in one of these processes. These two powders of nanoHA and alginate were used to prepare two different types of microspheres. Both powders and microspheres were characterised using FTIR, TEM, SEM, mercury porosimetry analysis and X-ray diffraction. Results indicated that pure hydroxyapatite HA and a mixture HA/ $\beta$ -TCP in the nanometer range, were obtained from HA-1 and HA-2 synthesis respectively. Macroporous microspheres with different characteristics but showing adequate shapes and sizes were obtained from these two types of hydroxyapatite.

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## CHAPTER IV– NANOHYDROXYAPATITE MICROSPHERES AS DELIVERY SYSTEM FOR ANTIBIOTICS: RELEASE KINETICS, ANTIMICROBIAL ACTIVITY, AND INTERACTION WITH OSTEOBLASTS

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**Abstract.** Severe periodontitis treatment, where massive alveolar bone loss occurs, involves bone defect filling and intensive systemic long-term antibiotics administration. This study aims at developing novel injectable drug delivery systems (nanohydroxyapatite microspheres) with the drug releasing capability for periodontitis treatment and simultaneously initiating the osteointegration process. Materials were characterized by XRD, SEM, inverted stand optical microscope analysis, and mercury porosimetry method. Amoxicillin, amoxicillin + clavulanic acid and erythromycin were the antibiotics used. Release properties during 28 days from the hydroxyapatite (HA) granules, and two types of nanoHA microspheres were investigated. Biocompatibility was assessed by cytotoxicity assays. HA granules were inadequate, releasing all antibiotic during the first hours. The concentration of antibiotics released in the first days from HA-2 was higher than from HA-1 microspheres, because of the increased porosity and surface area. The release profiles (fast initial release followed by long-term sustained release) of effective doses of antibiotics make these systems good alternatives for antibiotics delivery. Osteoblasts proliferated well on both types of microspheres, being cell growth enhanced in the presence of antibiotics. Erythromycin presented the most beneficial effect. Combining the sustained antibiotic release with the osteoconduction, resorbability, and potential use as injectable bone filling material of porous HA microspheres, these systems provide a forth fold beneficial effect.

**Keywords:** drug release; microsphere; cytotoxicity; antibacterial; nanoparticles; hydroxyapatite.

## 1. Introduction

Periodontitis is an oral disease that promotes, in its more severe form, massive alveolar bone loss [1, 2]. The usual treatment of this disease involves systemic administration of antibiotics in high dosages for long periods of time [3]. In the treatment of such infections, where vascularity is compromised, obtaining effective local antibiotic concentrations by parenteral administration is difficult [4], thus the delivery of an active antimicrobial agent directly to the site of infection represents a viable alternative treatment [5-12].

Several studies have reported the use of hydroxyapatite (HA) as a drugs carrier because of its adequate mechanical properties and composition similarity to mineral bone and suggest that it may be used as bone substitute [6, 13-16].

Biomaterials based on calcium phosphates are currently available as blocks and as granules as well as porous structures. Macroporous blocks are brittle and difficult to shape [15-17]. In addition, they cannot fit tightly to the surface or the defects, preventing the osteoconductive process [18]. On the other hand, granules present difficulties to handle and keep in place after implantation. These are limited to be used to fill cavities of extremity fractures, hence, an injectable and biodegradable support material would be ideal [19].

HA particle size is a relevant factor for *in vitro* cell activity, and is a parameter of great importance for injectability handling [19-22]. Desired characteristics of HA are fine and uniform particle size in the nanometer range, phase homogeneity, and reduced degree of particle agglomeration. Bone exhibits natural HA crystals with needle-like or rodlike shapes well arranged within the polymeric matrix of collagen type I. These natural nanoparticles, formed in physiological environment, have a more dynamic response, when compared to synthetic materials with larger size particle [20-24].

Nanohydroxyapatite microspheres intended to be used as injectable bone filling material or as enzyme delivery matrices can also be used as antibiotic releasing materials [25, 26].

The aim of this study consisted in attempting to use HA loaded with an antibiotic, to be used as a local drug delivery system, associating the drug release capability for the periodontitis treatment with the possibility of simultaneously initiating the process of osteointegration. Therefore three types of HA materials were tested: dense granules and two different types of nanohydroxyapatite microspheres, intended to be used as injectable bone filling materials.

The resident oral microflora is diverse and consists of a wide range of species of microorganisms. It is estimated that about 400 different species are able to colonize the mouth and any individual may typically harbor 150 or more different species. Among these, only a few are considered to be pathogens associated with periodontal disease [27]. The choice of antimicrobial agent must be based on the bacterial aetiology of the infection. And, as there are many bacteria involved in the periodontal disease, the choice of one single antimicrobial agent may not be suitable. However there

are several antibiotics extensively investigated for systemic use namely: ampicillin, amoxicillin, tetracycline, minocycline, erythromycin, clindamycin, and metronidazole [4].

In his study, amoxicillin with and without clavulanic acid and erythromycin, antibiotics already in use for oral and parenteral administration in the treatment of periodontitis [28], were tested to be used for controlled local delivery. The delivery systems tested (two different types of nanohydroxyapatite microspheres), were compared with dense granules behavior, and are expected to present more appropriate antibiotic release kinetics.

## **2. Materials and Methods**

### **2.1 Materials preparation and characterization**

#### *2.1.1 Preparation of HA granules*

The preparation methods for hydroxyapatite (HA) granules has been previously described [14]. Briefly, commercial HA (from Plasma Biotol, ref. P120 powder) was uniaxially pressed to cylindrical samples (0.5 g into 16-mm diameter mold) at 288 MPa and sintered. Samples were milled and sieved to obtain 75-106  $\mu\text{m}$  granules. This range of granules diameter allows for the occurrence of different sizes, improving the capacity to fill the periodontal pocket and maximizing the amount of antibiotic to be loaded and released.

#### *2.1.2 Synthesis of nanosized HA*

Aqueous solutions of calcium hydroxide ( $\text{Ca}(\text{OH})_2$ ) and orthophosphoric acid ( $\text{H}_3\text{PO}_4$ , 85%), both of analytical grade, were used as reactants for the preparation of two different types of HA nanoparticles. The first HA powder (HA-1) was synthesised according to the following procedure. Firstly, 1 L of an aqueous suspension of  $\text{H}_3\text{PO}_4$  (0.6M) was added drop-by-drop, slowly to a 1 L of an aqueous suspension of  $\text{Ca}(\text{OH})_2$  (1M) while stirring vigorously for about 2 h at room temperature. Concentrated NaOH was added until a final pH of 10.5 was obtained. The white solution obtained was washed using de-ionized water and dried in at 80  $^\circ\text{C}$  for 24 h. The other HA powder (HA-2) was synthesized in a similar fashion, with the difference of the sodium dodecyl sulphate addition (10 g) to the calcium solution.

#### *2.1.3 Alginate/ hydroxyapatite microspheres preparation*

The microspheres were prepared following a methodology developed in our group, and previously described [25, 29]. Briefly, pharmaceutical-grade sodium alginate (Protanal 10/60 LS) with a high  $\alpha$ -L-guluronic acid content (65-75%, as specified by the manufacturer) was kindly donated by Pronova Biopolymers and used without further purification. Na-alginate (1.5 g) was dissolved in 50 mL of distilled and de-ionized water until total homogenization, where HA powder (70  $\mu\text{m}$ ) was added to the alginate solution in a ratio of 1:4 w/w until complete dissolution. This paste was extruded drop-wise into a 0.1M  $\text{CaCl}_2$  crosslinking solution, where spherical-shaped particles instantaneously formed and were allowed to harden for 30 min. The size of the microspheres was controlled by regulating the extrusion flow rate (Encapsulation Unit VAr 1-J1, Nisco) using a single

syringe infusion pump (EW 47-900-00, Cole Palmer) and by applying a coaxial air stream. For the HA-1 sample, extrusion flow rate was 20 mL/h while for the HA-2 sample was 40 mL/h. At completion of the gelling period, microspheres were recovered and rinsed in water to remove  $\text{CaCl}_2$  in excess. Finally they were dried overnight in a vacuum oven at 30°C, and sintered at 1100°C for 1 h [30].

#### 2.1.4 Materials characterization

Materials were characterized by X-ray diffraction (XRD), scanning electron microscopy (SEM), transmission electron microscopy (TEM), inverted stand optical microscope analysis, and mercury porosimetry method.

### 2.2 Drug loading

Amoxicillin, erythromycin, and amoxicillin + clavulanic acid (7:1) solutions of concentrations 4, 2 and 4 mg/mL respectively, were prepared in tris-buffer solution (pH 7.4). Then, 0.5 g of HA particles were immersed in 10 mL of antibiotics solution at 37°C for 48 h. The drug solution was then removed and the particles were dried.

### 2.3 Release kinetics and bioactivity

The antibiotic loaded ceramics were immersed in 10 mL of simulated body fluid (SBF, a protein free solution with ionic concentrations and pH similar to the plasma of blood) at 37°C. To determine the concentration of amoxicillin and erythromycin released from the ceramic into the SBF, 2 mL of SBF were withdrawn and replaced by fresh 2 mL SBF after 1, 3, 6, 24 and 48 h. At 72 h, 50% of the SBF volume was replaced by fresh solution and from then onwards every 24 hours up to 28 days. The eluted SBF samples were frozen at -4°C for concentration measurements and microbiology assay. Control experiment using antibiotic-free ceramic samples was performed under the same experimental conditions (negative control). All tests were performed in triplicate.

The concentration and the bioactivity of the released antibiotic were checked using the diffusion method. *Staphylococcus aureus* ATCC 29213 was used to test erythromycin and amoxicillin + clavulanic acid, and *Escherichia coli* ATCC 25922 was used to test amoxicillin. *E. coli* was used to test amoxicillin and not erythromycin because it is only susceptible to the first. Oppositely, *S. aureus* is susceptible to erythromycin and can be used to test it. *Staphylococcus aureus* ATCC 29213, is a bacterial strain resistant to amoxicillin and susceptible to amoxicillin + clavulanic acid, therefore, due to the presence of  $\beta$ -lactamases. It can be used to test the delivery of this combination, as these two substances should be delivered at similar rates.

Nutrient agar plate inoculated with *S. aureus* and *E. coli* was used to create a  $10^6$  colony-forming units/mL suspension in NaCl to match 0.5 Mcfarland equivalence turbidity standard in ambient light. Bacteria suspension of 1.5 mL was incorporated in 250 mL of nutrient agar medium heated at 50°C. The agar was poured in sterile plates and left at room temperature. Samples of 70  $\mu\text{L}$  were deposited in triplicate into 10-mm stainless steel cylinders inserted into the agar. Samples were located in different areas in the plates and left at room temperature for about 30 min before

incubation at 37°C. After 18 h of incubation, the diameters of inhibition zones were measured and expressed as mean  $\pm$  SD as a function of time. The corresponding negative control samples (solution incubated with ceramic without antibiotic) were measured using the same methodology. In addition, standard solutions of known antibiotic concentrations were prepared and tested with the same method to calculate the unknown antibiotic concentration in the samples.

## 2.4 Cytotoxicity

Two different types of cytotoxicity testing were performed. Culture of MG63 human osteoblast cell line in the presence of different antibiotics concentrations (qualitative cytotoxicity), and direct growth and adhesion of MG63 cells on the surface of materials loaded with antibiotics. The growth of MG63 cells was evaluated using neutral red assay (NR), in which a colored reaction product is generated in viable cells. Thus, the absorbance values obtained are directly proportional to the number of living cells that are present. Therefore, changes in absorbance can be used to estimate cell proliferation.

### 2.4.1 Growth of human osteoblasts - Qualitative cytotoxicity

MG63 cells, expressing a number of features characteristic of osteoblasts were cultured at 37°C in a humidified atmosphere of a 5% CO<sub>2</sub> in air, in  $\alpha$ -minimum essential medium ( $\alpha$ -MEM) (Gibco), 10% foetal calf serum (FCS) (Gibco), 1% v/v fungizone (Gibco), 1% v/v penicillin/streptomycin (Gibco), and supplemented with 500 mL of ascorbic acid 50 mg/mL. The medium was changed every third day and, for sub-culture, the cell monolayer was washed twice with phosphate-buffered saline (PBS) (Gibco) and incubated with trypsin-EDTA solution (0.25% trypsin, 1mM EDTA; Gibco) to detach the cells. The effect of trypsin was then inhibited by adding the complete medium at room temperature. The cells were washed twice by centrifugation and re-suspended in complete medium and seeded in 24-well dishes at a density of 10<sup>4</sup> cells/well.

MG63 cells were cultured for a period of 10 days, in the above described experimental conditions as a control, and supplemented with: (i) amoxicillin 4, 10, 25, 100 and 200  $\mu$ g/mL; (ii) amoxicillin + clavulanic acid (7:1) 4, 10, 25, 100 and 200  $\mu$ g/mL; and (iii) erythromycin 4, 10, 25, 100 and 200  $\mu$ g/mL.

The quantification of viable cells was performed at days 1, 6 and 10 and assessed by neutral red assay (NR). Briefly, the culture medium was discarded and replaced by fresh medium containing 50 mg/mL NR (Sigma). Plates were incubated at 37°C for 3 h. At the end, the supernatant was discarded and cells were washed twice with PBS. The NR absorbed by the cells was extracted with 1% v/v acetic acid in 50% v/v aqueous ethanol. After being well homogenized on a shaker platform, the supernatants were transferred to another 96-well plate, and the optical density was read at 540 nm in a microplate spectrophotometer.

#### 2.4.2 Direct growth of MG63 cells on the surface of materials loaded with antibiotics.

MG63 cells were cultured at 37°C in a humidified atmosphere of a 5% CO<sub>2</sub> in air, in  $\alpha$ -minimum essential medium ( $\alpha$ -MEM) (Gibco), 10% foetal calf serum (FCS) (Gibco), 1% v/v fungizone (Gibco) and 0.5% v/v gentamicin (Gibco). Cultures were maintained until confluence and, at this stage, adherent cells were enzymatically released with trypsin and seeded in a 96-well dishes at a density of  $3,6 \times 10^4$  cells/cm<sup>2</sup>. Cells were cultured for periods up to 5 days, in the same experimental conditions previously described on the surface of TCPS (Control) and on the following sterilized materials (14 mg/well): HA granules, HA-1 microspheres and HA-2 microspheres previously immersed in solutions prepared as described earlier.

The quantification of viable cells was performed at days 1, 3 and 5 and assessed by neutral red assay (NR), using the above described procedure.

### 3. Results

#### 3.1 Materials characterization

Quantitative phase analysis of HA granules obtained by XRD showed that the only phase present was HA (99,7%). Granules size varied between 75 and 106  $\mu$ m.

Both microsphere types HA-1 and HA-2, were characterized using XRD, SEM, inverted optical microscopy, and mercury porosimetry analysis. These results are described briefly, as detailed materials characterization is not the aim of this study. More detailed materials characterization is published elsewhere [31]. HA-1 is composed by pure monoclinic HA, whereas HA-2 is composed by a mixture of monoclinic HA and a small percentage of  $\beta$ -TCP. Concerning porosity, HA-1 showed lower pore diameter than HA-2, 0.53 and 0.82  $\mu$ m, respectively. Similarly, HA-1 presented lower theoretical porosity than HA-2. In Figure 1 it can be seen as an example of HA-1 microspheres SEM images.

The average diameter of HA microspheres was  $450 \pm 25$   $\mu$ m ( $n=20$ ), and after the sintering process that generated the microspheres. By TEM observation it was possible to observe that HA crystallites presented nanometer size range (less than 100 nm in length) for both HA-1 and HA-2. These nanoparticles appeared as dispersed aggregates of crystallites.

#### 3.2 Releasing profile and biological activity

A linear relationship between the diameter of the inhibition zone and the standard concentration of the antibiotics tested was observed, being  $R^2 = 0.984$ ,  $0.991$  and  $0.983$  for amoxicillin, amoxicillin + clavulanic acid, and erythromycin, respectively. These calibration curves were used to calculate the amount of antibiotics released from the materials.

Figure 2 shows, as an example, the bacteria inhibition zones around the wells that were initially filled with 70  $\mu$ L of amoxicillin released from HA-1 microspheres after different immersion

periods. The bacterial inhibition assay revealed that all antibiotics released from the materials retained their biological activity. No inhibitory effect was detected for the control samples (immersed in antibiotic-free solution). This indicates that the inhibitory effect was due to the antibiotics being released from the materials.

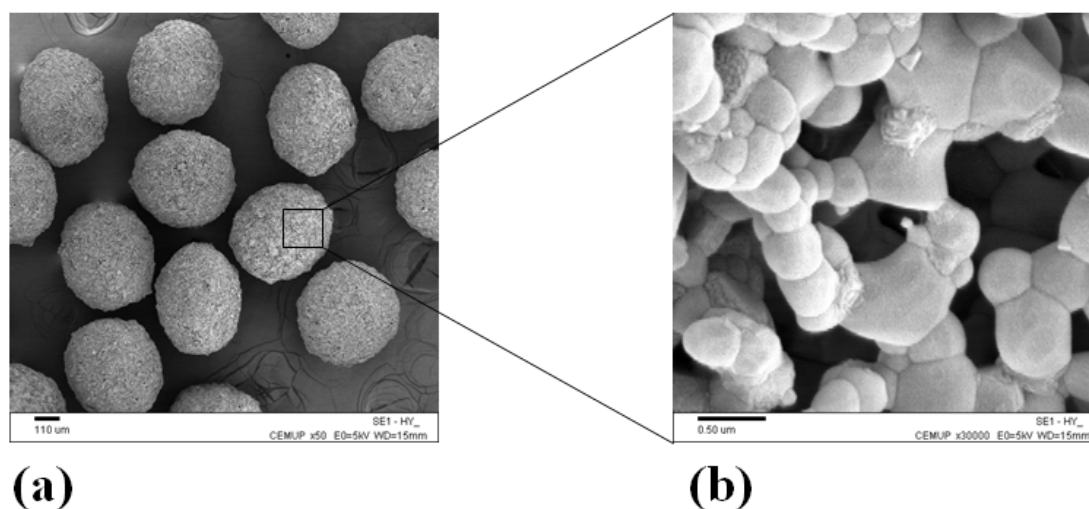


Figure 1. SEM micrograph of HA-1 microspheres (a, x50 and b, x30000)

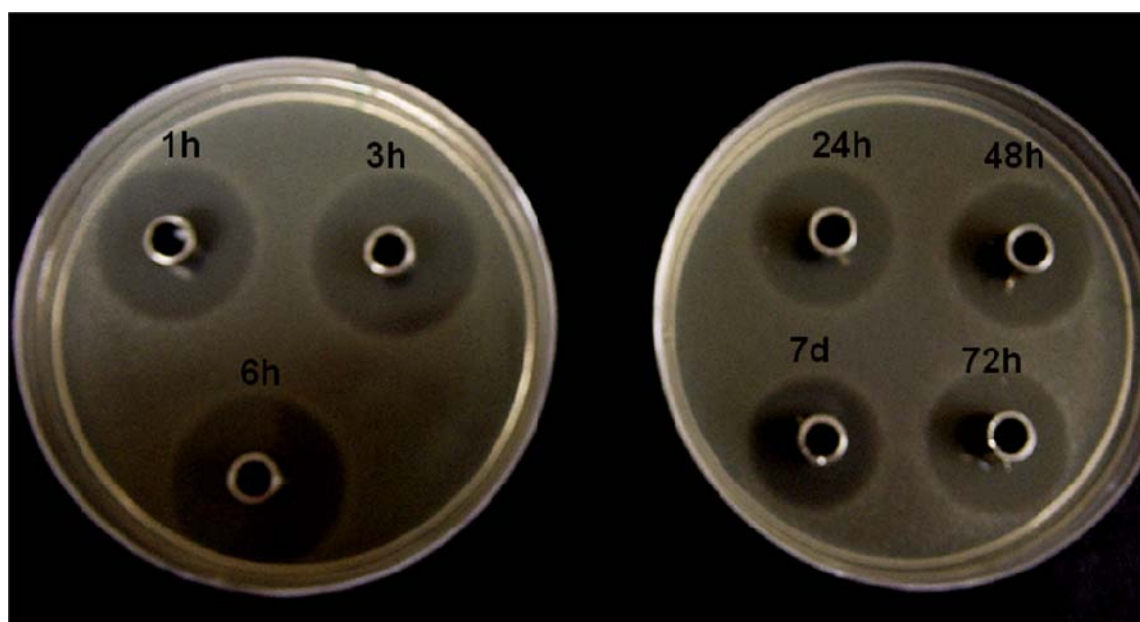


Figure 2. Bacteria inhibition zones around the wells that were initially filled with 70 µL of amoxicillin released from HA-1 microspheres after different elution periods.

Figures 3, 4, and 5 show the bacteria inhibition zone diameter (A) and the mean cumulative concentration of released amoxicillin, amoxicillin + clavulanic acid and erythromycin, respectively

(B), as a function of immersion time. The variation of the amount released antibiotic is inserted in Figures 3, 4 and 5(a).

For the HA granules, there was an initial burst of release for the three antibiotics. After 48h the released antibiotic stood below the minimal inhibitory concentration (MIC) for all three antibiotics tested. The MIC is the lowest concentration of a drug that prevents growth of a particular pathogen. For both, HA-1 and HA-2 microspheres, during the period of this study (672 h) the released antibiotics stood above the MIC.

Figure 3(a) shows that HA-1 microspheres exhibit a decrease in the concentration of released amoxicillin from 0 to 72 h, followed by a stable period with low variation in the released concentration. HA-2 microspheres have a higher and stable release rate in the early periods (till 240 h), followed by a release rate lower than HA-1. These results can be confirmed in Figure 3(b) where it may be seen that for both microspheres the releasing profile showed initial release bursts followed by sustained release of amoxicillin. On the period between 72 and 240 h, HA-2 microspheres presented a higher release rate, after which both materials had a similar behavior.

In what concerns the releasing behaviour of amoxicillin + clavulanic acid, Figure 4(a) shows that both types of microspheres have a decrease in the released concentrations of amoxicillin from 0 to 72 h, being HA-1 the one with a higher variation. Between 72h and 240 h a relatively stable period in terms of released antibiotic concentration occurred. However, because of the initial variation found for HA-1 microspheres, HA-2 had higher release concentration. After 400 h the two materials have nearly the same released concentration of antibiotic. In what concerns Figure 4(b), similarly to what happened with amoxicillin, it may be observed that for both types of microspheres the releasing profile showed initial release bursts followed by a sustained release of amoxicillin + clavulanic acid. However, HA-2 microspheres presented a higher release rate during all the tested period.

Concerning the behaviour of erythromycin, on Figure 5(a) it may be seen that both types of microspheres have similar behaviors in the early periods (between 0 and 72h). Both show a high decrease in the released concentration of erythromycin in the first 6 h, followed by a stable period with low variation in the released concentration. HA-1 microspheres have a decrease in the released concentration of erythromycin from 72 to 240 h, followed by a stable period with low variation in the released concentration. On the other hand HA-2 microspheres have a higher and stable release rate in the periods of 72 to 240h followed by a decrease in the release rate. Analyzing Figure 5(b), it can be easily seen that the main difference between the two types of microspheres occurred in the period of 72 to 576 h, being HA-2 the material with higher rate of released antibiotic.

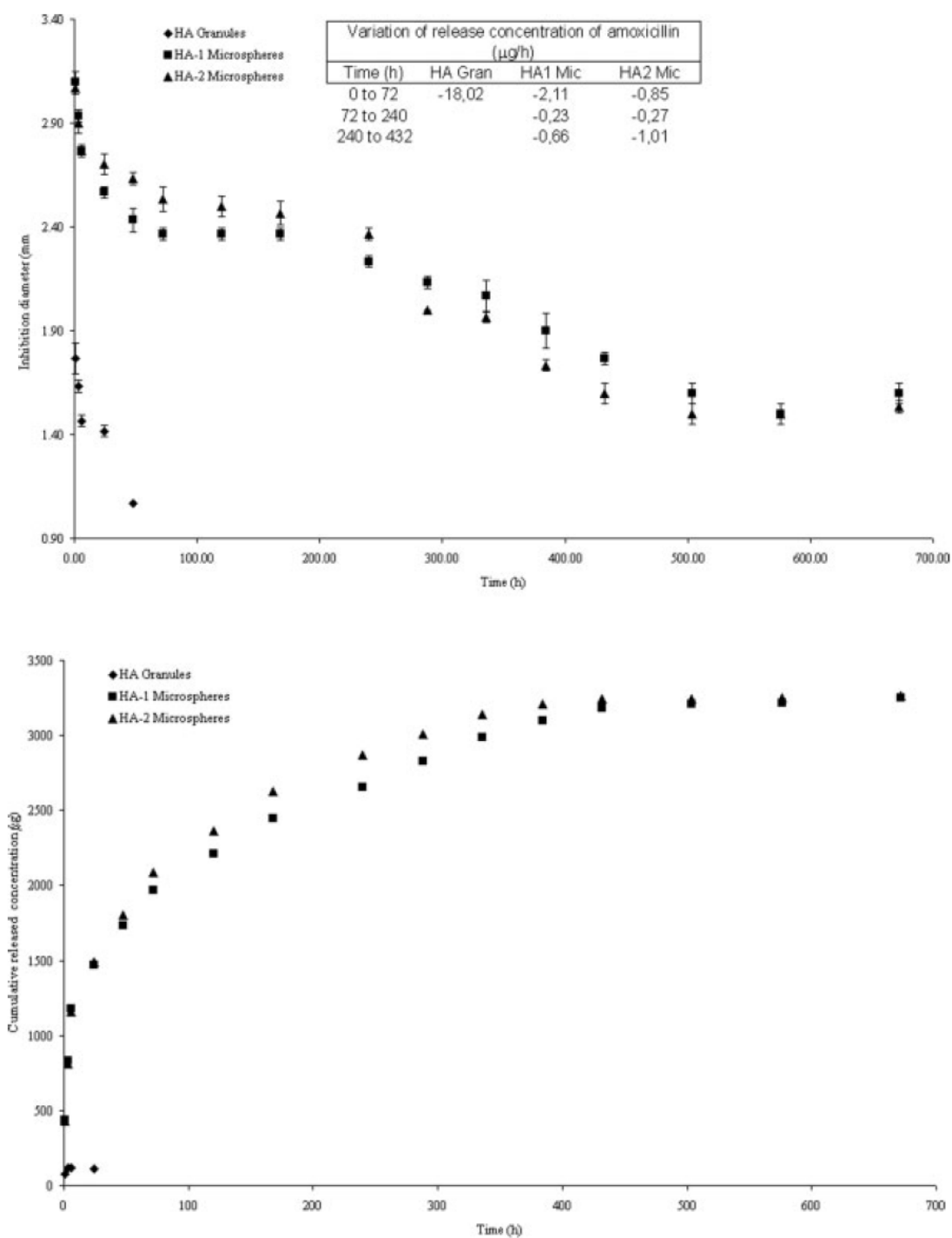


Figure 3. Bacteria inhibition zones diameter (A) and the mean cumulative concentration of release amoxicillin (B) as a function of release time. The variation of the released amoxicillin ( $\mu\text{g/h}$ ) is inserted in (A)

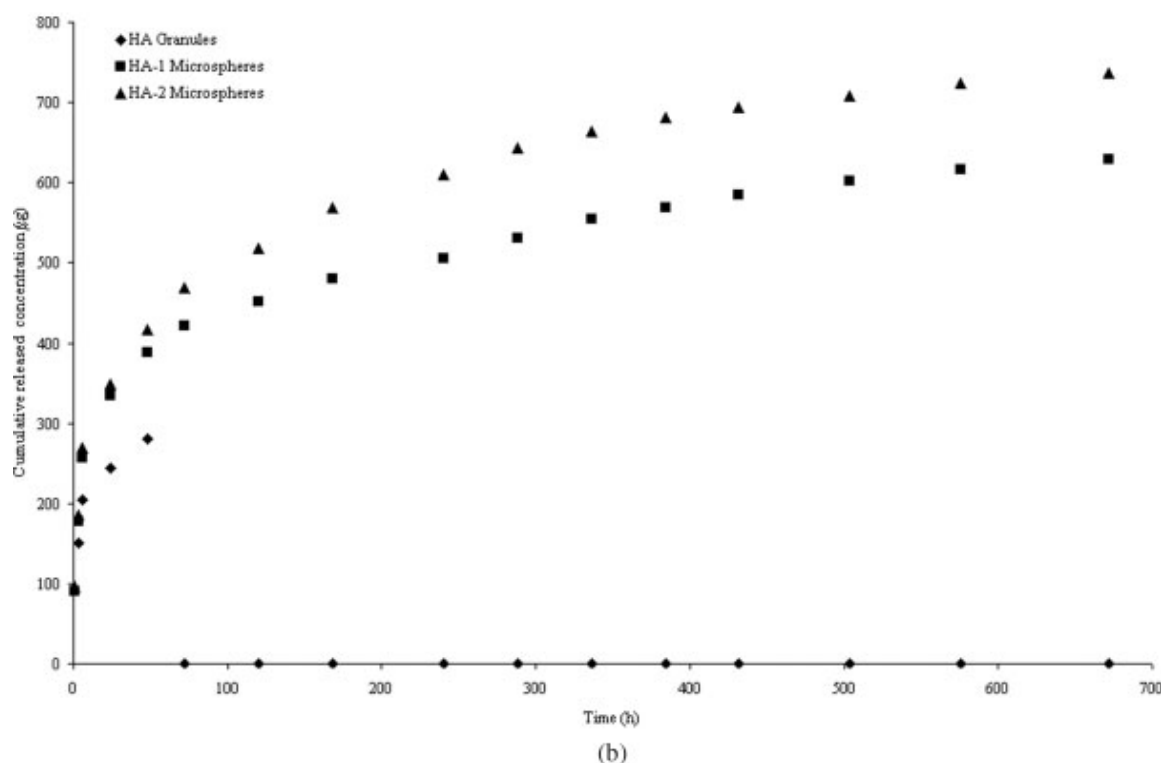
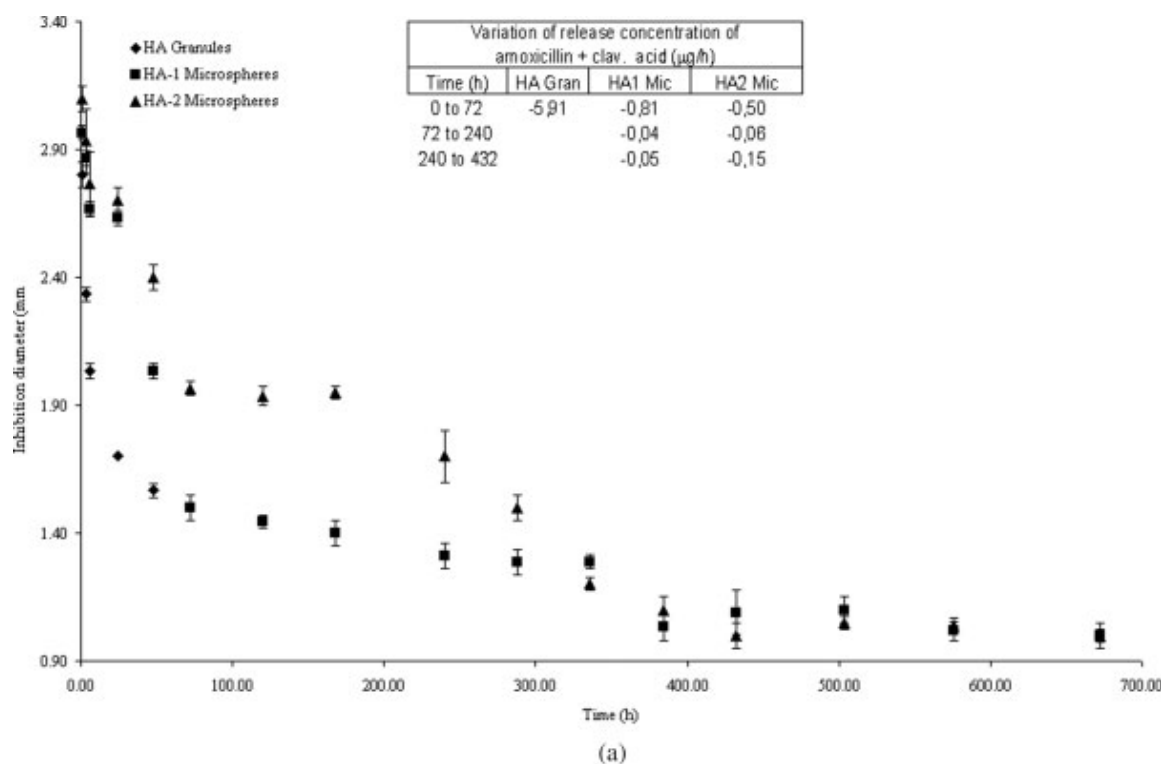


Figure 4. Bacteria inhibition zones diameter (A) and the mean cumulative concentration of release amoxicillin + clavulanic acid (B) as a function of release time. The variation of the released amoxicillin + clavulanic acid ( $\mu\text{g/h}$ ) is inserted in (A)

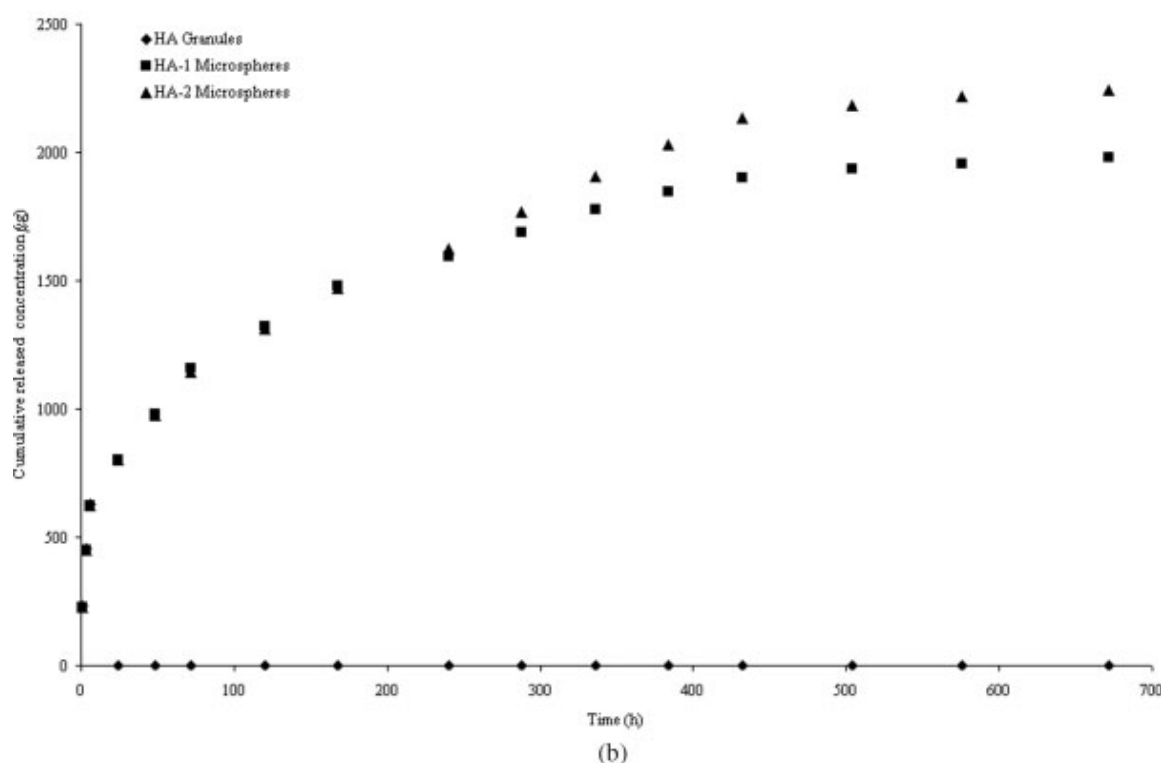
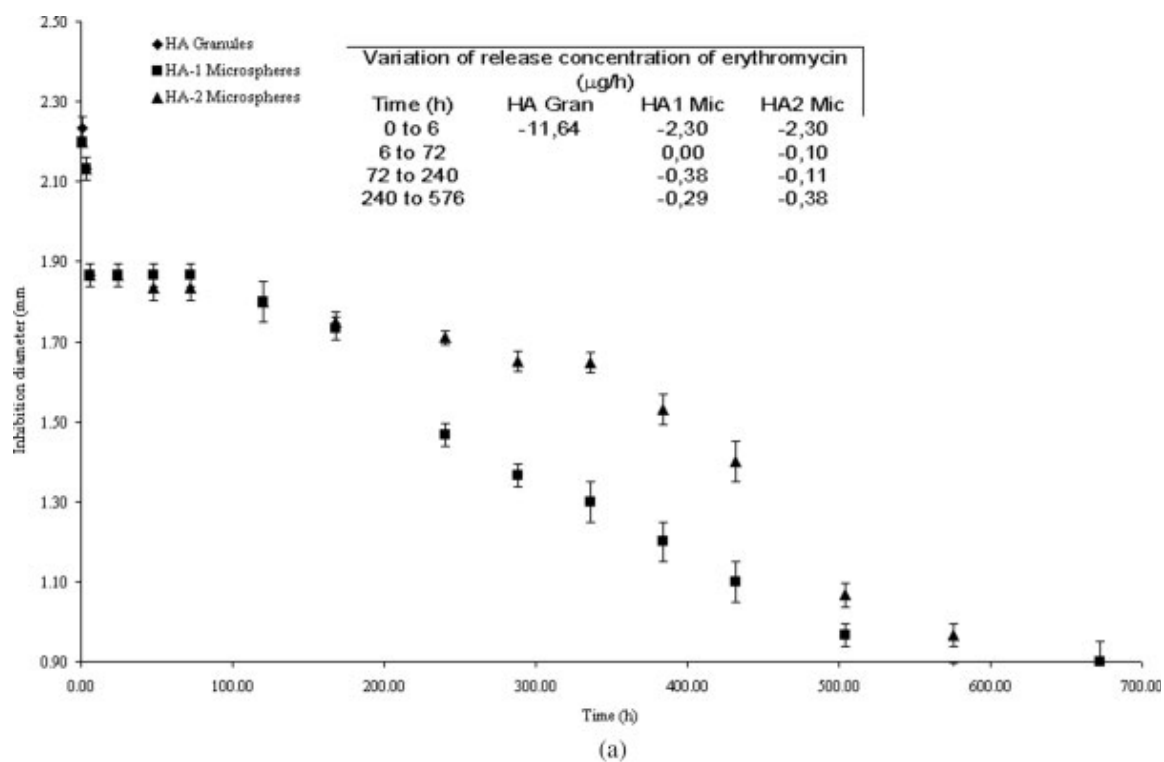


Figure 5. Bacteria inhibition zones diameter (A) and the mean concentration of released erythromycin (B) as a function of release time. The variation of the released erythromycin ( $\mu\text{g/h}$ ) is inserted in (A)

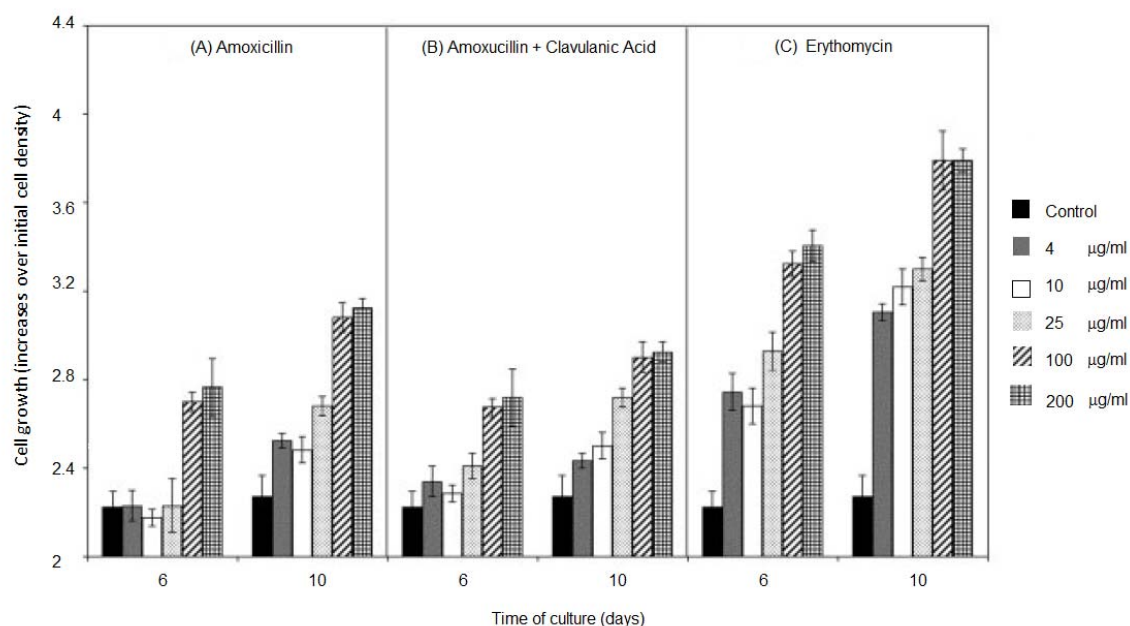


Figure 6. Effects of the presence of different concentrations (4, 10, 25 100 and 200 µg/ml) of amoxicillin (A), amoxicillin + clavulanic acid (B) and erythromycin (C) on the growth of MG63 cells. MG3 cells were grown on TCPS with and without (control) antibiotics supplement, as described in materials and methods. Results are expressed as the average increase in the number of cells relative to the numbe present initially on day 0.

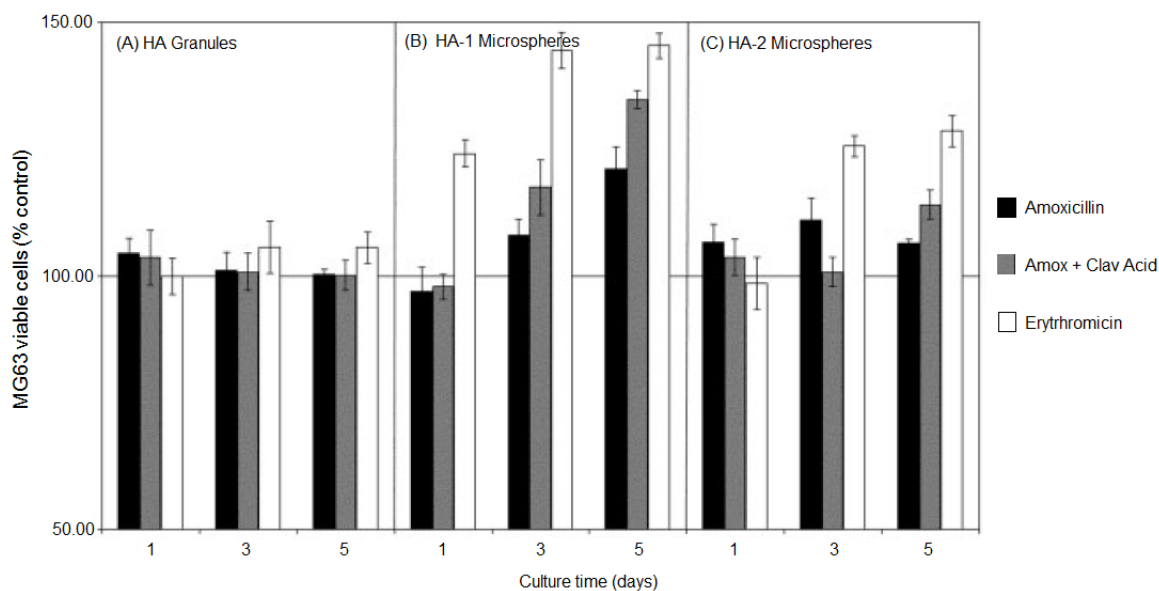


Figure 7. Effect of amoxicillin (A), amoxicillin + clavulanic acid (B) and erythromycin (C) adsorbed on HA granules, HA-1 and HA-2 microspheres, relative to the level expressed by control cells, grown on the same material without adsorbed antibiotic (defined as 100%)

### 3.3 Cytotoxicity

As described above, the growth of MG63 cells was evaluated using neutral red assay, in which a colored reaction product is generated in viable cells. Thus, the absorbance values obtained are directly proportional to the number of living cells that are present. Therefore changes in absorbance can be used to estimate cell proliferation.

Triplicate culture experiments were performed. The results are shown as the arithmetic means  $\pm$  the standard deviation (SD). Analysis of the results was carried out using SPSS software applying the Student's *t*-test, with a significance level of  $p = 0.05$ .

#### 3.3.1 Growth of human osteoblasts - Qualitative cytotoxicity

The results in Figure 6 show that MG63 cells cultured on plastic culture dishes proliferate over a period of 10 days. Although all cultures had the same number of cells on day 1 (results not shown), at day 6 the growth of MG63 cells was elevated on cultures supplemented with antibiotics.

In what concerns to culture supplemented with amoxicillin, an increase in the antibiotic concentration is directly related with an increase in cell number. The difference is more marked at day 10 for all concentrations, and at day 6 for 100 and 200  $\mu\text{g/mL}$ . For the amoxicillin + clavulanic acid supplemented cultures a very similar phenomenon was observed, although the differences between day 6 and day 10 as well as between different antibiotic concentrations were less obvious. Significant statistical differences on the cell growth comparing with control ( $p < 0.05$ ) were seen on 100 and 200  $\mu\text{g/mL}$  supplemented cultures with clavulanic acid.

The difference between the control and antibiotic-grown cells was more pronounced with the erythromycin supplemented cultures, whose growth was markedly higher than for control cells (Figure 6). These differences were evident at days 6 and 10 for all the tested concentrations. The growth of erythromycin supplemented cells for day 10 was approximately 37, 42, 45, 67 and 67% greater, respectively, than for the cells cultures in control conditions. In fact significant statistical differences on the cell growth comparing with control ( $p < 0.05$ ) were seen on erythromycin supplemented cultures independently on the concentration used.

#### 3.3.2 Direct growth and adhesion of MG63 cells on the surface of materials loaded with antibiotics.

Results concerning neutral red assay in cultures growing on materials (HA granules and the two types of microspheres HA-1 and HA-2) with and without antibiotic are presented in Figure 7. The results are presented as a percentages compared with control, being the control the material without antibiotic adsorbed. MG63 cells were also cultured on plastic culture dishes and proliferate over a period of 5 days for all the described conditions.

On HA granules the behavior of cells was similar with and without the presence of antibiotic.

For both types of microspheres the presence of antibiotics clearly enhanced cell proliferation. For HA-1 microspheres all three tested antibiotics clearly have shown benefits in cell proliferation and

are statistically different from control. However, erythromycin clearly have the more pronounced effect, particularly for the initial period (day 1 and 3) the behavior of erythromycin microspheres is statistically different from the other antibiotics ( $p<0.05$ ). There is no statistic differences between amoxicillin and amoxicillin + clavulanic acid.

On HA-2 microspheres there is no significant differences between control microspheres and amoxicillin with and without clavulanic acid ( $p<0.05$ ). However, erythromycin microspheres presented enhancement in cell growth statistically different ( $p<0.05$ ) from both antibiotic free and other studied antibiotics microspheres.

#### **4. Discussion**

Penicillins act by inhibition of bacteria cell wall synthesis. They are narrow spectrum and bactericidal antibiotics. Among penicillins, amoxicillin has been favored for treatment of periodontal disease because of its considerable activity against several periodontal pathogens. The molecular structure of penicillins includes a  $\beta$ -lactam ring (with antimicrobial activity) that may be cleaved by bacterial enzymes ( $\beta$ -lactamases). Some  $\beta$ -lactamases have a high affinity for clavulanic acid, a  $\beta$ -lactam molecule without antimicrobial activity. To inhibit bacterial  $\beta$ -lactamase activity, clavulanic acid has been added successfully to amoxicillin. This combination has been used for systemic periodontal therapy [4].

Erythromycin is a macrolide antibiotic, which has an antimicrobial spectrum similar to or slightly wider than that of penicillin, and is often used for people who have an allergy to penicillins. Structurally, this macrocyclic compound contains a 14-membered lactone ring with 10 asymmetric centres and two sugars (L-cladinose and D-desoamine), making it a compound very difficult to produce via synthetic methods. Erythromycin prevents bacteria from growing, by interfering with their proteins synthesis. Erythromycin binds to the 23s rRNA molecule in the 50S of the bacterial ribosome, blocking the exit of the growing peptide chain thus inhibiting the translocation of peptides [4].

Therefore, amoxicillin with and without clavulanic acid as well as erythromycin, commonly used antibiotics in systemic treatments of periodontitis, can be possible alternatives for local delivery systems.

Results of this study suggest that both types of HA microspheres are characterized by a highly porous structure (Fig 1), providing large surface area for drug loading and allowing for a sustained release profile over the period of study. HA granules presented an initial burst, releasing the entire antibiotic in the first hours.

Quantitative phase analysis of HA granules obtained by XRD showed that the only phase present was HA (99.7%). Granules size varied between 75 and 106  $\mu\text{m}$ , this range of granule diameter allows for the occurrence of different sizes, improving the capacity to fill the periodontal pocket and maximizing the amount of antibiotic to be released.

Both microspheres, presented a release profile of zero-order from 432 h till the end of the study characterized by a sustained release. The release kinetics of antibiotics from the microspheres was determined by differences in composition and porosity of the materials, being the main difference observed in the early periods (0 to 240 for amoxicillin and amoxicillin + clavulanic acid, and 0-432 for erythromycin). HA-1 presented lower porosity than HA-2, therefore, because of the higher amount of antibiotic adsorbed on an increased surface area material, the release rate of HA-2 was higher than for HA-1, for the above mentioned period.

$\beta$ -TCP is known to have higher dissolution rate than HA in the physiological environment, thus increasing *in vivo* resorbability [15, 32, 33]. Therefore, similarly to what was found in other studies in the literature [7], the presence of  $\beta$ -TCP in HA-2 and not in HA-1 is related to the adsorption/release behaviour of antibiotics, indicating that the presence of small amounts of  $\beta$ -TCP might have a beneficial effect in the sustained release of the studied antibiotics.

The release kinetics of erythromycin from the microspheres was different from that of amoxicillin and amoxicillin+ clavulanic acid (being the releasing profile of the former two very similar). Erythromycin presented a more effective sustained release, presenting a very high release concentration till 432 h. In accordance with other authors [12, 13, 16], this fact can be explained by differences in the chemical structure between antibiotics, giving rise to different chemical binding (adsorption) between antibiotics and the materials.

The porous structure provided by these nano-HA microspheres, with high surface areas, facilitated sustained release of the tested antibiotics from both types of microspheres. The release kinetics was superior to that reported for other carriers, which presented an initial burst release followed by a very slow release after 24 h, usually below MIC values [18, 34-39]. These release profiles enable the HA-microspheres to fulfil an important criterion of any ideal drug delivery system, where initial high doses of drug are required for effective attack on bacteria, followed by a sustained release. The initial doses provide large concentration of antibiotic needed in the period after surgery; on the other hand sustained release provides continuous dosing of antibiotic for long-term treatment.

The aim of the cytotoxicity tests was to examine whether the presence of antibiotics (amoxicillin, amoxicillin + clavulanic acid, and erythromycin) adsorbed to the materials affected the growth of MG63 cells. This cell line has previously been used in biocompatibility studies, because it exhibits a number of features similar to those of typical human osteoblasts [40]. The culture of MG63 cells supplemented with different concentration of the three antibiotics showed that there was an increase in cell number as compared with similar cells cultured without antibiotic. This effect was more marked for erythromycin supplemented cultures, than for the other two antibiotics, indicating that the presence of these antibiotics, particularly erythromycin, may have a beneficial effect in cell proliferation.

The present study also showed that cultures of MG63 cells on HA-1 and HA-2 microspheres, where amoxicillin, amoxicillin + clavulanic acid, and erythromycin were adsorbed, presented higher percentages of viable cells than these cells on the same materials without antibiotic. These results correlate well with those found for the MG63 cultures supplemented with the same antibiotics, once

that, as it may be seen on Figure 5, the improvement on cell proliferation is concentration dependent. Therefore, the higher antibiotics release rate of HA-1 microspheres when compared with HA-2 in early periods, gave rise to a higher enhancement in cell proliferation on HA-1 than on HA-2, if we consider the antibiotics effect.

In fact, the total amount of viable cells is very similar for HA-1 and HA-2 microspheres once that because of the higher surface area of HA-2 compared to HA-1 in the absence of antibiotics, the former presented lowed values of viable cells. In both cytotoxicity tests, erythromycin showed a higher beneficial effect in osteoblasts proliferation.

## **5. Conclusion**

Microspheres prepared from two types of HA nanocrystallites aggregates have shown to have chemical compositions, porosities and surface areas that provided adequate conditions for the sustained release profile of amoxicillin, amoxicillin + clavulanic acid, and erythromycin. Bactericidal tests have indicated that during the period of this study the released antibiotics had inhibitory effect on bacteria. HA granules did not present a good alternative as a delivery system for the tested antibiotics. The concentration of antibiotics released in the first days from HA-2 was higher than from HA-1 microspheres, because of the increased porosity and surface area. The release profiles (fast initial release followed by a long-term sustained release) of effective doses of antibiotics indicated that both alternatives were good alternatives for a delivery system of the studied drugs.

Osteoblastic cells proliferate well on the surface of both types of microspheres, being cell growth enhanced by the presence of antibiotics. Erythromycin was the antibiotic presenting a higher beneficial effect.

Combining the sustained antibiotic release with the osteoconductive properties, resorbability of the porous HA microspheres, and potential use as injectable bone filling material, these systems provide a forth fold beneficial effect. These microspheres may therefore be considered as potentially good alternative systems to act as carriers for antibiotics and to enhance bone regeneration while treating periodontitis.

## **Acknowledgments**

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## CHAPTER V - COMPARATIVE STUDY OF NANOHYDROXYAPATITE MICROSPHERES FOR MEDICAL APPLICATIONS

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**Abstract.** The present study concerns the preparation, physical and *in vitro* characterisation of two different types of hydroxyapatite (HA) microspheres, which are intended to be used as drug delivery systems and bone regeneration matrices. Hydroxyapatite nanoparticles (HA-1 and HA-2) were prepared using the chemical precipitation synthesis with H<sub>3</sub>PO<sub>4</sub>, Ca(OH)<sub>2</sub> and a surfactant, SDS (sodium dodecyl sulfate), as starting reagents. The HA powders were dispersed in a sodium alginate solution and spherical particles were obtained by droplet extrusion coupled with ionotropic gelation in the presence of Ca<sup>2+</sup>. These were subsequently sintered to produce HA-1 and HA-2 microspheres with a uniform size and interconnected microporosity. Both powders and microspheres were characterised using FTIR and X-ray diffraction. Moreover, SEM and mercury intrusion porosimetry were used to analyse the microspheres and TEM was used to analyse the powders. Results showed that pure hydroxyapatite (HA) and mixtures of HA/β-TCP in the nanometer range and needle-like shape were obtained for HA-1 and HA-2 powders, respectively. Neutral red, scanning electron microscopy and confocal microscopy were used to evaluate the

behaviour of osteoblastic-like MG-63 cells cultured on HA microspheres surfaces for 7 days. Results showed good adhesion and proliferation of osteoblasts on the HA microspheres surface. Cells built bridges between adjacent microspheres, forming microspheres-cells clusters in both types of materials.

## 1. Introduction

Many biomaterials have been studied to repair bone defects or restore bone tissue functionality. During the last two decades, extensive research on ceramic materials like calcium phosphates, has shown that these materials are suitable as bone substitutes due to their biocompatible, bioactive, biodegradable and osteoconductive characteristics (1-5) and when implanted *in vivo*, they are non-toxic and they do not induce any antigenic response (6).

Bone exhibits natural hydroxyapatite crystals with needlelike or rodlike shapes, well arranged within the polymeric matrix of collagen type I. These natural nanoparticles formed in physiological environment have a more dynamic response when compared to synthetic material with larger particle size (7). In order to prepare fine HA powders, many chemical processing routes have been employed, including hydrothermal reactions (8-10), sol-gel synthesis (11-13), pyrolysis of aerosols (14,15) and micro-emulsion (16,17), biomimetic process (18,19), and chemical precipitation (20-24), which is the most used alternative.

Recently, sodium alginate has been presented as an interesting material for biomedical applications, showing capability to be processed under mild conditions (25-28). Alginate, seaweed extract composed of chains of alternating  $\alpha$ -L-guluronic acid and  $\beta$ -D-mannuronic acid residues, is considered by far the most widely used polymer for immobilization and microencapsulation technologies. Sodium alginate is water soluble and may polymerize into a solid matrix. Its processing into microspheres is compatible with the presence of proteins and even bacteria or viruses. Alginate microspheres are an inexpensive, reliable and available agent for antigens encapsulation for example in several animal diseases treatment. Besides, alginate microspheres are stable at low pH and can be prepared to a wide variety of sizes (29). The simple and versatile aqueous based gel formation of sodium alginate in the presence of divalent cations such as calcium has been used to immobilize cells (30), for hybrid artificial organs (31), hemoglobin carriers (32), macromolecular delivery (33-35), and drug delivery systems (35).

This work describes the preparation and initial characterisation of novel hydroxyapatite microspheres intended to be used as a biomaterial for bone regeneration or as enzyme delivery matrices. The purpose of this work is to do the morphological and chemical composition characterisation and the *in vitro* biological assessment of microspheres based on synthesised nanophased hydroxyapatite obtained by two different procedures.

## **2. Materials and Methods**

### **2.1 Synthesis of nanosized Hydroxyapatite (HA)**

Aqueous solutions of calcium hydroxide ( $\text{Ca(OH)}_2$ ) and ortho-phosphoric acid ( $\text{H}_3\text{PO}_4$ , 85%), both of analytical grade, were used as reactants for the preparation of two different types of HA nanoparticles. HA powder 1 (HA-1) was synthesised according to the following procedure: firstly, 1L of an aqueous suspension of  $\text{H}_3\text{PO}_4$  (0.6M) was slowly added, drop by drop, to a 1L of an aqueous suspension of  $\text{Ca(OH)}_2$  (1M) while stirring vigorously for about 2h at room temperature. Concentrated NaOH was added until a final pH of 10.5 was obtained. The white solution obtained was washed using de-ionized water and dried in oven at  $80^\circ\text{C}$  for 24 h. HA powder 2 (HA-2) was synthesized in the same way, with the difference of the addition of sodium dodecylsulfate addition (10g) into the calcium solution.

### **2.2 Preparation of HA microspheres**

Microspheres were prepared using a methodology previously described (32, 34). Briefly, pharmaceutical-grade sodium alginate (Protanal 10/60 LS) with a high  $\alpha$ -L-guluronic acid content (65-75%) was kindly donated by Pronova Biopolymers and used without further purification. 1.5g of Na-alginate was dissolved in 50mL of distilled and deionized water until total homogenization, where upon nanophased hydroxyapatite powder was added to the alginate solution in a ratio of 2:8 w/w until thorough dispersion. This paste was extruded using a droplet generator device (Encapsulation Unit Var 1-J1, Nisco) into a 0.1M  $\text{CaCl}_2$  crosslinking solution, where spherical-shaped particles instantaneously formed, and were allowed to harden for 30 min. The size of the microspheres was controlled by regulating the extrusion flow rate, using a single syringe infusion pump (EW 47-900-00, Cole Palmer) and by applying a coaxial air stream. The extrusion flow rate for HA-1 was two fold the flow rate for sample HA-2. At completion of the gelling period, microspheres were recovered and rinsed in water to remove the excess  $\text{CaCl}_2$ . Finally they were dried overnight in a vacuum oven at  $30^\circ\text{C}$ , and sintered at  $1100^\circ\text{C}$  for 1h (36)

### **2.3 Characterisation of nanosized HA**

#### *2.3.1 Infrared spectroscopy*

Chemical characterisation was performed using Fourier transformed infrared (FTIR) spectroscopy. For this purpose, hydroxyapatite was reduced to powder and analyzed as KBr pellets using a PerkinElmer System 2000 spectrometer.

#### *2.3.2 Transmission electron microscopy – TEM*

The size and morphology of the HA powders was determined using a Transmission electron microscope (TEM, Zeiss model EM 902A) at an accelerating voltage of 80kv. A HA solution was prepared by dispersing 0.5mg of HA in 1mL of ethanol. This was placed in an ultrasonic bath overnight, to ensure thorough dispersion. A drop of the solution, agitated for 1min in vortex, was

placed on a Formvar carbon-coated copper 400 grid and left for 1 min. Excess liquid was removed with filter paper and the grid was allowed to dry for 10 min. TEM images were acquired and observed with Axion Camera Zeiss and AxionCs 40AC Zeiss, v 4.2.0.0 software.

#### *2.3.3 X-ray diffractometry – XRD*

X-ray diffraction characterisation of HA-1 and HA-2 powders was conducted using a Panalytical X'Pert PRO alpha-1 with a RTMS X'Celerator detector. It used Ni-filtered Cu K $\alpha$  radiation over the 2 $\theta$  range of 10-90° at a scan rate of 2.4° /min and with a sampling interval of 0.002° at 40 mA and 45 kV.

### **2.4 Characterisation of nanoHA microspheres**

#### *2.4.1 Inverted stand optical microscope analysis*

The size of the microspheres was estimated using an inverted stage microscope (Olympus PME3-ADL) with an eye piece lens equipped with an micrometer rule, with an accuracy of 10  $\mu$ m. The average diameter was determined from 20 microspheres for each material.

#### *2.4.2 Infrared spectroscopy*

Chemical characterisation was performed using Fourier transformed infrared (FTIR) spectroscopy. For this purpose, nanoHA microspheres were reduced to powder and analyzed as KBr pellets using a PerkinElmer System 2000 spectrometer.

#### *2.4.3 Scanning electron microscopy analysis – SEM*

The microspheres morphology was analyzed by digital imaging and scanning electron microscopy (SEM). Using a JEOL JFC-100 fine coat ion sputter device, samples were sputter coated with gold. Then they were studied with a JEOL JSM-6301F microscope at an accelerating voltage of 5 kV.

#### *2.4.4 Mercury porosimetry method*

Mercury intrusion porosimetry equipment (Quantachrome Poremaster model No. 60) was used to determine surface area, pore number fraction, and porosity from both types of microspheres. Samples were dried at room temperature; about 1.8g dried microspheres were used in this determination and Helium was used for gas adsorption. The data reported were obtained using Quantachrome Poremaster for Windows, version 3.0.

#### *2.4.5 X-ray diffractometry – XRD*

X-ray diffraction characterisation of HA microspheres was conducted using a Panalytical X'Pert PRO alpha-1 with a RTMS X'Celerator detector. It used Ni-filtered Cu K $\alpha$  radiation over the 2 $\theta$  range of 10-90° at a scan rate of 2.4° /min and with a sampling interval of 0.002° at 40 mA and 45 kV.

## 2.5 Biological Assessment

MG-63 cells were cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air, in  $\alpha$ -MEM (GIBCO 22561-021), 10% fetal bovine serum (FBS), 1% fungizone and 0.5% gentamicin. For the subculture, the cell monolayer was washed twice with phosphate buffered saline and incubated with trypsin-EDTA solution (0.05% trypsin, 0.25% EDTA) for 5 min at 37°C to detach the cells. Cells were re-suspended in culture medium for re-seeding. Microspheres were sterilized at 120°C for 20 min, placed on a 96 PS culture plate (14 mg of microspheres in each well, *c.a* 1cm<sup>2</sup> of surface area) and incubated with PBS overnight. Cells were then seeded on microspheres pre-conditioned with  $\alpha$ -MEM with 10%FBS, 1% fungizone and 0.5% gentamicin. MG-63 cells were cultured ( $2 \times 10^4$  cells/cm<sup>2</sup>) up to 7 days under controlled conditions (cells seeded on 96 TCPS plates) and on the surface of both type of microspheres. The control cultures and seeded microspheres were evaluated throughout the incubation time (7 days) for cytotoxicity (Neutral Red) and observed by confocal microscopy (CSLM) and scanning electron microscopy (SEM). Medium was substituted by fresh one every other day.

### 2.5.1 Cytotoxicity tests (*Neutral Red*)

Cytotoxicity of HA-1 and HA-2 microspheres was investigated on MG-63 osteoblast-like cells, which express a number of features characteristic of relatively immature osteoblasts (34) using Neutral Red assay. A 0.4% w/v solution of neutral red dye tested for cell cultures (Color Index 50040) in distilled water was prepared and stored at 4°C (stock solution). The working solution was prepared immediately before use by diluting the stock solution in complete medium to yield a final concentration of 50  $\mu$ g/mL. Five replicates were performed for each type of microspheres. The negative control was microspheres in culture medium in PS 96 wells culture plates. The positive control was MG-63 in culture medium in 96 wells TCPS culture plates. After incubation for 1, 3, 5, and 7 days at 37°C, the supernatant was discarded, 0.150 mL of neutral red dye solution were added to the wells for 3 h at 37°C. The supernatant was removed, the wells washed twice with PBS and 0.1 mL of lysing solution (50% ethanol in 1% acetic acid) was added. The color intensity of each well was read at 540 nm with reference to 620nm, the optical densities of the replicates were averaged and the viability read in negative control was discounted.

### 2.5.2 Cell morphology (*SEM and CSLM*)

For SEM, microspheres-cells constructs were washed twice with PBS and fixed with 1.5% glutaraldehyde in 0.14% cacodylate solution for 30min at room temperature. They were dehydrated by suspending in successive ethanol solutions (50%, 70%, 80%, 90%, and 100%) for 10min in each solution. After dehydration, the samples were transferred to hexamethyldisilazan and air-dried at room temperature overnight. Finally, the microspheres-cells constructs were sputter coated with gold and examined under a JEOL JSM-6301F microscope at accelerating voltage of 5kV.

For CSLM, samples were washed twice with PBS, fixed in 4% v/v formaldehyde (methanol-free; Polyscience) for 15 min, permeabilized with 0.1% v/v Triton X-100 for 5 min, and incubated in 10 mg/mL bovine serum albumin and 100  $\mu$ g/mL RNase for 1h at room temperature. F-actin

filaments were stained with Alexafluor-conjugated phalloidin (Molecular Probes) for 20 min at RT in dark, and nuclei were counterstained with 10  $\mu\text{g/mL}$  propidium iodide (Sigma) for 15 min. Finally, samples were washed with PBS, mounted in Vectashield® and stored at 4°C in dark. CLSM images were acquired on a BioRad MRC 600 microscope.

### 3. Results

The FTIR spectra of powders and microspheres using HA-1 and HA-2 materials are represented in Figure 1. It may be seen that crystallinity increased from HA-1 powder to HA-1 microspheres, especially on the phosphate bands at 560-633  $\text{cm}^{-1}$  and around 1030  $\text{cm}^{-1}$ . Occluded water gives rise to a band at 1420-1650  $\text{cm}^{-1}$  observed in the HA-1 powder and vanished after the sintering process for HA-1 microspheres. The presence of the  $\text{CO}_3^{2-}$  group is observed in the HA-1 powder and lost in the spectra of microspheres, which suggests that  $\text{CO}_3^{2-}$  could be not into the crystalline network but on the surface of the material. This last sample presented higher crystallinity than the original powder material. Both HA-1 and HA-2 materials can be easily recognized as non-stoichiometric hydroxyapatites. In the spectra of the HA-2 powder a bands appears at 472 (v2), 563 (v4), 605, and 960 (v1)  $\text{cm}^{-1}$  that correspond to the  $\text{PO}_4^{3-}$  group. On the other hand, bands are present at 1642, 1454 (v3), 1420 and 875  $\text{cm}^{-1}$  due to the presence of carbonate group, that is missing after the sintering process similarly to HA-1 materials. The O-H stretching OH- is observed in the band at 3571  $\text{cm}^{-1}$  and the O-H stretch H<sub>2</sub>O is shown in the band 3432  $\text{cm}^{-1}$ . HA-2 microspheres are characterized by sharp, well-resolved bands at 3571, 602 and 571  $\text{cm}^{-1}$ . The most intense feature in the IR spectra is the 1041  $\text{cm}^{-1}$  band with some shoulders at 1118, 971 and 945  $\text{cm}^{-1}$  suggesting the presence of two phases; HA and  $\beta$ - TCP. The band at 1089  $\text{cm}^{-1}$  corresponds to P-O stretching vibrations. The broad bands at 3497 and 1992  $\text{cm}^{-1}$  is due to the presence of both water and structural hydroxyl groups. The bands at 3572 and 633  $\text{cm}^{-1}$  have been assigned to stretching and bending vibrations of the structural hydroxyl group.

Figure 2 shows the morphology of the nanoparticles after the sintering process, of both materials, that generate the microspheres. From TEM images in Figures 2a and 2b some agglomerates of HA and the needle like shape of the particles in both materials may be seen. HA-1 and HA-2 samples present small agglomerates where the nanometer size (less than 100nm in length) of the HA particles is observed.

The alginate/hydroxyapatite beads were formed by dropping the paste into an aqueous solution of calcium ions. During the sintering process, the alginate was burned off and the hydroxyapatite particles became associated, maintaining the original spherical shape. The average diameter of HA microspheres varied depending on the type of HA used, thus, for HA-1 samples it was  $471 \pm 23 \mu\text{m}$  ( $n=20$ ) whereas for the HA-2 samples it was  $407 \pm 42 \mu\text{m}$  ( $n=20$ ). HA-2 microspheres shrank to ca. 19% of their original size after the sintering process, whereas the shrinkage for HA-1 microspheres was of ca. 28%.

SEM images of the microspheres are presented in Figure 3. After the sintering process their size is decreased due to alginate burn-off, even though their original shape is maintained. HA microspheres are depicted in Figures 3a and 3b, illustrating their spherical shape as well as their

uniform size. Higher surface roughness is evidenced in HA-2 microspheres when compared to HA-1 (Figures 3c and 3d). Interconnectivity seems to be higher in HA-2 than in HA-1 microspheres. This can be due to the thickness of the walls that surround pores (Figures 3e and 3f).

Data obtained from mercury intrusion porosimetry analysis, which are in line with the above results (Table 1), do not show significant differences between both types of microspheres related to the pore surface area, apparent density or total intraparticle porosity. Porosity does not appear to be a significant HA-2 feature in comparison with HA-1 microspheres, however, evident differences about diameter, pore volume and pore fraction number are found, all being higher for HA-2.

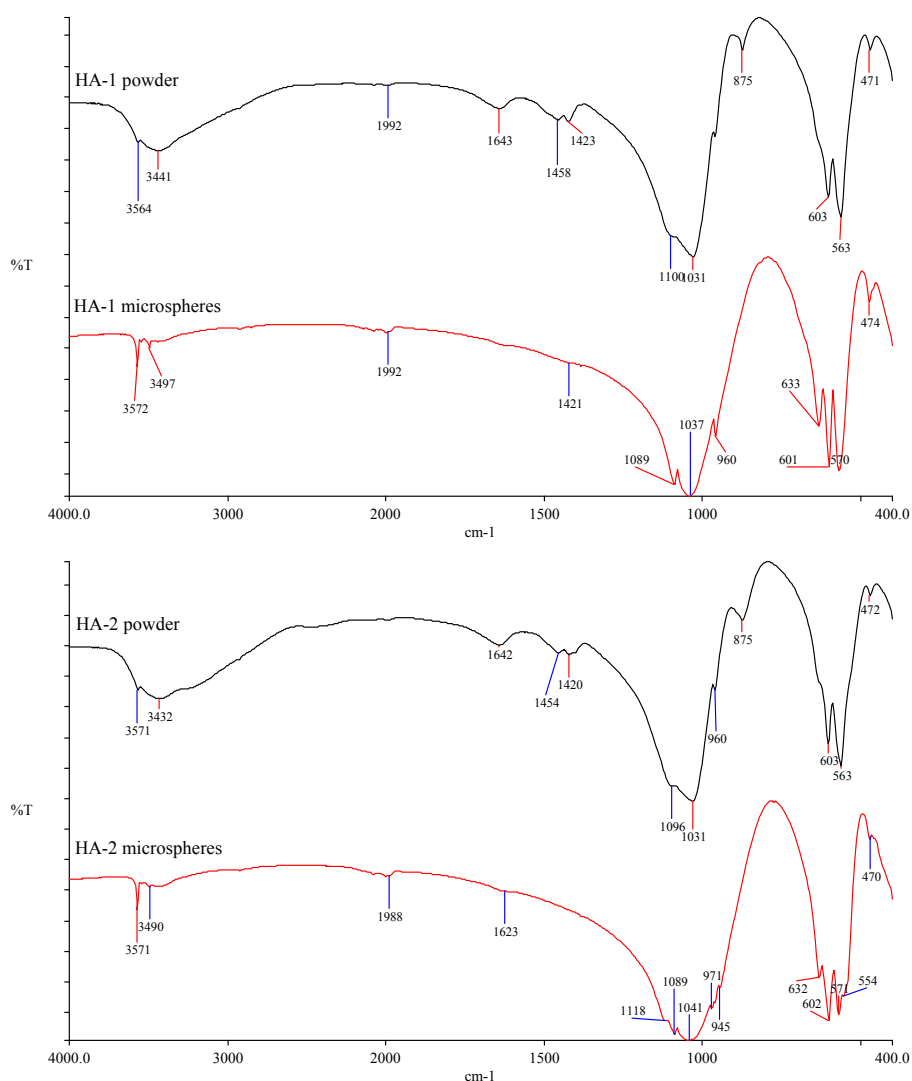


Figure 1. FTIR spectra of HA-1 and HA-2 samples (powders and microspheres)

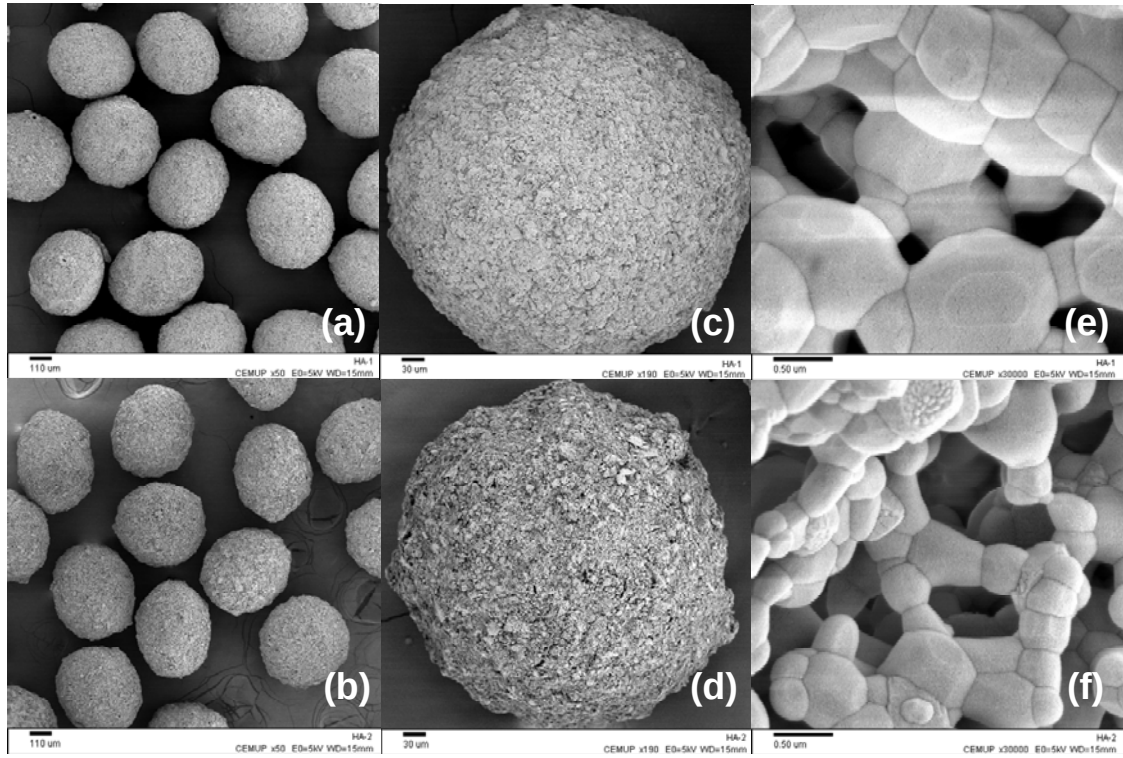
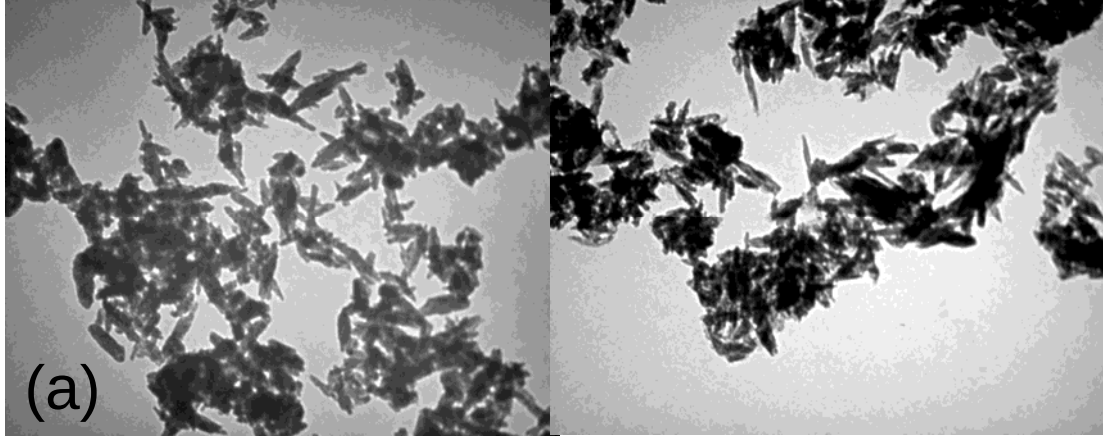


Figure 3. SEM micrographs of HA-1(a,c,e) and HA-2 (b,d,f) microspheres: a,b (x50) c,d (x190) and e,f (x30000).

XRD patterns of all samples are shown in Figure 4. HA-1 powder is represented by the pattern in Figure 4a and some undefined humps can be seen, due to the non-sintered state. Figure 4a and pattern c present many similarities even though after the sintering process they revealed the presence of different phases. The pattern in Figure 4b shows clearly the peaks corresponding to pure monoclinic HA, however, Figure 4d shows a mixture of monoclinic HA and  $\beta$ -TCP.

Table 1. Mercury porosimetry

| Determinations / Samples                     | HA-1    | HA-2    |
|----------------------------------------------|---------|---------|
| Diameter average ( $\mu\text{m}$ )           | 0.5311  | 0.8211  |
| Pore volume ( $\text{cc/g}$ )                | 0.1507  | 0.1876  |
| Pore surface area ( $\text{m}^2/\text{g}$ )  | 0.4958  | 0.5444  |
| Pore number fraction                         | 0.00196 | 0.00336 |
| Total surface area ( $\text{m}^2/\text{g}$ ) | 1.9308  | 1.4023  |
| Apparent density ( $\text{g/cc}$ )           | 1.7653  | 1.6726  |
| Total intraparticle porosity (%)             | 45.063  | 47.571  |
| Theoretical porosity (%)                     | 45.052  | 45.871  |

In the present work, the cytotoxicity tests of HA microspheres were performed using human MG-3 osteoblast-like cells line. Results presented in Figure 5 showed that there is continued growth during the culture time using both HA-1 and HA-2 microspheres. Cells seeded on TCPS, used as a control, showed the same trend, but with a higher growth rate than both microspheres. The control presented less number of viable cells in the first day probably due to the less available surface area compared to the microspheres. The HA-1 microspheres presented a slight increment in the number of viable cells in comparison with HA-2 microspheres at the end of the culture, and higher growth rate as well.

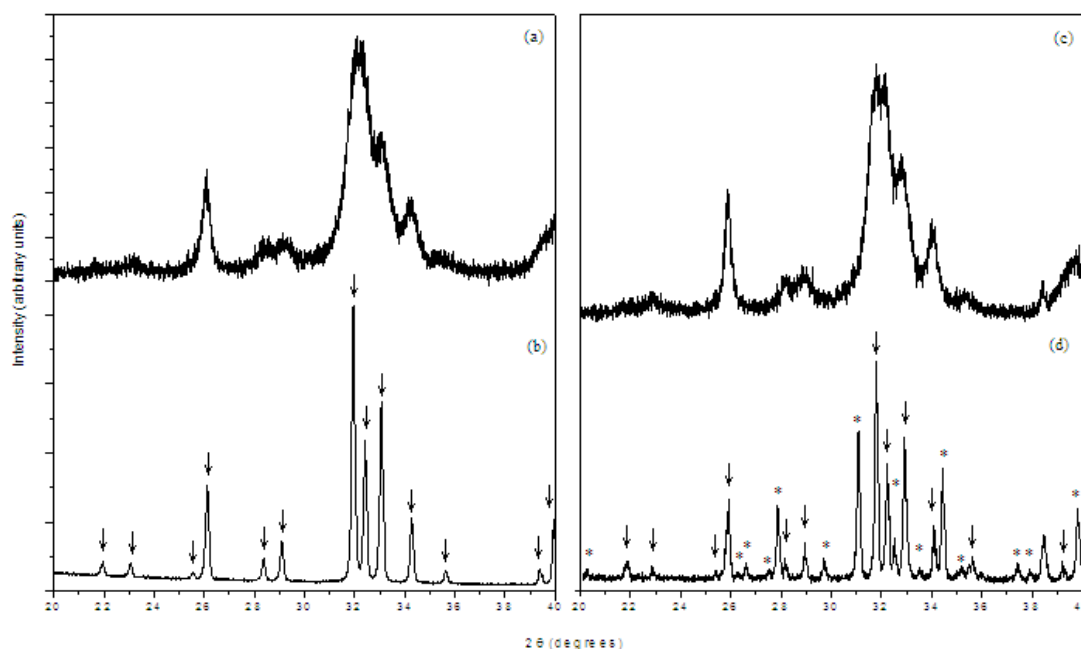


Figure 4. X-ray diffraction of powders and microspheres of HA-1 and HA-2. (a) HA-1 powder, (b) HA-1 microspheres, (c) HA-2 powder, (d) HA-2 microspheres. ↓: monoclinic HA, \*:  $\beta$ -TCP.

SEM images of MG-63 osteoblast cells cultured for 7 days on the surface of HA-1 and HA-2 microspheres based on nanosized hydroxyapatite are presented in Figure 6. The surface of both HA-1 and HA-2 microspheres is almost completely covered by layers of cuboidal cells which became more dense along the entire time of culture, forming confluent monolayers. Cells built bridges between neighbouring microspheres (HA-1 and HA-2 microspheres) forming microspheres–cells clusters (See Figure 7). CLSM images in Figure 8 show HA-1 and HA-2 microspheres uniformly colonized by osteoblasts cells after 5 days of culture.

#### Statistical analysis

Data are presented as mean  $\pm$  standard deviation ( $n=5$ ) and were analyzed using the Student t-test for independent samples. Differences between groups were considered statistically different when  $p < 0.05$ .

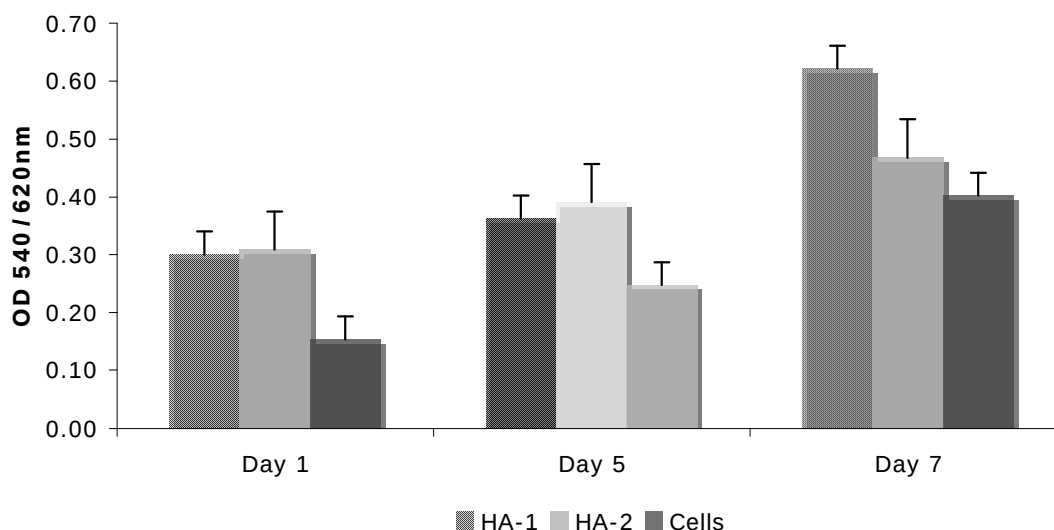


Figure 5. MG-63 cells culture on microspheres based on hydroxyapatite nanosized

## 4. Discussion

In this work nanoHA microspheres synthesis was carried out, using two different types of nanosized hydroxyapatite, and a subsequent characterisation at morphological and physical level was performed. HA microspheres were prepared using the droplet extrusion process combined with ionotropic gel formation in the presence of calcium ions. The methodology used to prepare HA microspheres has a remarkable advantage as it allows the preparation of spherical-shaped particles with an adequate and uniform size (34, 36-39).

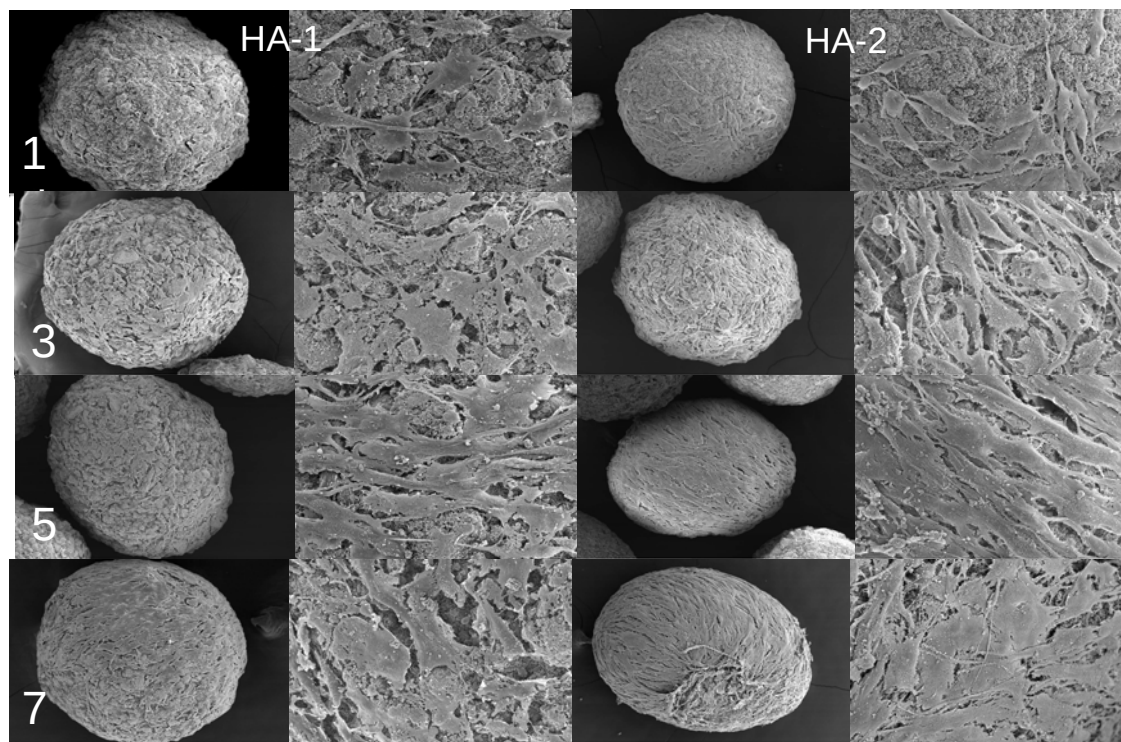


Figure 6. SEM micrographs of 1 to 7 days of MG-63 cells culture on microspheres HA-1 and HA-2 (x180 and x1000).

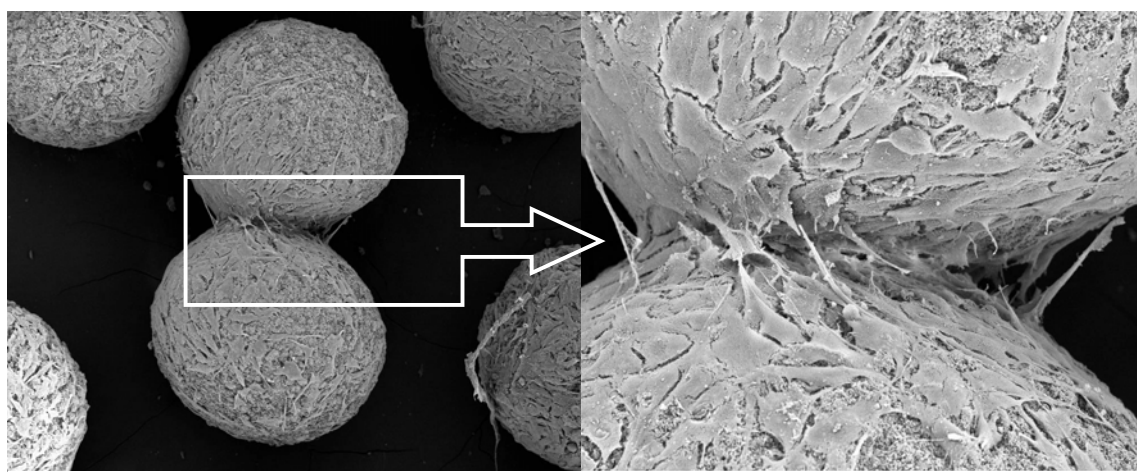


Figure 7. SEM micrographs (left x110 and right x1000). Notice that osteoblast cells built bridges between neighbors microspheres. This image corresponds to HA-1 construct after 3 days.

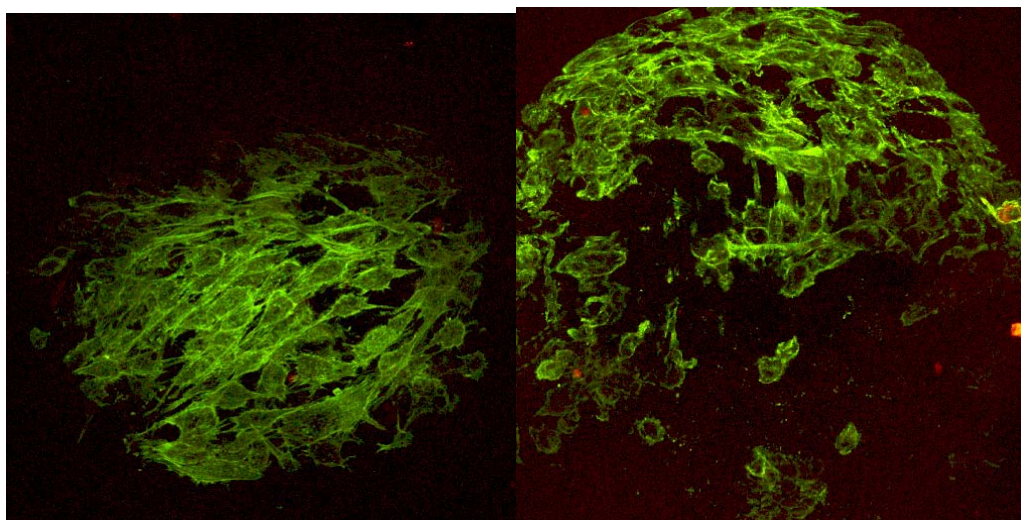


Figure 8. CLSM images of HA-1 (left, x100) and HA-2 microspheres (right, x100) respectively after 5 days of MG63 culture.

The microspheres described in this work are composed of two types of hydroxyapatite nanoparticles, both processed by chemical precipitation, and differing in the SDS addition. The synthesis of hydroxyapatite can take place through a variety of processes. One such method is the wet chemical route, where hydroxyapatite is precipitated from either an acid-based reaction or a reaction between various salts. Among the different synthesis methods, the wet or precipitation method of synthesizing HA has proved to be popular and for this reason this preparation method has been extensively published. The chemical precipitation method involves the addition of phosphate ions to a solution of calcium ions, thereby precipitating HA as well as other calcium phosphates such as  $\beta$  tricalcium phosphate,  $\beta$ -TCP.

Many chemical processing routes have been employed to prepare fine and agglomerate-free ceramic HA. For that reason several dispersants have been successfully used to prepare HA particles (40, 41) with smaller size and higher surface area (42). In the present work, a surfactant (SDS) assisting the chemical precipitation method was found to be able to promote the formation of nanosized HA crystal and at the same time control the agglomeration of particles.

Sodium dodecylsulfate (SDS –  $C_{12}H_{25}NaO_4S$ ) is an ionic surfactant and probably the most researched anionic surfactant compound. The molecule has a tail of 12 carbon atoms the attached to a sulfate group, giving the molecule amphiphilic properties. In the current study SDS could have worked by disrupting inter-particles bonds, thereby de-agglomerating them. Also, anions of SDS could effectively impart a negative charge on the particles and create an electrostatic repulsion creating more isolate particles. This same fact could explain the presence of two different phases in the HA-2 material based on SDS: the electrostatic repulsion could have limited the complete reaction between reagents and favour the production of  $C_{10}(PO_4)_6(OH_2)$  and  $\beta$ - $Ca_3(PO_4)_2$ . Furthermore, the presence of SDS into the calcium phosphates preparation could have filled some spaces that after the burn-out process were empty, creating higher porosity and interconnectivity in HA-2 more than in HA-1 microspheres (Figures 3e and 3f).

Even though the role played by SDS in the production of HA-2 material is still unknown, it is not part of the aim of this work and it will need further studies.

The characterisation analyses included FTIR, TEM, SEM, mercury porosimetry and XRD and *in vitro* tests. FTIR analysis indicated that the characteristic spectra bands of both ceramics, HA-1 and HA-2 powders, are maintained in the microspheres, suggesting the sodium alginate did not induce subsequent modifications in the ceramics structures, for instance the addition of  $\text{CO}_3^{2-}$  group, may be due to alginate, in the HA lattice, probably due to the fast sintering cycle which provides higher porosity in comparison with longer cycles, or it may also derive from the contact with the atmosphere during HA processing. Therefore, alginate may have played two roles in the microspheres preparation: as a vehicle to form spherical shape particles and as pores inducing agent in the sintering process, all of this without any modification on HA. It is important to point out the multiple binding sites on the microsphere surface that could allow the attachment of certain macromolecules such as enzymes or antibiotics, among others. This feature may enhance its role as a drug delivery system component to be used in biomedical applications.

Attractive advantages such as avoiding the use of organic solvents of difficult removal, the manufacturing spherical particles with uniform size, and the possibility of producing particles with different porosities (28,36) are some characteristics related to microspheres prepared using the method referred here. Besides, in the present work nanophasic HA was used to prepare microspheres causing a decrease in diameter and therefore a reduced surface area; in contrast to previous studies where micrometric commercial HA and calcium-titanium-phosphate (CTP) were used as raw materials (34).

The use of nanostructured HA in the microspheres introduced a new question mark about how would bone cells behave when in contact with this new type of microspheres, since existing literature related to microspheres based on nanosized particles is scarce. Even though, recent works have indicated the benefits of using nanoHA in several biomaterials different to microspheres. To follow some few examples: adhesion of osteoblasts on non-functionalized with RGD nanoparticulate calcium phosphate was similar to conventional HA functionalized with RGD (43); fibrous scaffolds fabricated by electrospinning from polycaprolactone (PCL) solutions containing nanoparticles of hydroxyapatite promoted much better adhesion and proliferation of cells than the corresponding TCPS (44); biocompatibility testing of nanoparticles dispersed within a viscous polysaccharide (dextran sulfate) porous sponge showed retention of human bone marrow stromal cells viability, production of extracellular matrix and alkaline phosphatase expression (45); investigations about the effect of nanometer HA on the proliferation and osteogenic differentiation of periodontal ligament cells showed that nanoHA has better ability to promote the activity of osteogenetic differentiation for periodontal ligament cells, when compared with dense HA (46).

The cell culture tests with MG-63 were carried out in this work aiming at analyzing possible differences in behaviour resulting from the significant changes in HA crystallites size, and at comparing the two types of material, HA-1 and HA-2 as substrates for cells.

The present studies suggest that features such as composition and roughness can modulate osteoblast responses to these scaffolds *in vitro*, including cell proliferation, attachment, and three-

dimensional structure of the forming osteoblast multilayer network. Differences in composition (nanophasic HA and HA/ $\beta$ TCP) and evident roughness (see Fig. 3,) higher in HA-2 than HA-1 are the most relevant characteristics, since pore diameter or porosity do not present significant differences.

After 7 days of culturing, the distribution of cells over the HA microspheres appeared similar, suggesting that the cells on both HA-1 and HA-2 were proliferating with cuboidal and well-attached morphology colonizing uniformly both type of microspheres, even though HA-1 microspheres presented a slight increment in the number of viable cells in comparison with HA-2 microspheres at the end of the culture as well as higher growth rate.

Albeit, several authors (47-49) have shown that surface roughness is an important parameter in the basic biologic responses improving cell attachment and proliferation; in this study HA-1 microspheres presented slightly higher cell viability than HA-1, whereas HA-2 microspheres presented higher roughness than HA-1. This fact could be related to the chemical composition (HA or HA/ $\beta$ -TCP) revealing that this factor would have more influence than roughness on the cell response.

## **5. Conclusions**

Preparation and initial characterisation of two different types of hydroxyapatite nanosized microspheres (HA-1 and HA-2) intended to be used as drug delivery systems and bone regeneration matrixes were studied in the present work. SEM images showed that MG-63 cells, cultured for 7 days, attached and spread through the available surface of these two types of porous microspheres with some orientation, exhibiting adequate osteoblasts morphology. Neutral Red assay results revealed higher cell viability in the HA-1 than in HA-2 microspheres.

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## CHAPTER VI - SPARC (SECRETED PROTEIN ACIDIC AND RICH IN CYSTEINE) IN COLLAGEN/CALCIUM PHOSPHATES SCAFFOLDS FOR BONE ENGINEERING

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**Abstract.** A new biomaterial to be employed in bone engineering was designed. Seven different types of scaffolds were prepared based on collagen, calcium phosphates, nanohydroxyapatite and SPARC (secreted protein acidic and rich in cysteine). Physical (pore diameter, compression test and calcium concentration analysis) and *in vitro* (cell viability, DNA content measurement and histology analysis) characterizations were carried out. Results presented that SPARC played an important role in the preparation of this new type of scaffolds for tissue engineering due to its calcium and collagen binding sites, and its performance *in vitro* suggests a remarkable influence of SPARC on cellular adhesion and proliferation destined for osteogenesis.

### 1. Introduction

In bone biology is known that the organic matrix, called osteoid, is the one third of bone in weight and is formed mainly by proteins where collagen represents 90% of bone [1] of which 95% is collagen type I, 5% collagen type V and there are type III and XII as well in spite in small amounts. Collagen fibers are stabilized by hydrogen and piridoline bridges (aminoacid-aminoacid and hydroxylisins-lysins, respectively). Albeit, collagen has not calcium affinity and consequently, other proteins (such as Osteonectin, also known as SPARC) are involved in the mineral deposition. Glycoproteins are modified by phosphorylation and sulfation in addition of N- and O- linked oligosaccharides depending on the type of tissue [2]. The glycoproteins group represents 25% of non-collageneous proteins and Osteonectin (ON), Alkaline phosphatase (ALP) and RDG proteins are part of this group [3, 4].

SPARC, also called BM40, with a molecular weight of ~43kD, represents around 10% of non-collagenous proteins, exhibiting high affinity for collagen type I, calcium, and hydroxyapatite due to its two high affinity calcium-binding sites (EF-hands) and twelve low calcium-affinity sites [2-7], which is very unusual. SPARC binds to collagen to mediate deposition of hydroxyapatite and influences the progression of cells through the cell cycle binds to platelet-derived growth factor (PDGF) [2]. It seems that SPARC is involved in the regulation of cell adhesion to the matrix, becoming important in the normal mineralization process [5]. Immunocytochemical studies have localized this bone protein within cytoplasm of endosteal osteoprogenitor cells, osteoblasts, newly buried osteocytes and mineralized bone [8]. It has been identified by immunohistochemistry in osteoblast (odontoblast and ameloblast) and at points of association between proteoglycans and collagen fibers [4] suggesting that its major role is to confer stability on the ECM. By extension, SPARC is presumed to exert some control over the orientation and growth of mineral crystals in osteoid [8].

According to the literature, SPARC plays important rolls in different scenarios such as bone morphogenesis, cellular migration and proliferation until bone remodeling [9-11]. Concerning morphogenesis, SPARC was found to be expressed by mesenchymal stem cells and osteoblasts as they begin the process of endochondral ossification [12]; in fracture healing, SPARC has been detected immediately after injury as periostial cells begin proliferating and depositing ECM [13]; and also it has been suggested that SPARC might affect bone turn-over through its effects on both osteoblasts and osteoclasts [9]. In addition, SPARC might serve as a local regulator of chondrocyte response to PDGF, thus serving to limit the response to growth factors [14]; and it can be an useful marker of cell differentiation in bone and mineralizing cartilage [15]. However, some authors have attributed to SPARC two functions as antagonist such as de-adhesion and anti-proliferation [16-19]. The aim of the present work is the development of a new biomaterial based on collagen, calcium phosphates, nanoparticulated HA, and SPARC for bone engineering, using the osteoconductive and osteoinductive properties of those materials; and its physical and *in vitro* characterization to verify its applicability in this field.

## 2. Materials and methods

### 2.1 Synthesis of HA

Aqueous solutions of calcium hydroxide,  $\text{Ca(OH)}_2$  (Merck, Germany) and ortho-phosphoric acid,  $\text{H}_3\text{PO}_4$ , 85% (Aldrich, Germany) both of analytical grade, were used as reactants on the HA powder synthesis according to the following procedure: 100 ml of an aqueous suspension of  $\text{H}_3\text{PO}_4$  (0.12M) was slowly added, drop by drop, to a 100 ml of an aqueous suspension of  $\text{Ca(OH)}_2$  (0.2M) and 100  $\mu\text{l}$  of sodium dodecylsulphate solution -SDS (Amresco, Solon, OH) in 6.26  $\mu\text{l/ml}$  concentration, while stirring vigorously for about 2 h at room temperature (Figure 1). Concentrated NaOH (Aldrich, Germany) and HCl (Merck, Germany) were added until a final pH of 10.5 was obtained. The white solution obtained was washed using de-ionized water and dried in oven at 80°C for 24 h.

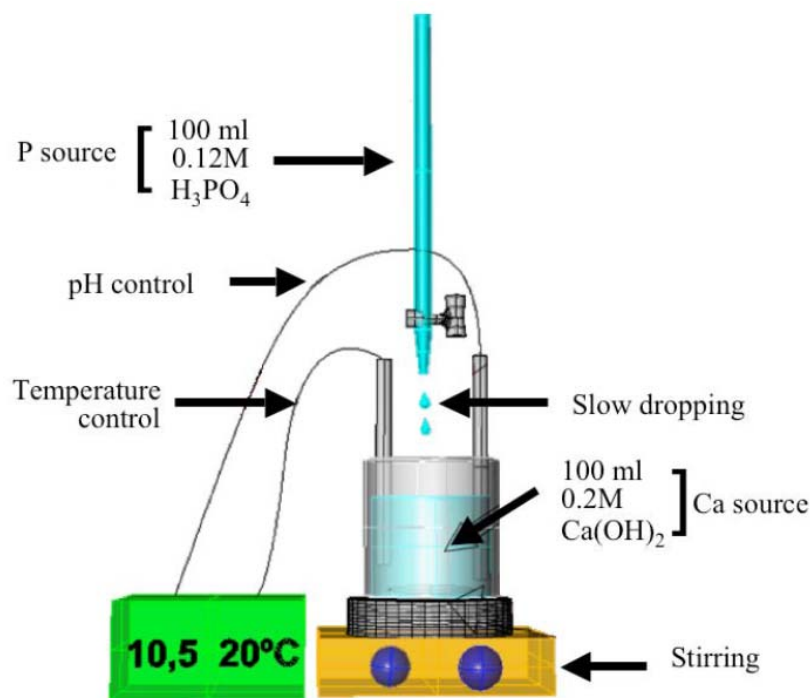


Figure 1. Set-up nanoparticulate hydroxyapatite synthesis.

## 2.2 Preparation of collagen scaffolds

Briefly [20], porous scaffolds were produced by freeze-drying (sublimation of the ice crystals produces the porous scaffold structure [21]) a porcine-derived collagen type I/III slurry (Geistlich Biomaterials, Wouhussen, Switzerland) forming 2 mm thick sheets. Disks, 8 mm in diameter, were sterilized and lightly cross-linked by de-hydrothermal treatment under a vacuum of 50 mTorr and 105°C for 24 h. Collagen scaffolds were hydrated sequentially using 4 ml of solution per scaffold of 100%, 80% and 50% ethanol solutions, and then materials were rinsed twice with sterile water.

From now onwards, seven groups of scaffolds will be mentioned and they appear described in Table 1. These groups of samples were produced according to Figure 2: scaffolds were impregnated using 20  $\mu\text{l}$  of SPARC (Sigma, St Louis, MO) in 4  $\mu\text{g}$  of protein/ml of PBS per each side, and let to rest each side for 30 min.  $\text{Ca(OH)}_2$  (Riedel-de-Haden, Hanover, Germany) and  $\text{H}_3\text{PO}_4$  (Riedel-de-Haden, Hanover, Germany) solutions in concentration of 66.36 mM and 39.74 mM, respectively; were mixed in 1:1 ratio immediately before use, 20  $\mu\text{l}$  of mixture solution were pipetted to each side, and let rest for 20 min. A 20  $\mu\text{l}$  of HA nanoparticles in suspension was added to each side of every scaffold at varying concentrations (1:1, 1:3, 1:5 w/w; groups G5, G6, G7 and G7\*) and let to rest 20 min each side. In a further crosslinking, matrices were immersed for 1 h in a solution (2ml/scaffold) containing 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC, Sigma, St Louis, MO) and N-hydroxysuccinimide (NHS, Sigma St. Louis MO) at a ratio of EDAC:NHS:carboxylic acid (10:4:1) [22]. EDAC and NHS concentrations are based on mean

scaffold mass of 0.002 g and 0.0012 mmol carboxylic acid groups per gram of collagen [23]. After freeze-drying process, samples were hydrated and stored at 4°C until cell culture.

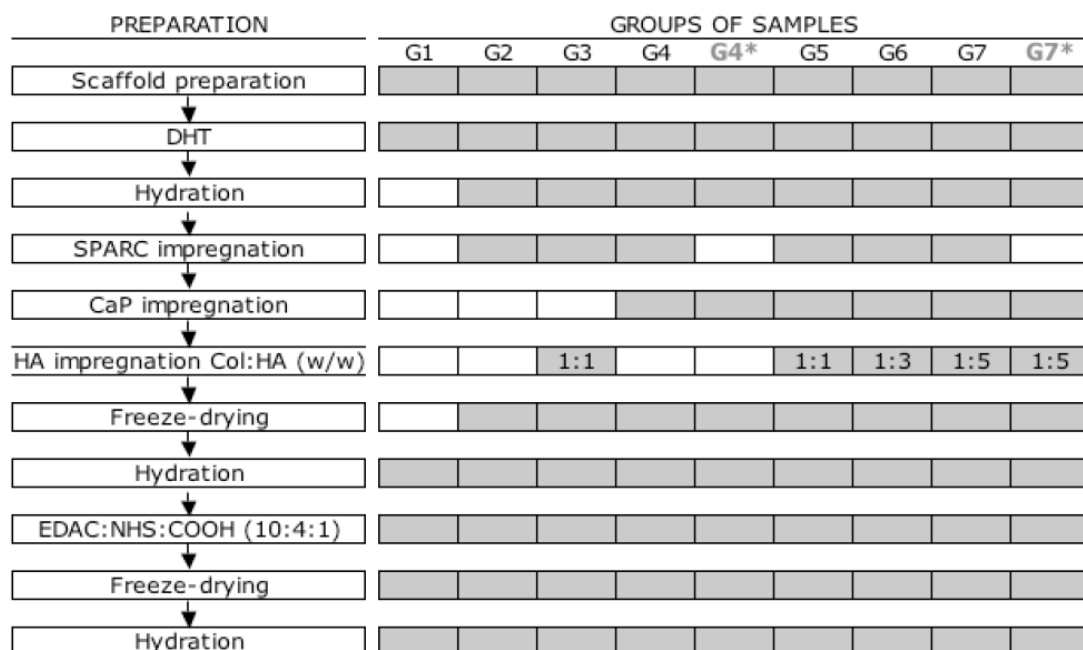


Figure 2. Multi-step process for Col/SPARC/CaP scaffolds preparation G4\* and G7\* groups were prepared for ICP analysis.

## 2.3 Physicochemical characterization

### 2.3.1 Analysis of pore structure

To determine the average pore size of the collagen scaffolds, eight specimens of unseeded samples from collagen group (G1) were routinely embedded using the JB-4 embedding kit (Polysciences, Warrington, PA) and microtomed sections of 6 µm of thickness were stained with Aniline Blue. From digitized light micrographs, pore size and porosimetry percentage were assessed using ImageJ 1.40g free software [24].

### 2.3.2 Trace Element Analysis

Three specimens for each condition were dissolved in conc. HCl, and according to the initial concentration of calcium and phosphorus ions present in each condition, samples were diluted in a 2% of HNO<sub>3</sub> solution to obtain concentrations in the range of 10 – 100 ppm for each element. Quantification was performed using an ICP-OES (Inductively Coupled Plasma – Optical Emission Spectrometer, Activa-S, Horiba Jobin Yvon, NJ). Atoms in the plasma emitted light to detect phosphorus and calcium at 213 nm and 317 nm, respectively. The intensity of the energy emitted at the chosen wavelength was proportional to the element concentration in the analyzed sample, and the values obtained were compared with standards in the range of concentration above mentioned.

Table 1. Groups of scaffold used in the present study. G4\* and G7\* groups were fabricated as controls for ICP analysis.

| GROUP | ABBR.  | COMPOSITE                                     |
|-------|--------|-----------------------------------------------|
| 1     | C      | 1% w/v Collagen I/III (Col)                   |
| 2     | CS     | Col + SPARC                                   |
| 3     | CSHA1  | Col + SPARC + HA (Col:HA = 1:1w/w)            |
| 4     | CSCaP  | Col + SPARC + CaP ([Ca]=66.36mM; [P]=39.74mM) |
| 4*    | CCaP   | Col + CaP ([Ca]=66.36mM; [P]=39.74mM)         |
| 5     | CSPHA1 | Col + SPARC + CaP + HA (Col:HA = 1:1w/w)      |
| 6     | CSPHA3 | Col + SPARC + CaP + HA (Col:HA = 1:3w/w)      |
| 7     | CSPHA5 | Col + SPARC + CaP + HA (Col:HA = 1:5w/w)      |
| 7*    | CPHA5  | Col + CaP + nHA (C:nHA = 1:5w/w)              |

### 2.3.3 Mechanical compression testing

Five specimens of each condition were pre-soaked in distilled water and their mechanical properties analyzed, using a Zwick/Roell Z2.5 static materials tester (Zwick GmbH & Co., Ulm, Germany) with integrated testing software (testXpert, Zwick). The uniaxial compression tester used an unconfined cell employing 20 N of load at a crosshead speed of 0.5 mm/s until 75% of sample compression. The stain – stress curves were plotted for each group of scaffolds: nominal stain (%) and standard force (N) were obtained directly, and the stress (Pa) was calculated from standard force (N) and the scaffold area (m<sup>2</sup>).

## 2.4 In vitro cell culture studies

### 2.4.1 Seeding of GBMSCs on scaffolds

Goat Bone Marrow Stem Cells (GBMSCs) were isolated from the iliac crest of Spanish female adult goats. Cells were cultured until confluence and sub-cultured at P1 and P2 with basic culture medium (alpha-MEM supplemented with 10% of FBS and 1% of penicillin/streptomycin) before seeding onto the scaffolds. These were produced in sterilized conditions and pre-incubated in culture medium overnight on 2% of agar layer, to avoid cell adhesion on the bottom of the wells. GBMSCs were seeded on scaffolds (n=6 per group) in 24-well plates coated with 1ml of 2% agarose to avoid cell adhesion on the bottom of the culture dish. Cells were seeded at 10<sup>6</sup> cells/ per side of each scaffold, pipetting 20 µl of cellular suspension in each side and resting 30 min before addition to the other side. Constructs and un-seeded controls were incubated at 37°C in a humidified

atmosphere of 5% v/v CO<sub>2</sub> in air for 7 and 14 days in osteogenic medium (alpha-MEM supplemented with 10% FBS, 1% penicillin/streptomycin, 50 µg/ml of L-ascorbic acid 2-phosphate, 100 nm of dexamethasone and 10 nm of β-Glycerol phosphate). Osteogenic medium was added to each sample (0.5 ml/well) 2 h post-seeding, followed by another 1 ml 17 hours later. Media were changed every 2-3 days until the cultures were finished at 2 weeks.

#### *2.4.2 Diameter contraction*

The diameter of the cell-seeded scaffolds and un-seeded controls were measured at various points during the 2 weeks culture period using circular templates ranging from 1 to 10 mm diameter in 0.5 mm increments. The change in diameter of the matrices was expressed as a percent reduction in the scaffolds diameter based on the starting diameter.

#### *2.4.3 Cell viability*

The number of viable cells was measured by MTS assay (CellTiter 96<sup>R</sup> AQueous Assay, Promega, Madison, WI). The principal mechanism of this assay is that metabolically active cells react with a tetrazolium salt in MTS agent to produce a soluble formazan dye, which can be absorbed at 490 nm wavelength. Each sample (*n*=6 per group) was rinsed with PBS, followed by incubation in 300 µl of culture medium and 60 µl of MTS for 4h at 37°C in a humidified atmosphere of 5% v/v CO<sub>2</sub> in air. Aliquots of 100 µl from culture medium and samples were pipetted in an opaque 96-well culture plate. Absorbance at 490 nm of the content in each well was measured by a spectrophotometric plate reader (Wallac Victor<sup>2</sup> 1420 Multilabel Counter, Perkin Elmer Life Sciences). The background values from culture medium were subtracted from the obtained samples values.

#### *2.4.4 Cell proliferation evaluation*

Cell-seeded scaffolds (*n*=6), reserved for DNA content assessment were rinsed with PBS and stored at -20°C until they were assayed. After lyophilization, samples were digested overnight in 500 µg/ml proteinase K solution (Sigma Chemical, Saint Louis, MO), at 60°C. Fluorometric (Wallac Victor<sup>2</sup> 1420 Multilabel Counter, Perkin Elmer Life Sciences) assessment of DNA content in digested samples was performed using Quant-iT<sup>TM</sup> PicoGreen dsDNA kit (Molecular Probes, Eugene, OR), which is a sensitive fluorescent nucleic acid stain for quantifying double-stranded DNA in solution, at 485 nm / 535 nm and 1.0 seconds. Prior to DNA quantification constructs were thawed and standards (ranging between 0 and 2 µg/ml) were prepared per each well of an opaque 96-well plate. Twenty microliters from digested samples, diluted 1:10 ratio in Tris-EDTA buffer, were added to 80 µl of Tris-EDTA buffer, and 100 µl of PicoGreen working dye solution were dispensed to each well. The background fluorescence of the matrix was accounted subtracting the mean value obtained for non-seeded scaffolds from the value of each seeded sample. A standard curve was created and sample DNA values were read off from the standard graph.

#### *2.4.5 Histology studies*

After fixation in formalin and subsequently embedment in paraffin, cell-seeded scaffolds were cut to 6 µm thickness and stained routinely with Harris hematoxylin solution (Sigma Aldrich, St Louis,

MO) and eosin Y solution aqueous (Sigma Aldrich, St Louis, MO). Coverslips were applied to the glass slides with Cytoseal (Sigma Aldrich, St Louis, MO).

#### 2.4.6 Statistical Analysis

For statistical analysis, the samples sizes ( $n$ ) refer to the number of scaffolds from each condition used in a particular experiment. Error is reported in figures as the mean  $\pm$  standard deviation (SD). These values were calculated using the 1-way ANOVA statistical technique. This technique assesses the variance between groups, which is then compared against the error variance to test whether it is significantly different. The confidence interval used was 95%.

### 3. Results and discussion

#### 3.1 Analysis of pore structure

Morphometric analysis of the pore structure indicated a pore diameter of  $208 \pm 65 \mu\text{m}$  and porosity of  $89 \pm 3.9 \%$  (Figure 3).

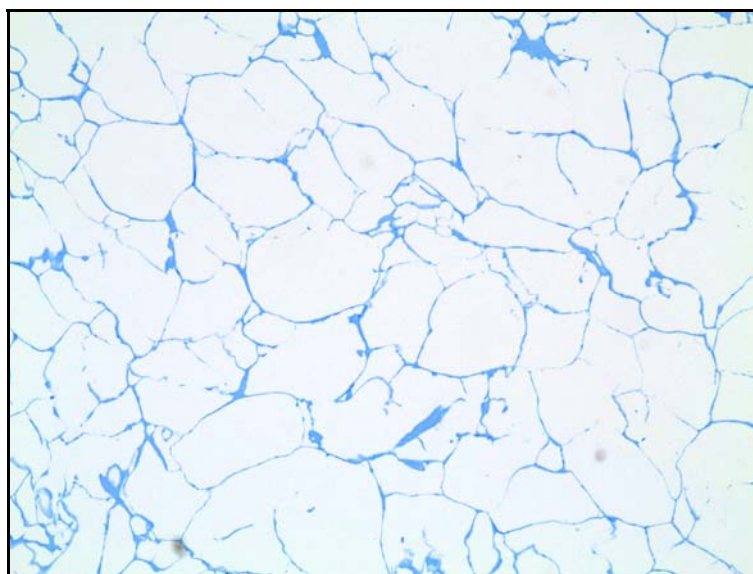


Figure 3. Microtomed section stained with Aniline Blue of  $6 \mu\text{m}$  of thickness, fom collagen scaffolds (G1). 4x, Pore diameter  $208 \mu\text{m}$  and apparent density of  $2\text{E}-5 \text{ g/mm}^3$ , that corresponds to 89% of porosity

#### 3.2 Trace Element Analysis

The quantitative validation was accomplished using inductively coupled plasma (ICP) emission spectroscopy as a destructive method for the determination of the calcium and phosphates content of the scaffolds.

Calcium content in the scaffolds groups (G3, G4, G5, G6 and G7) with the presence of SPARC was notably higher than in groups (G1, G4\* and G7\*) without the addition of SPARC, that were the control samples (Figure 4) as expected due to the high affinity for calcium that SPARC has in two of its four domains.

In Figure 4, G1 and G2 do not show a significant difference because G2 has not any addition of HA that could be attached to the SPARC domains, and for that reason G1 without addition of protein has the same result as G2 in ICP.

Even though G4 in comparison to G4\* has the presence of SPARC, both groups have the presence of calcium phosphates; however none of them showed an increment in the content of calcium or phosphate ions. This fact suggests that calcium ions were attached to the SPARC domains, and phosphate ions did not react with the calcium ions to form HA *in situ* inside to the scaffold. The reason could be that the time of contact between calcium and phosphates ions and/or their concentrations were not enough.

A similar fact is verified for the groups G3 and G5. Although the calcium phosphate solution was not added to the G3 group, G5 presented the same performance as G3, suggesting that the calcium and phosphate ions measured by ICP could belong mainly to HA.

An interesting comparison may be identified from groups G4\*, G7 and G7\*. G4\* and G7\* differed in the absence and highest content of HA, respectively. However, there is not a significant difference when the protein is not added. On the other hand, G7 and G7\* had a huge difference in the ICP results due to the presence of protein in the first one and total absence in the second, suggesting that calcium and phosphate ions attachment depend exclusively on the presence of SPARC on the scaffolds.

G5, G6 and G7 groups contained an increasing concentration of HA nanoparticles and, as expected, G5 and G7 were the groups with the lowest and highest content of both ions, respectively. G7, with 1:5 (w/w) collagen/HA, presented the best performance among all scaffolds prepared for this study. Generally speaking, HA has a Ca/P ratio of 1.67; however, the ICP results presented higher Ca /P ratios for G3, G5, G6 and G7 than the rest of the groups which showed very low ratios. The Ca/P average ratio was  $2.17 \pm 0.06$  for G3, G5, G6 and G7 groups and for G4 and G4\* was  $0.6 \pm 0.06$ . Subtracting, a Ca/P ratio around 1.6 is obtained, suggesting that extra calcium ions are provided by the calcium phosphates solution. Following this line of thought, calcium ions from the solution could be attached faster to the protein than phosphate ions avoiding the *in situ* HA production.

Nevertheless, the highest value of HA content that can be attached to the SPARC protein is still unknown, also, the fact of how this attachment is performed, requires further studies to answer these questions.

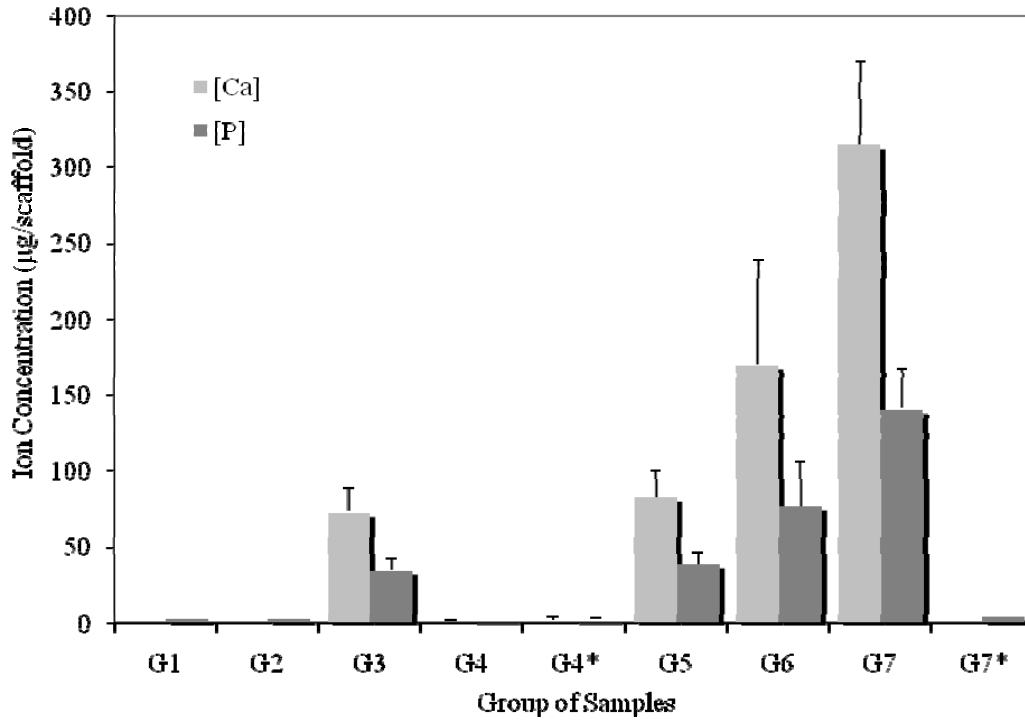


Figure 4. Calcium and phosphorous content into scaffolds (n=3). Controls: G1, G4\* and G7\*.

### 3.3 Mechanical compression testing

The strain-stress curves for these types of scaffolds (G1-G7) exhibit a characteristic of low-density behavior and open-cell foams with distinct linear elastic. The curve is divided in three main zones (Figure 5a): linear elastic, the zone where the loads are released and the sample will return to its original configuration; collapse plateau, considered the region where the material will be deformed permanently; and finally densification where there is a complete pore collapse throughout the material [21]. From the initial linear regime the linear elastic modulus or Young's modulus ( $E^*$ ) were calculated for all compression test and they were determined via linear regression (Figure 5b). According to Young's modulus ( $E^*$ ) values, samples can be divided in three major groups (Figure 5b). The first group is that of collagen samples (G1) that are clearly the most susceptible materials to deform at lower values ( $G1 = 6.75 \pm 0.06$  Pa) of stress in comparison to the other six groups of scaffolds. The second group is composed by samples G2 to G6 where the Young's modulus are not significant different between them ( $G2 = 24.96 \pm 0.49$  Pa;  $G3 = 26.96 \pm 4.13$  Pa;  $G4 = 28.71 \pm 0.86$  Pa;  $G5 = 28.34 \pm 4.48$  Pa); however, this group shows the highest  $E^*$  values, meaning that the tendency to deform is lower than for the two other groups. The third one is composed by samples G6 and G7 with an intermediate value for the Young's modulus ( $G6 = 19.81 \pm 2.26$  Pa;  $G7 = 19.41 \pm 3.05$  Pa).

G2 scaffolds show higher resistance to deform than G1 due probably to the presence of SPARC, and this resistance is even higher in the G3 to G5 samples with calcium phosphates and hydroxyapatite particles added to the protein. This finding suggests that SPARC could increase the

resistance of the collagen scaffolds, in addition, there is a boundary where the load of calcium phosphates and HA could have a reverse effect on the resistance to deformation, that is verified in G6 and G7 samples with lower  $E^*$  values, where higher loads of phosphates could subtract resistance to the scaffold that could deform easier at higher stress.

### **3.4 Diameter contraction**

The change in diameter of the matrices was expressed as a percent reduction in scaffolds diameter based on the starting diameter. Two analyses may be performed from the measurement of scaffolds diameter (cell-seeded samples and un-seeded controls) during the culture period, immersed in osteogenic medium. Contractions of the scaffolds can be observed in comparison to their own controls and between different samples (Figure 6).

G1, G2 and G4 controls maintained their original diameter during the complete experience, aside from scaffolds that contained HA such as G3, G5, G6 and G7; and precisely these last samples from the early beginning of the culture, decreased their diameter until approximately 10% after 14 days.

G1 maintained its original diameter until day 5 when it suffered a radical contraction (50%) in comparison to its control, it can be suggested that until day 5 there was a colonization stage increasing the cellular density and after that the cellular networks were created, thus forcing the scaffold contraction. A similar situation can be observed for G2 as well. G3, G5 to G7 controls and samples exhibited a similar behavior along the experience: a slow rate of contraction in the first week and an abrupt decrease in the diameter by the end of the second week.

Generally speaking, it was observed that contraction for cell-seeded samples decreased when the amount of particles into the scaffold increased, and thus, controls presented an increasing contraction percentage from G1 to G7 with the increasing of retained solids.

### **3.5 Cytotoxicity assessment**

The number of viable cells from each sample ( $n=6$  per group) was measured by MTS assay and the background values from culture medium were subtracted from the obtained samples values (Figure 7). At the beginning of cell culture scaffolds without HA particles inside (G1, G2 and G4) shown higher cell viability than those without HA. However, after two weeks of culture, cell viability increased (no significant difference among them) in all samples except collagen scaffolds. These facts suggest that the protein, calcium phosphates and nanoparticles of HA could induce a significant enhancement in cellular viability on scaffolds loaded with those agents at the end of 14 days of cell culture.

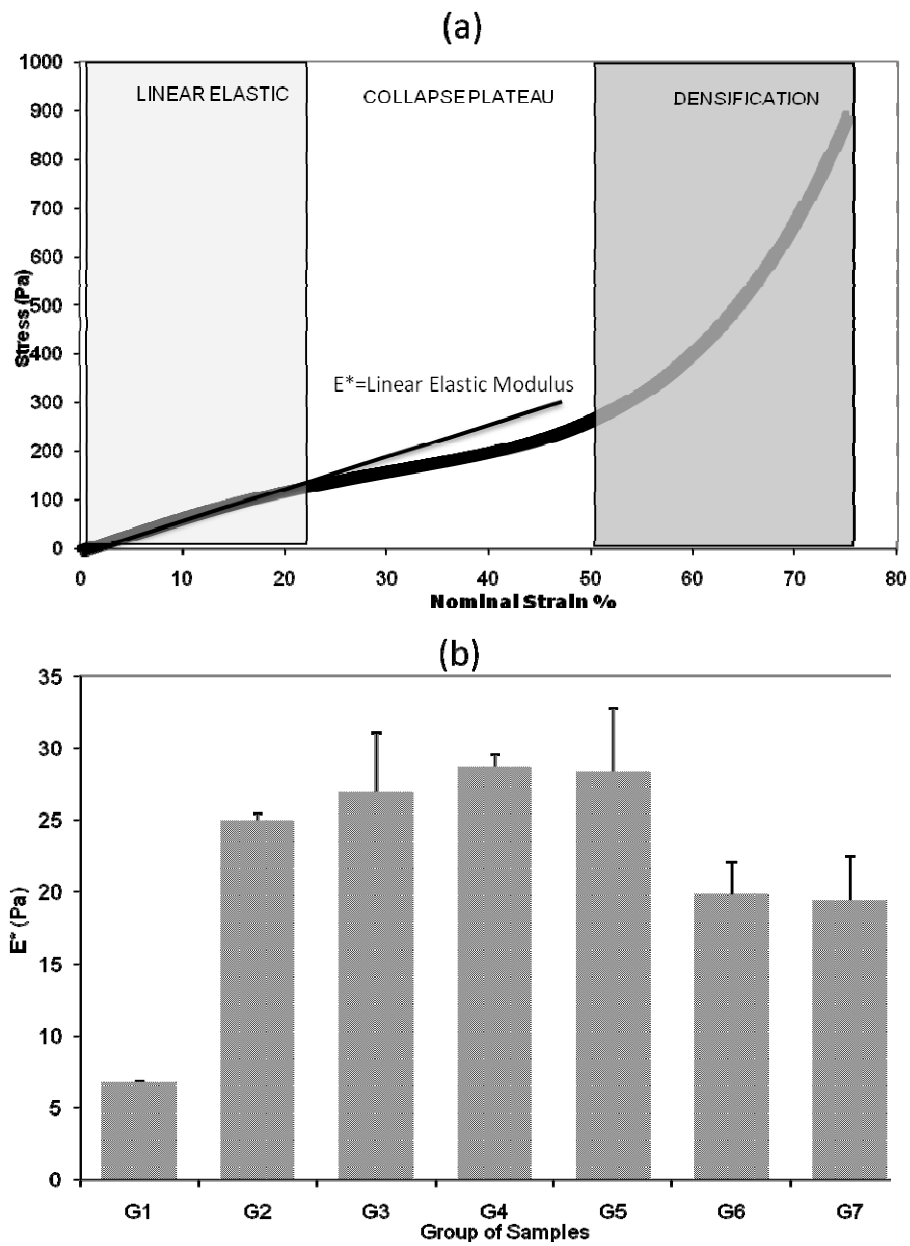


Figure 5. Different zones in the characteristic strain-stress curve (a). Linear elastic modulus values ( $\pm$ SD) for all groups of samples ( $n=5$ ) (b).

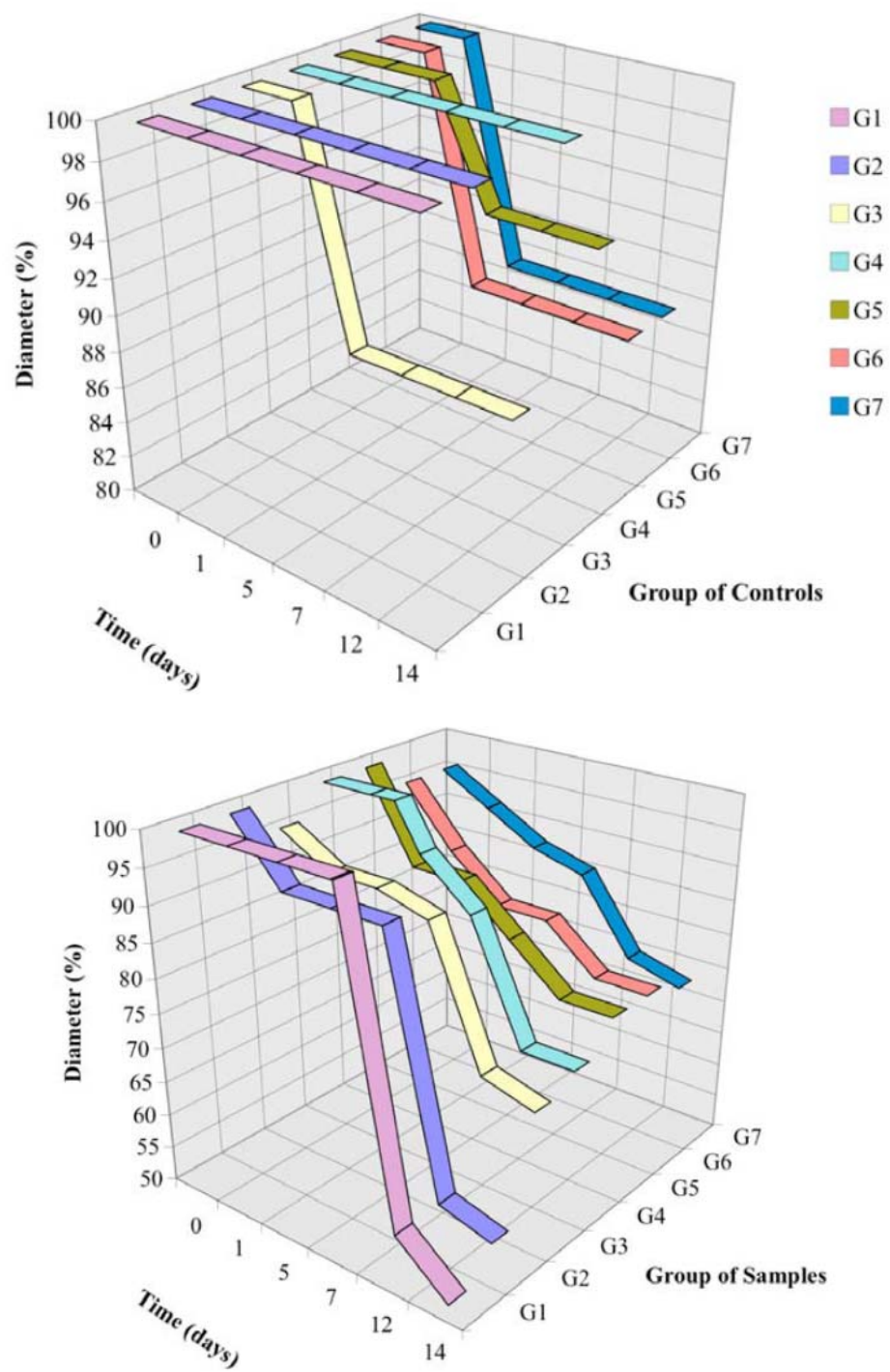


Figure 6. Diameter contraction in percentage of G1-G7 scaffolds ( $n=3$ )

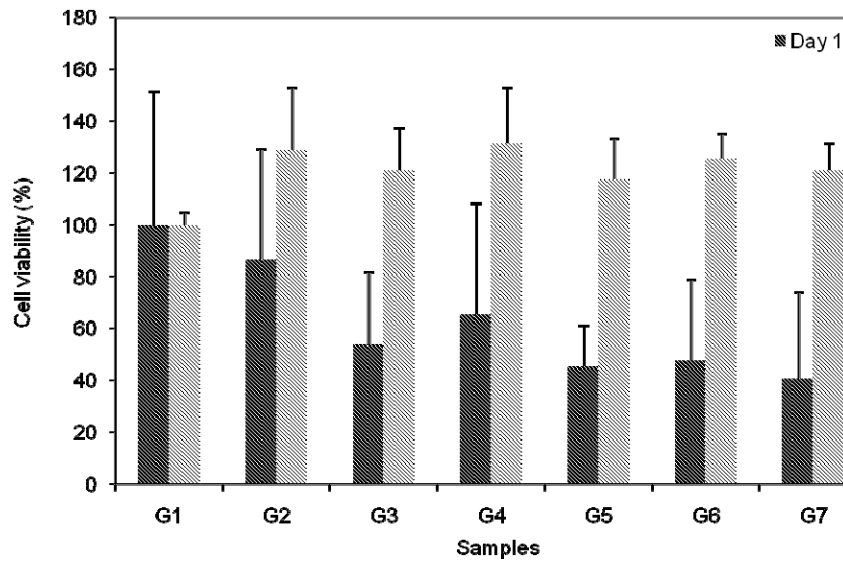


Figure 7. Cell viability after 1 day and 2 weeks of GBMSCs in osteogenic medium (n=6, mean  $\pm$ SD)

### 3.6 Cellular proliferation

Cell-seeded scaffolds ( $n=6$ ) were tested using a DNA content assessment kit. The background fluorescence of the matrix was accounted subtracting the mean value obtained for non-seeded scaffolds from the value of each seeded sample, and a standard curve was created and sample DNA values were read off from the standard graph (Figure 8). No significant effect of the presence of SPARC, calcium phosphates or HA particles on the DNA content of the constructs was determined (ANOVA,  $p = 0.996$ ), indicating a similar number of cells in scaffolds from each of the seven groups after 2 weeks in culture.

### 3.7 Histology studies

Cell-seeded scaffolds from G1 to G7 samples were cut and stained routinely with Harris hematoxylin solution and eosin Y solution aqueous, and were afterwards observed using a light microscope. Micrographs from G1 and G7 samples are shown in Figure 9. At the end of 2 weeks of culture, cells migrated and filled some large pores located in the central region of G1 scaffolds (Figure 9a), meanwhile, G7 scaffolds shown higher extracellular matrix even though not suggesting a larger proliferation than G1 samples (Figure 9b).

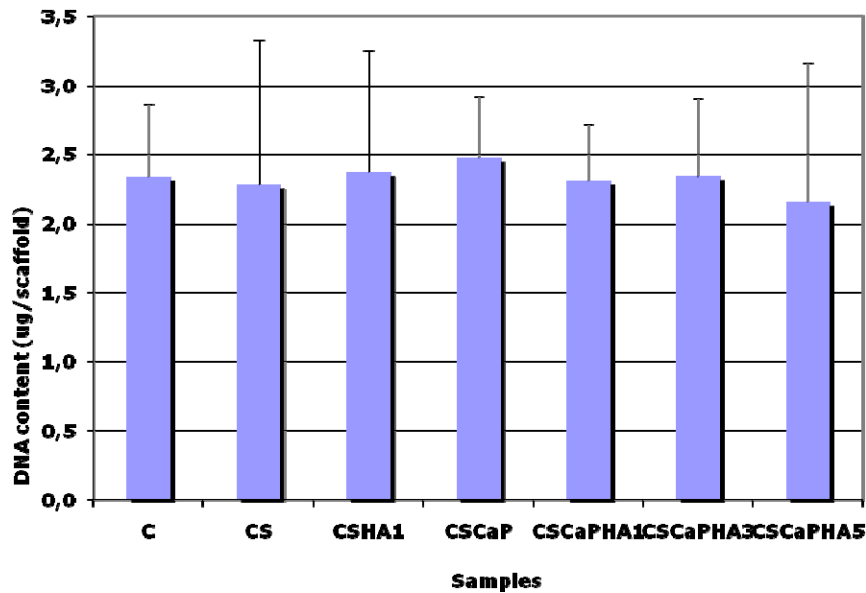


Figure 8. DNA content of GBMSCs cultured on the scaffolds 2 weeks of culture in osteogenic medium ( $n=6$ , mean  $\pm$ SD)

#### 4. Conclusions

A basic strategy of bone tissue engineering [25, 26] employs a three-dimensional porous scaffold to deliver bone-inducing factors or osteogenic cells to the defect site. Such engineering constructs of scaffolds with cells and/or factors are expected to substitute autogenous bone graft by providing similar components. Several materials have been investigated such as hydroxyapatite (HA) and collagen due to their attractive compositional analogy to bone matrix. Moreover, and in particular of this study, a secreted protein acidic and rich in cysteine (SPARC) has been added to the porous matrix based on collagen type I, nanoparticles of HA and calcium phosphates (CaP).

SPARC was selected to be incorporated to the matrix because of its interesting role in bone biology such as morphogenesis, remodeling and cellular proliferation; and the results obtained during this study explicitly indicated its paramount roll in:

- The calcium ions binding: High affinity of calcium ions was shown effectively by SPARC in the group of samples, where the protein and CaP and/or HA were incorporated (G3, G5, G6 and G7). Low calcium and phosphate ions concentrations, obtained by ICP analysis in samples G1 and G2, could be due to PBS rinse during the scaffolds preparation;

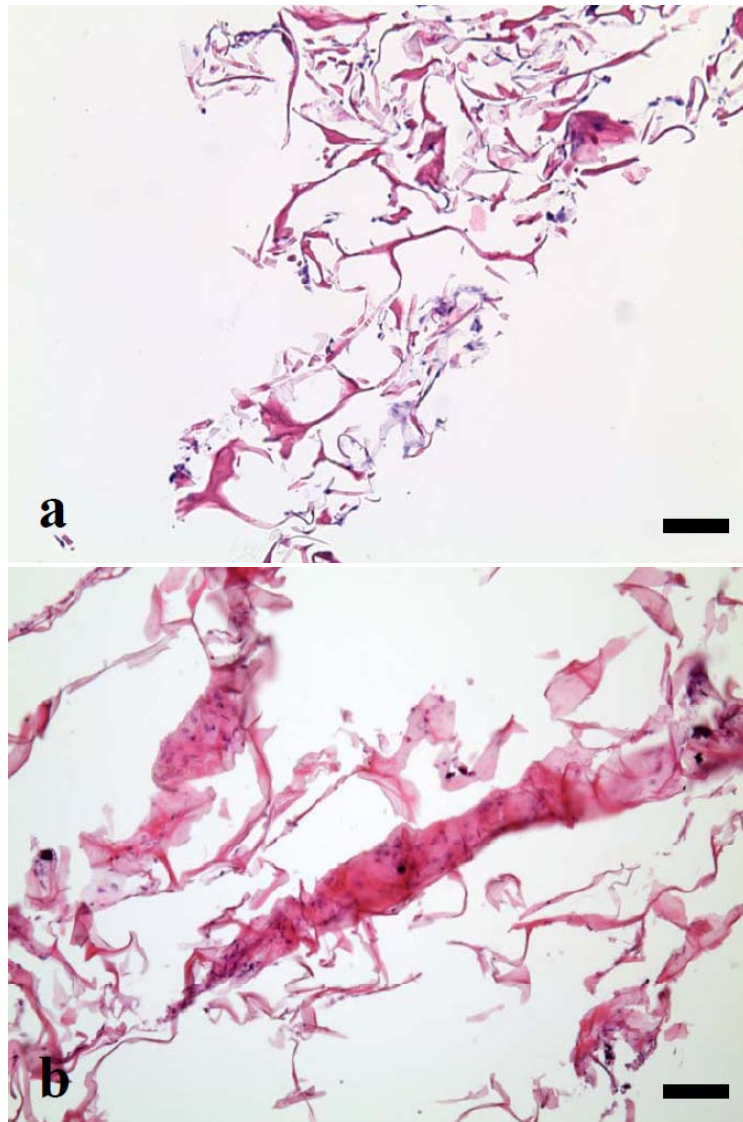


Figure 9. Light micrograph of central region of goat bone marrow stem cells seeded on (a) G1 and (b) G7 scaffolds at 14 dys. H&E stain; scale bars, 100  $\mu$ m.

- Increasing notably the mechanical performance;
- Producing less diameter contraction during cell culture; and
- Increasing cellular viability of bone marrow stem cells after two weeks.
- 

Additional work is needed to elucidate how the SPARC attachment to the components of the porous matrix occurs.

This new biomaterial may represent a novel approach to bone tissue engineering, as a strategy applying principles and materials that nature employs to develop its biological processes, and to be used for biomedical applications such as bone defect repair and biomimetic synthesis.

## Acknowledgements

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## ANEX II

### Histological study of goat bone marrow stem cells culture (GBMSCs) and scaffolds based on collagen I/III

#### 1. Introduction

Previously to *in vivo* experiments, an *in vitro* assessment was conducted to evaluate the performance of the proposed biomaterial at the cellular level. Experiments were carried out using goat bone marrow stem cells (GBMSCs) on biomaterial based on collagen I/III for 3 and 5 weeks.

Next specific goals were considered at the beginning and during the experience:

- To evaluate the crosslinking degree (EDAC:NHS:carboxylic acid=5:2:5) used in the processing of the biomaterial to identify it as *strong*, *adequate* or *weak*, where, *strong*: the entrance of cells to the scaffold is not allowed, *adequate*: a balance between cellular proliferation maintaining the shape and structure of the sponge for longer, *weak*: scaffold collapsed.
- To study the effect of 3D culture on the osteogenic differentiation of GBMSCs

#### 2. Materials and Methods

##### 2.1 Isolation and cell culturing of GBMSCs

After anaesthesia, the pelvic areas of six female and male goats, six months old, was shaved, disinfected, and a skin incision was made over the long axis of the iliac crest. The iliac was freed from overlying muscle and fat tissue by sharp and blunt dissection and the bone was exposed. After rinsing, a syringe and attaching a biopsy needle with 5000 IU/ml heparin, a bone marrow sample of ~10ml was taken from the iliac crest. The marrow was collected in 30ml medium to which 1500 IU heparin was added and transported to the laboratory on ice. At the end of the first passage (P0), the cells were cryopreserved in 10% of dimethyl sulphoxide (DMSO). Cryopreserved cells were thawed and replated at a density of  $2 \times 10^6$  cells in 150 cm<sup>2</sup> flask with expansion culture medium – DMEM low glucose supplemented with 10% fetal bovine serum (FBS) and 1% P/S (penicillin/streptomycin), see Figure A1. When cells were near confluence, they were washed firstly with collagenase type II and subsequently with phosphate-buffered saline (PBS), every step for 10 minutes in incubation, and finally cells were enzymatically released by means of a 0.25% trypsin-EDTA solution and replated  $2 \times 10^6$  cells in 150 cm<sup>2</sup> flask. After P2 cells were released as described earlier and seeded in 24 wells tissue culture with osteogenic culture medium–  $\alpha$ -MEM supplemented with 10% FBS, 1%P/S, 50  $\mu$ g/ml of L-Ascorbic acid 2-Phosphate (0.173M), 100nM dexamethasone and 10mM  $\beta$ -Glycerol phosphate (4.629 mg/ml). Cells were cultured at a density of 500.000 cells/well ( $2.5 \times 10^5$  cell/cm<sup>2</sup>) at 37°C in a humid atmosphere with 5% CO<sub>2</sub>. Osteogenic medium was changed every other day until 5 weeks.

## **2.2 Collagen scaffolds preparation**

Porous scaffold were produce from a porcine-derived collagen type I/III slurry (Geistlich Biomaterials, Wolhusen, Switzerland) to form sheets ~2.5mm in thickness. Morphometric analysis of the pore structure showed a pore diameter of  $190 \pm 25 \mu\text{m}$  and porosity of  $88 \pm 25\%$ . Disks with 8mm of diameter were sterilized and firstly cross-linked by dehydrothermal treatment under a vacuum of 50mTorr and 105°C for 24h, afterwards, a second cross-linking were performed a solution containing 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC, Sigma Chemical, St. Louis, MO) and N-hydroxysuccinimide (NHS, Sigma) in ratio of 5:2:5 (EDAC:NHS: carboxylic acid) [1]. EDAC and NHS concentrations were calculated based on mean scaffold mass of 2.2mg and 1.2 mmol carboxylic acid groups per gram of collagen [2]. Collagen scaffolds were hydrated sequentially using 4ml of solution per scaffold of 100%, 80%, and 50% ethanol solutions, and then scaffolds were rinsed twice with sterile water for 48h and stored at 4°C until cell culture.

## **2.3 Seeding GBMSCs on scaffolds and cell culture**

Scaffolds were soaked in complete medium for 30 minutes, then briefly blotted on sterile filter paper to remove excess medium. Each scaffold was seeded with P2 GBMSCs by pipetting a suspension of  $2 \times 10^6$  cells in 20  $\mu\text{l}$  medium onto one surface of the scaffold. After 10 minutes, the scaffolds were flipped and an additional 20  $\mu\text{l}$  of cell suspension was added to the opposite side, for a total of  $4 \times 10^6$  cells per scaffold. Cell-seeded matrices were placed in 12-well plates coated with 1.5 ml of 2% (w/v) agarose (Cambrex BioScience, Rockland, ME) to prevent the cells from attaching to the bottom of the culture dish, and incubated at 37°C in 5% CO<sub>2</sub>. osteogenic medium was added to each sample (0.5 ml/well) 1 hour post-seeding, followed by another 0.5 ml 16 hours later. Medium was changed every 3 days until the cultures were terminated at 3 weeks and 5 weeks. Non-cell-seeded scaffolds were cultured in parallel under similar conditions (viz. osteogenic medium).

## **2.4 Histology and Histochemical studies**

After fixation in formalin and subsequently embedded in paraffin scaffolds-cells constructs and pellets samples were cut into 4 $\mu\text{m}$  thin slices and stained routinely with Harris Hematoxylin Solution (Sigma Aldrich) and Eosin Y Solution Aqueous (Sigma Aldrich), afterwards specimens were mounted with Cytoseal (Sigma Aldrich). After deparaffinization and re-hydration of the tissue sections, calcium was stained with Alizarin Red Solution and cytoplasm was stained by the use of Fast Green solution. Sections were washed with deionised water, dehydrated in acetone and air-dried, rinsed in xylene and finally mounted. Sections were histochemical stained with Alkaline Phosphatase (ALP) staining of the adherent cell population that exhibited alkaline phosphatase enzyme and those areas that had mineralized. Safranin-O is a cationic dye that binds to sulfate glycosaminoglycans (chondroitin 6-sulfate or keratin sulfate) staining pink, Fast green acts as a light counter-stain and Hematoxylin stains cell nuclei dark purple. As a control, Safranin-O staining was used to confirm that chondrogenesis did not take part in the cell culture time.

### 3. Results

GBMSc culture was carried out to evaluate a decisive condition that is crosslinking density: high density will not allow the cellular colonization and subsequent cellular proliferation. On the other hand, low density will bring stability and mechanical problems to the biomaterial after being implanted or even in *in vitro* tests. Previous experience in our laboratory found various densities of crosslinking: EDAC:NHS:Carboxylic acid (1:1:5, 5:2:5, 10:4:1; EDAC1, EDAC2, and EDAC3, respectively) for similar sponges for cartilage tissue engineering, EDAC2 was chosen for this study as a intermediate in the range.

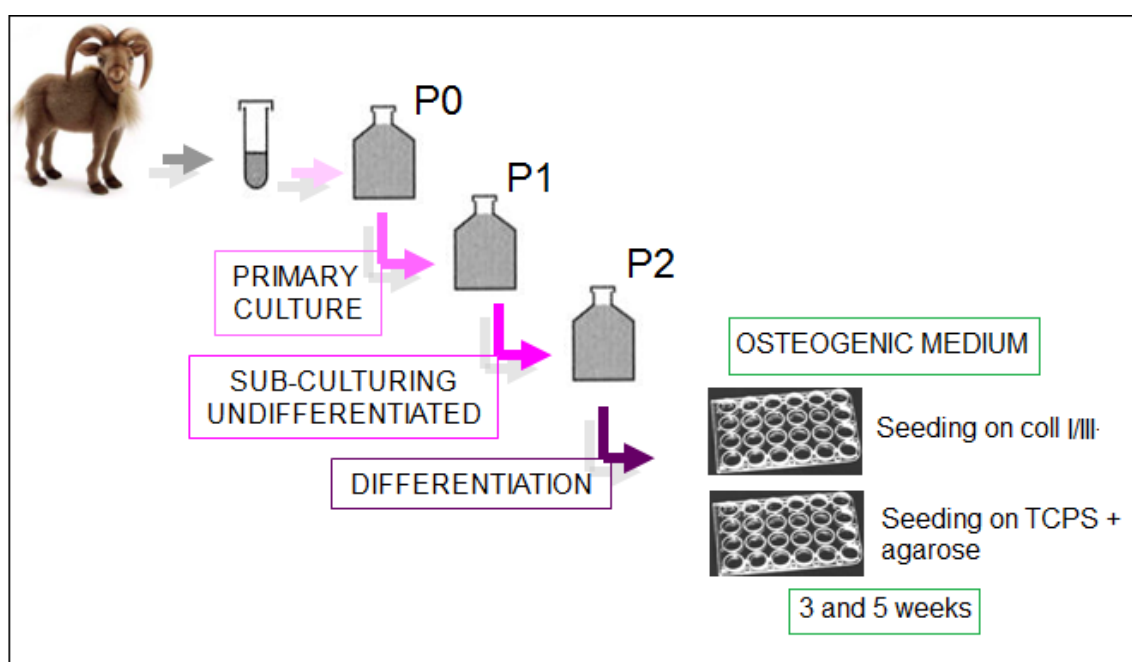


Figure A1. Scheme about goat bone marrow stem cells' culturing. After isolation of 6 goats, cells were culture to primary culture (P0) and sub-cultured until osteoblasts differentiation (P2) and finally they were seeded on sterile scaffolds and TCPS, used as controls in a cellular density of  $4 \times 10^6$  cells/scaffold.

According to observations of the histological images of the sponges after 3 weeks (Figure A1a) and 5 weeks (Figure A1b), the cells grew on the surface of the sponge without being able to enter into the scaffold. Hence the observed cell micro-masses adhered only to the surface, being the same result found for both endpoints of the cell culture.

As opposed to this, the controls grown (Figure A2) in cellular micro-masses that grew in monolayer and then, due to the lack of space an elevated population, these layers roll on themselves to form cell pellets which were evaluated using histological and immunohistochemistry techniques.

In the literature one can find different methods and examples of 3D cell culturing [3-4]. In this case, it was shown that many cells were found inside and on the surface of the micromasses. Although, there are no data that verified stem cells differentiation to bone cells or their performance to form new bone; in the histological observation were found osteoblasts, osteoclasts, bone lining cells and osteocytes within this sort of cellular pellets (Figure A4). Probably, longer cultures may eventually

decay due to lack of means of transport of nutrients such as oxygen that is the blood vessels function in cell culture using porous biomaterials. Furthermore, the production of calcium phosphate deposits is scarce over 5 weeks of culture.

#### 4. Discussion

Effects of varying the characteristics of another “pillar” of tissue engineering – biomaterial scaffolds – are evaluated. *In vivo*, interactions between cells and extracellular matrices play a critical role in regulating cellular processes, such as migration, contraction, and biosynthesis. In a similar manner, the behavior of cells *in vitro* can be greatly influenced by numerous characteristics of biomaterial scaffolds into which they are seeded, such as the chemical composition, architecture, or mechanical properties of the scaffold [5].

The chosen density for this test revealed to be too high for this biomaterial in particular, due to the strengthening of collagen fibers by the action of EDAC and NHS, making it difficult for the cell to access to the scaffold, due to pores shrinkage and resistance to contraction once colonized by cells. This contraction is important in cell proliferation because it allows for cells to grow and expand towards the inner portions of the scaffold.

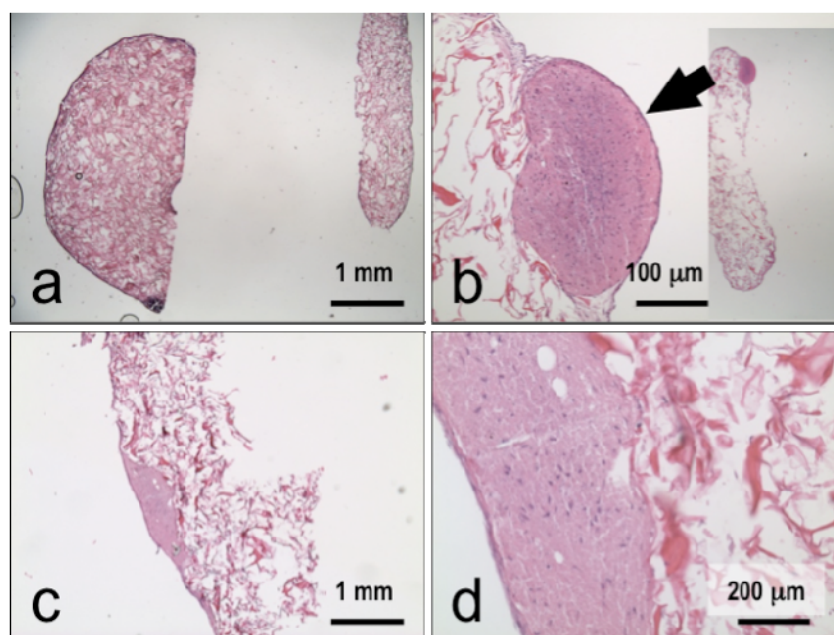


Figure A2. Light micrographs of osteoblast-seeded scaffolds following 3 weeks (a, b) and 5 weeks (c, d) culture period. (a) osteoblasts only growing on scaffold surface, (b) cell concentration on scaffold surface, (c) and (d) higher population of cells conforming an expanded mass on scaffold surface where a oriented growth in layers parallel to surface is observed. In any case, cells could not colonize the inner portions of the scaffold due to the high crosslinking density (5:2:5) chosen for this test.

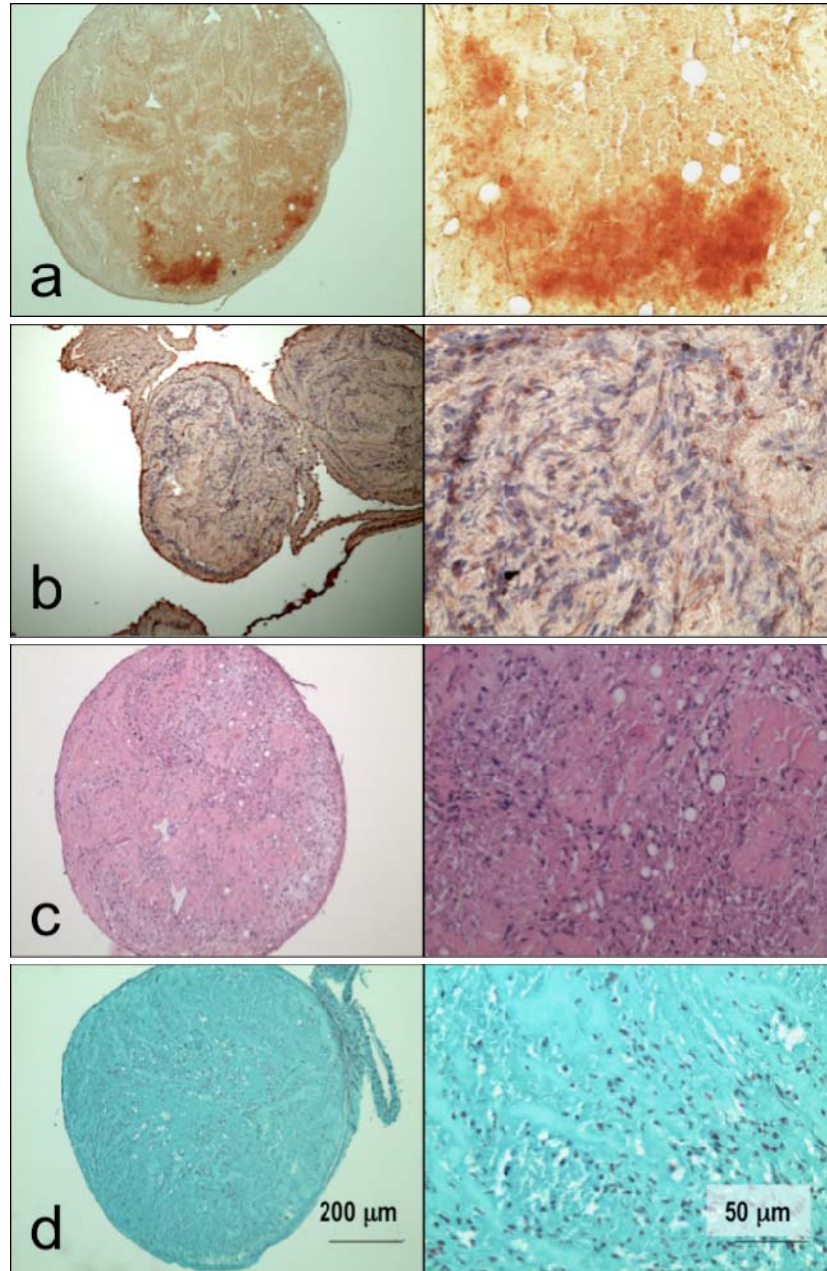


Figure A3. Light micrographs of histology and immunohistochemistry of osteoblast-seeded scaffolds following 3 5 weeks. (a) Alizarin Red technique indicated positive result and it is observed calcium phosphate deposits inside the scaffold, (b) ALP by immunohistochemistry where extracellular matrix formation is observed, (c) H&E staining indicating inside and superficial cell proliferation, (d) Negative Safranin-O staining to verify that stem cells did not differentiate into chondrocyte cells and did not produce cartilage tissue. Images were taken from central regions of the constructs, which appeared uniform throughout.

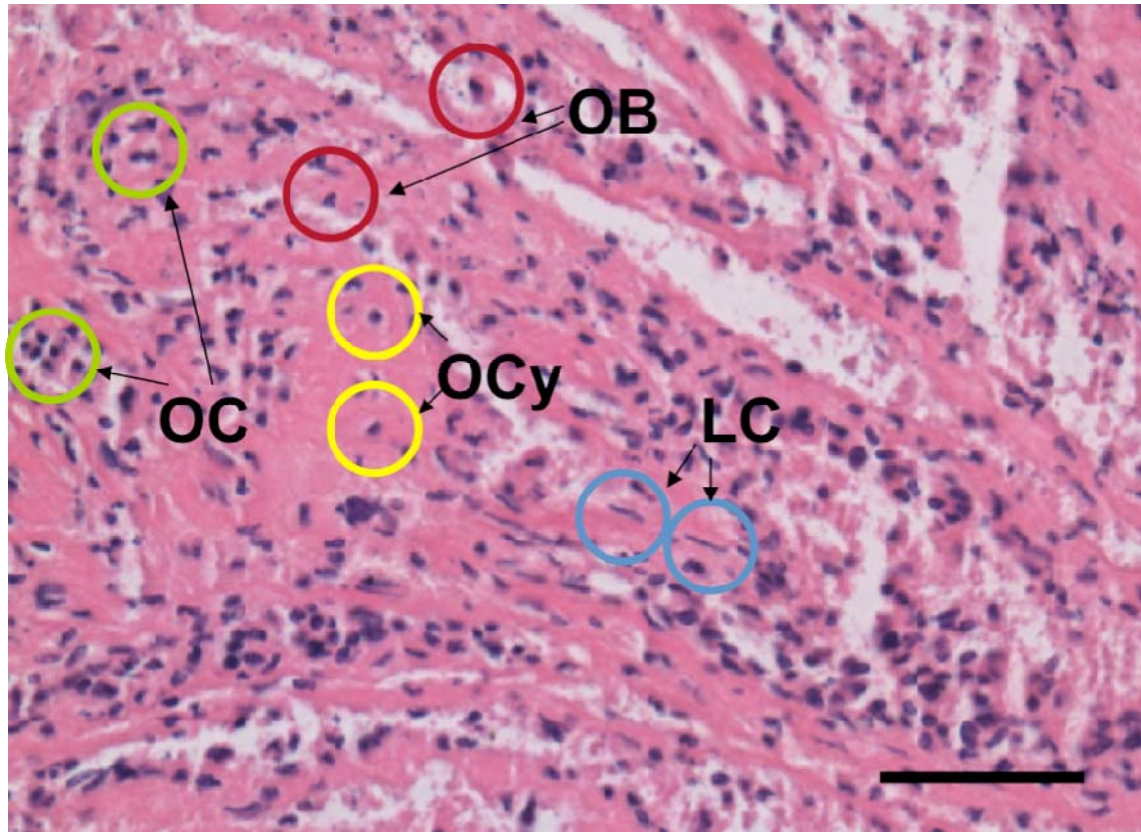


Figure A4. Light micrographs where different types of bone cells over 5 weeks of cell culture in osteogenic medium are observed. OB: osteoblasts, OC: osteoclasts, OCy: osteocytes, LC: Bone lining cells. H&E, Scale bar: 100  $\mu$ m.

## 5. Conclusions

In next *in vitro* experiments using collagen I/III a milder crosslinking density must be used in order to have bone cells colonizing and proliferating inside the scaffold, or in case of *in vivo* experiments, so that surrounding bone cells might enter to initiate bone regeneration. Three-dimensional culturing is not a viable way to induce new bone *in vitro* due to its lack of blood vessels that transport oxygen and other nutrients for better viability and performance of older and deeply located cells.

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## CHAPTER VII - BONE REGENERATION USING A SCAFFOLD BASED ON COLLAGEN I/III, SPARC AND HIDROXYAPATITE NANOPARTICLES: AN IN VIVO STUDY

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**Abstract.** This *in vivo* study analysed the osteoconductive and osteoinductive properties of hydroxyapatite and collagen scaffold for bone regeneration procedures. The biomaterial was processed from nanophased hydroxyapatite aggregates uniformly included in collagen type I/III macroporous sponges. Implants were placed subcutaneously and intraosseously in rats, for up to six weeks follow up. Histological studies were carried out for specimens at different timepoints. Results showed strong evidence of osteoconductive and osteoinductive properties that make this new biomaterial as an interesting alternative to promote bone tissue regeneration.

### 1. Introduction

Osteoinduction and osteoconduction are two concepts of paramount importance in tissue engineering, because they are included in the desired properties of potentially interesting bone engineering scaffold materials. Osteoinduction is defined as the ability to cause pluripotent cells, from a non-osseous environment, to differentiate into osteoblasts toward bone formation [1-3] in location where normally healing would not occur through the formation of bone tissue. On the other hand, osteoconduction supports ingrowth of capillaries and cells from the neighbouring host bone into a three-dimensional structure to form new bone at locations where normally healing would occur through bone formation, but where above critical sized defects would inhibit such growth to occur naturally [4].

Adjustment to irregular wound site (malleability), good bony apposition, promotion of bone ingrowth, absorbability with biocompatible components, are properties that a desired material must

hold as they are considered as crucial in designing a matrix biomaterial for bone regeneration [5-7]. For this reason, this study selected materials with adequate track record to be selected with such aim. Thus, hydroxyapatite (HA) is by far, one of the most widely used materials in the design of biomaterials for bone regeneration due to its ceramics nature and its importance in the bone's architecture [8-11]. Besides, HA has enhanced interest as a drug delivery carrier due to its osteoconductivity and biocompatibility [12-16]. However, much attention has focused on nano-structured hydroxyapatite (nanoHA) because it can enhance the formation of new bone tissue by increasing osteoblast adhesion, proliferation, osteointegration, and, deposition of calcium-containing minerals on its surface [12-19]. Also, nanoHA is one of the main compounds in natural bone and can be used directly for new bone formation [20].

SPARC, also called BM40, exhibits high affinity for collagen type I, calcium, and hydroxyapatite, due to its two high affinity calcium-binding sites (EF-hands) and twelve low calcium-affinity sites [21-26], which is very unusual. SPARC binds to collagen to mediate deposition of hydroxyapatite and influences the progression of cells through the cell cycle binds to platelet-derived growth factor (PDGF) [24].

According to the literature, SPARC plays important roles in different scenarios such as bone morphogenesis, cellular migration and proliferation until bone remodelling [27-29]. Concerning morphogenesis, SPARC was found to be expressed by mesenchymal stem cells and osteoblasts as they begin the process of endochondral ossification [29]; in fracture healing, SPARC has been detected immediately after injury as periosteal cells begin proliferating and depositing ECM [28].

On the other hand, collagen is biocompatible and osteoinductive, acting as an excellent delivery system for macromolecules such as bone morphogenetic proteins (BMPs) and other growth factors [30-31]. When associated to HA particles forming a biocomposite, it prevents the HA dispersion in implants, resulting in an easily moldable biomaterial [32].

In this work, a new biomaterial based on nanophased hydroxyapatite and collagen was processed, aiming at achieving adequate osteoconduction and osteoinduction capabilities that might make it a useful alternative to be used bone regeneration scaffolds. In vivo experiments using a rat model, implanted this biomaterial subcutaneously and intraosseously in order to evaluate biocompatibility and bone ingrowth.

## **2. Materials and Methods**

### **2.1 Nanophased hydroxyapatite aggregates/type (I/III) collagen composite scaffolds processing**

Briefly [18], aqueous solutions of calcium hydroxide ( $\text{Ca}(\text{OH})_2$ ) and orthophosphoric acid ( $\text{H}_3\text{PO}_4$ , 85%), both of analytical grade, were used as reactants for the preparation of HA nanoparticles. HA powder was synthesized according to the following procedure: first, 1 L of an aqueous suspension of  $\text{H}_3\text{PO}_4$  (0.6M) was slowly added, drop by drop, to a 1 L of an aqueous suspension of  $\text{Ca}(\text{OH})_2$  (1M), to which sodium dodecylsulphate was previously added; while stirring vigorously for about 2 h at room temperature. Concentrated NaOH was added until a final pH of 10.5 was obtained. The white solution obtained was washed using deionized water and dried in an oven at 80°C for 24 h.

In the nanoHA/collagen sponge processing, an acid suspension of a porcine-derived collagen type I/III slurry (Geistlich Biomaterials, Wouhusen, Switzerland) was blended for 90 minutes at 4°C. A

cooling system maintained the temperature of the suspension at 4°C throughout the entire mixing process to avoid fiber denaturation. The nanoHA slurry was then degassed under vacuum (50 mTorr) for 60 minutes to remove the many the visible air bubbles created during mixing. After degassing, the slurry was prepared for freeze-drying by first, pouring the slurry into plastic pans at room temperature and then placing them into a freeze-dryer. The temperature of the freeze dryer was decreased at a constant rate from 20°C to -40°C over a period of 60 minutes. The temperature was held constant at -40°C for 60-120 minutes, and then sublimated at 100-200 mTorr, 0°C for 17 hours [33-34].

Secondly, collagen scaffolds were stiffened, using a physical crosslinking process called de-hydrothermal treatment (DHT). This process took place inside a vacuum oven at 50 mTorr. The vacuum was maintained at 105°C for 24 hours to induce covalent crosslinks between the polypeptide chains of the collagen fibers. Completed scaffolds [35] were impregnated using 20 µl of SPARC (Sigma, St Louis, MO) in 4 µg of protein/ml of PBS per each side, and let to rest each side for 30 min. Ca(OH)<sub>2</sub> (Riedel-de-Haden, Hanover, Germany) and H<sub>3</sub>PO<sub>4</sub> (Riedel-de-Haden, Hanover, Germany) solutions in concentration of 66.36 mM and 39.74 mM, respectively; were mixed in 1:1 ratio immediately before use; 20 µl of mixture solution were pipetted to each side, and let to rest for 20 min. 20 µl of HA nanoparticles in suspension were added to each side of every scaffold (1:5 w/w) and let to rest for 20 min each side. In a further crosslinking, matrices were immersed for 1 h in a solution (2ml/scaffold) containing 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC, Sigma, St Louis, MO) and N-hydroxysuccinimide (NHS, Sigma St. Louis MO) at a ratio of EDAC:NHS:carboxylic acid (10:4:1) [36]. EDAC and NHS concentrations were based on mean scaffold mass of 0.002 g and 0.0012 mmol carboxylic acid groups per gram of collagen [37]. After freeze-drying process, samples were sterilized until implantation.

## 2.2 Animal experimentation and operative procedure

After approval by the Research Committee of the Puerta de Hierro University Hospital and in compliance with the conditions and guidelines described in the protocol for Use of Laboratory Animals from this Institution, Wistar rats weighing between 200-250 g (8 weeks old) went through surgical procedure to produce an intraosseous and subcutaneous implantation trials (Table I). The animals were deeply anesthetized with Halothane during all the surgical processes. The animals were housed in a 12 h light/12 h dark schedule at 22°C, and fed *ad libitum*. At the end of the selected period of time, the rats were sacrificed by CO<sub>2</sub> asphyxiation and the samples were retrieved.

After trichotomy and antisepsis of lower limbs, the knee joint was exposed using a medial parapatellar incision and the patella was reflected aside. Abbocaths were used to ream the shaft of the femur in stages starting from 18Ga diameter and increasing in size. Therefore pre-processed implants made of collagen / nanoHA composite sponges were placed. Afterwards, a capsule suture with 3.0 reabsorbable threads was made, followed by the closure of the skin wound with 3.0 silk thread. After surgery, animals received analgesic drugs.

## 2.3 Histology

Specimens were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 24 h at 4°C, decalcified in 5% EDTA with 4% paraformaldehyde in 0.1 M phosphate buffer for 3,5 h at 4°C, then they were de-hydrated and embedded in paraffin. Three to three and a half µm sections were stained using Hematoxylin and Eosin (H&E) according to routine protocols, Masson's Trichromic stain (TC) [38] for histological evaluation and histomorphometry, Reticulin silver impregnation (RET) to

detect collagen and reticular fibers [39], PAS (Periodic Acid-Schiff) -stained for the detection of glycogen and Von Kossa technique (VK) for calcium detection. As controls, three unimplanted intact Col I/III, ColI/III + nanoHA, ColI/III nHA + DHT, Col I/III + EDAC and ColI/III nanoHA + DHT + EDAC scaffolds were embedded in paraffin and prepared similarly for the microscopic analysis.

Table 1. Design of experiments.

| <b>Samples</b>                            | <b>Endpoints (days)</b> |                 |                     |                 |
|-------------------------------------------|-------------------------|-----------------|---------------------|-----------------|
|                                           | <b>Subcutaneous</b>     | <b><i>n</i></b> | <b>Intraosseous</b> | <b><i>n</i></b> |
| Col I/III                                 | 7                       | 2               | 30                  | 2               |
| Col I/III + nanoHA (without crosslinking) | 5                       | 3               | 30                  | 6               |
|                                           | 15                      | 7               | 45                  | 4               |
| Col I/III + nanoHA (with crosslinking)    | 15                      | 2               | NA                  | NA              |

### 2.3.1 Microscopic studies

The preparations were studied using routine microscopy (Nikon Eclipse E600 light microscope) with light background, eventually it was used polarized white light, and a video camera equipped with Nikon DXM 1200. The selected images were captured and reviewed with the capture program and image analysis Nikon ACT-1.

### 2.3.2 Statistical Analysis

Statistical Analysis was carried out using Mann-Whitney U test were used. A value of  $p < 0.05$  was considered significant.

## 3. Results

### 3.1 Histological findings

Samples analysis was based on histology studies, that were divided between controls and implanted samples. These were subdivided in subcutaneous implants and intraosseous implants that were carried out with the aim of verifying the scaffold biomaterial osteoinductive and osteoconductive properties, respectively.

### 3.2 Control Samples Histology

Control samples without implantation were studied using routine histology techniques. Histology studies from controls without crosslinking (specifically DHT and EDAC), showed that collagen networks are formed by two types of fibers: fine and thick ones that stain green and red with TC (Fig 1), meaning that thicker collagen fibers have lost their expected ability to stain green –instead stained red-, probably due to procedures performed on raw material from its origin. In order to find the reason why collagen fibers stained red instead green, it was first made PAS staining and results were verified with Alcian blue staining at pH 2.5 indicating positive presence of mucoproteins in collagen I/III scaffolds.

Mineralized crosslinked control samples (Col I/III+nanoHA+DHT+EDAC) were stained with VK and small mineral agglomerates were detected, with variable size associated to nanoHA presence (Fig 2).

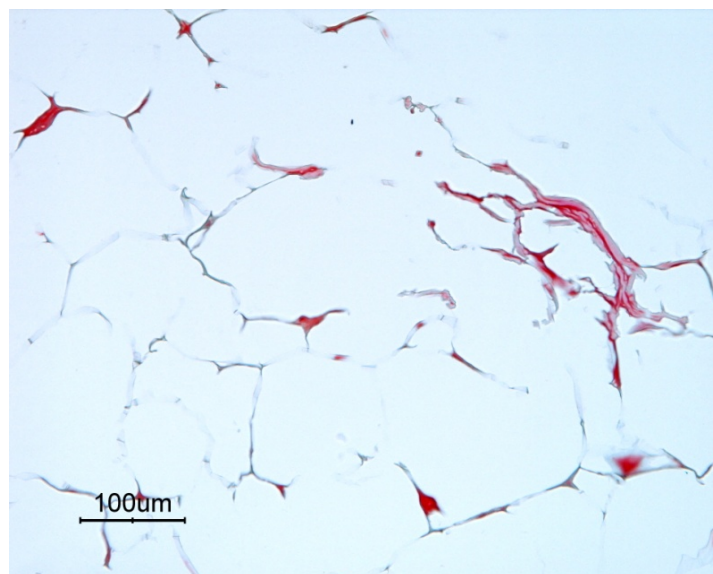


Figure 1. Collagen I/III control scaffolds were studied using Masson's Trichrome acid stain differentiating collagen I to III fibers in the network, where fine and thick fibers that stain green and red, representing col I and col III respectively, were observed.

### 3.3 Subcutaneous implanted samples - histology

After implantation, biomaterials act as a foreign body, triggering a foreign body reaction (FBR) that begins as wound healing, including accumulation of exudate at the site of injury, infiltration of inflammatory cells to debride the area, and the formation of granulation tissue [40]. Rather than the resorption and reconstruction that occurs in wound healing, FBR is initially characterized by the formation of foreign body giant cells, that can be observed in Figure 3 after 7 days of subcutaneous implantation, where these giant cells are the products of macrophage fusion - hallmark of the foreign body reaction- and they can fuse to form larger foreign body giant cells composed of up to a few dozens of individual macrophages [41]. In collagen I/III + nanoHA samples, macrophages phagocytose small nanoHA aggregates that could be observed as randomly distributed in the collagen network (Fig 4).

Secondly, it is expected to observe encapsulation of the foreign object that refers to the firm collagen shell deposited around the biomaterial, effectively isolating it from the host tissues as a protective measure against the foreign body [42] that can be detected in Figure 5 (H&E, 7 days) at low magnifications in the light microscope. Increasing the magnifications (Fig 6), is possible to observe high number of fibroblasts that are in charge of forming granulation tissue, accompanied by emerging blood vessels. Besides proliferating, fibroblasts produce collagen and proteoglycans so as to form the granular tissue. At later stages, fibrosis and scarring are visible (Fig 7) after 15 days of implantation, as a final healing response to biomaterial, including persistence of collagen increase and FBR (Fig 8). For this stage, for mineralized crosslinked samples with DHT and EDAC, an intense inflammatory response was observed affecting the whole biomaterial and the surrounding tissues and scar fibers proliferation (data not showed).

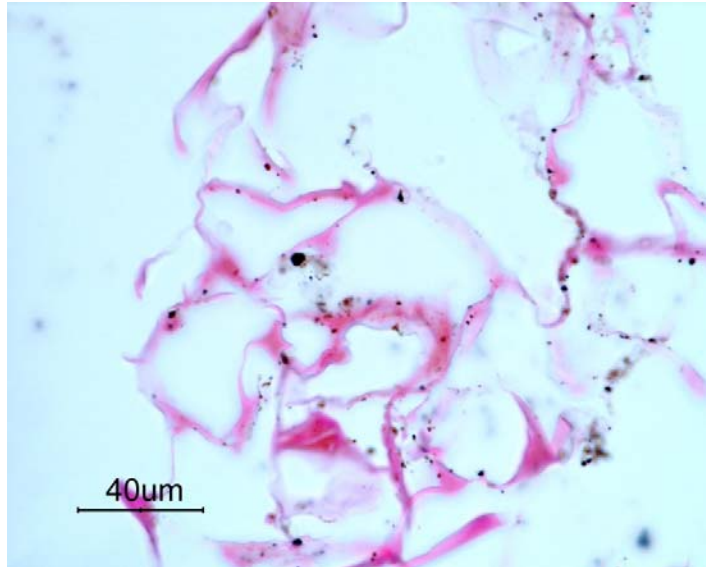


Figure 2. Small and well dispersed aggregates of small particles (black arrows) containing calcium (probably nanoHA aggregates) are observed along the collagen matrix. Mineralized and crosslinked control scaffolds (Collagen I/III + nanoHA) stained with Von Kossa.

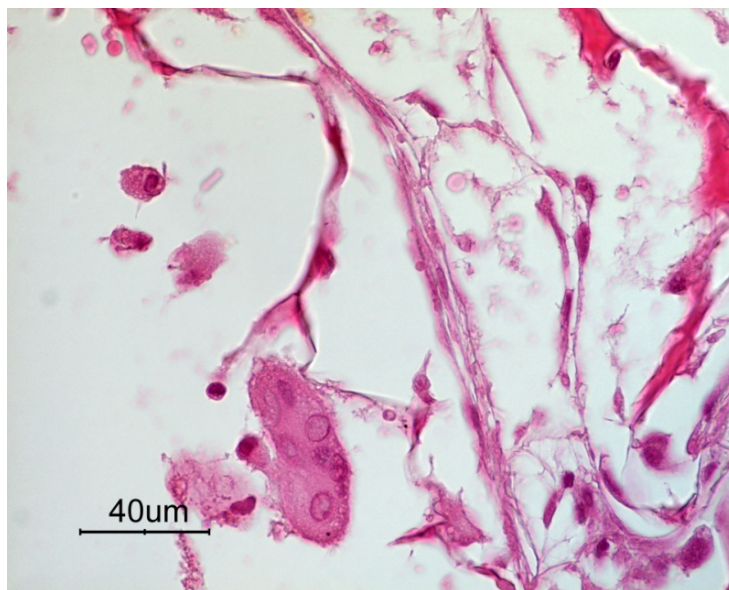


Figure 3. Foreign body giant cells formation (black arrow) announcing the initial stages of foreign body reaction (FBR) of mineralized scaffold (Col I/III+nanoHA) after 7 days of subcutaneous implantation (H&E).

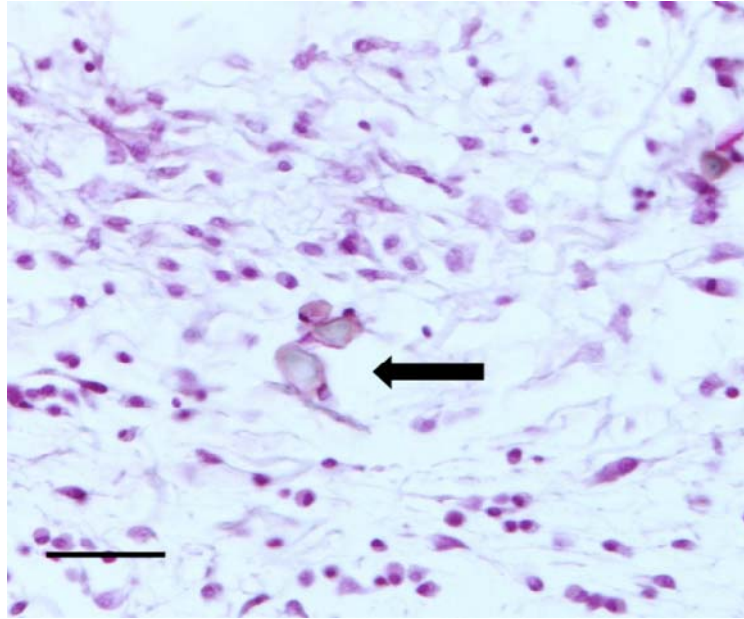


Figure 4. Foreign body giant cells formation (black arrow) announcing the initial stages of foreign body reaction (FBR), after 7 days of implantation subcutaneously. Scale bar: 40mm (H&E).

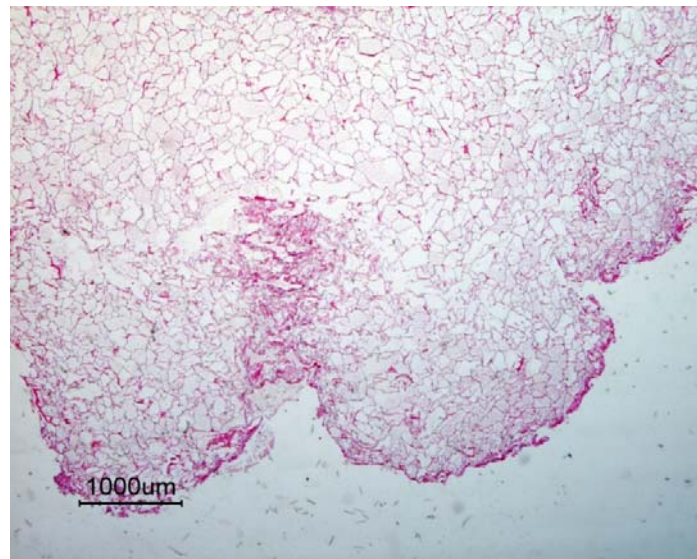


Figure 5. Firm collagen layer surrounding the mineralized scaffold created by neighbouring cells as a protective measure against the foreign body after 7 days of subcutaneous implantation (H&E).

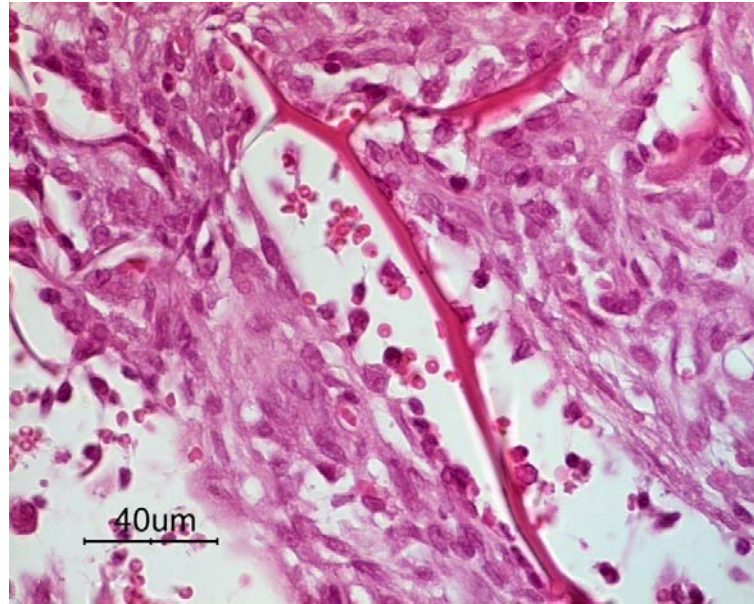


Figure 6. Fibroblasts proliferation conforming granulation tissue near new blood vessels, after 7 days of subcutaneous implantation (H&E).

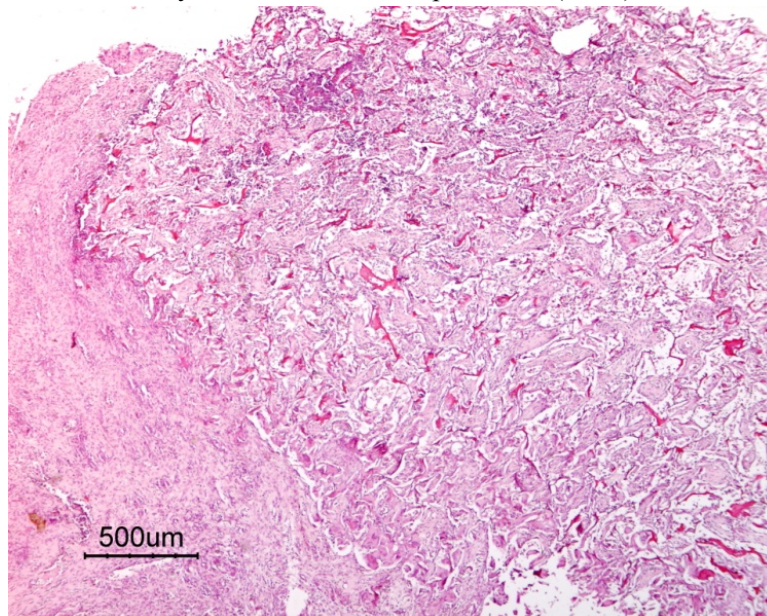


Figure 7. Processes of fibrosis and scar formation are fully visible after 15 days of subcutaneous implantation (H&E).

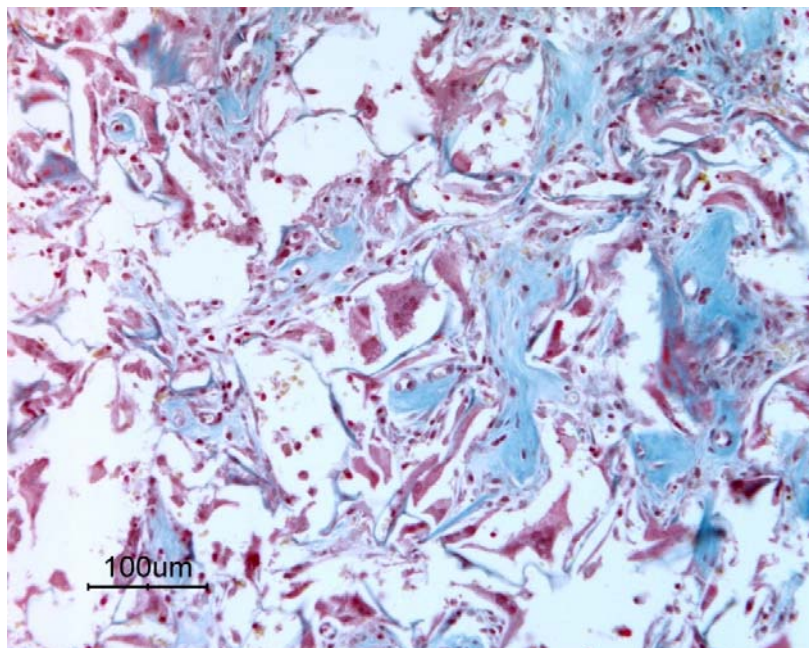


Figure 8. At later healing stages after implantation, the collagen persistence is evident as well as the foreign body reaction (FBR) after 15 days of subcutaneous implantation (TC).

### 3.4 Intraosseous implanted samples - histology

Col I/III + nanoHA samples were implanted intraosseously with endpoints of 15, 30 and 45 days; however, the samples appearance had common characteristics, suggesting that time factor had little effect. In each case there are three regions of the wounds (Fig 9): a deeper area (A1) in contact with bone marrow, an intermediate area (A2) and a top area (A3). Thus, in the deeper area there were osteoclasts and osteoblasts in large quantities, establishing extensive contact with compact or trabecular bone and depositing calcium phosphate mineral along the thicker fibers of collagen. Bone cells, of spongy bone type, are elongated and cover elongated collagen thick fibers. On the other hand, cartilage and cartilage/bone tissue formation is suggested due to the presence of chondrocytes cells around this area. This fact should be expected in a natural regeneration process of trabecular bone. In the top area of the wounds, the inflammation reaction is severe suggesting that regeneration process has not occurred yet but it indicates that this process is progressing from the deepest area, and that there was not time enough for the process to be completed.

Uncrosslinked mineralized samples; intraosseously implanted for 45 days showed the presence of osteoclasts and osteoblasts in well-organized structures divided by fibrosis trabecula, which suggests a typical new bone formation (Fig 10). At this endpoint, it was observed that a structured bone between fibers with new blood vessels was appearing (Fig 11), besides old bone in the presence of increasing osseous medulla and newly organized bone and newly formed collagen fibers, as observed in Figure 12. After 45 days of implantation, the so called A2 area (intermediate area) is ascending towards the top, including more newly formed bone, until reaching A1 area, which persists, although diminished, at this endpoint.

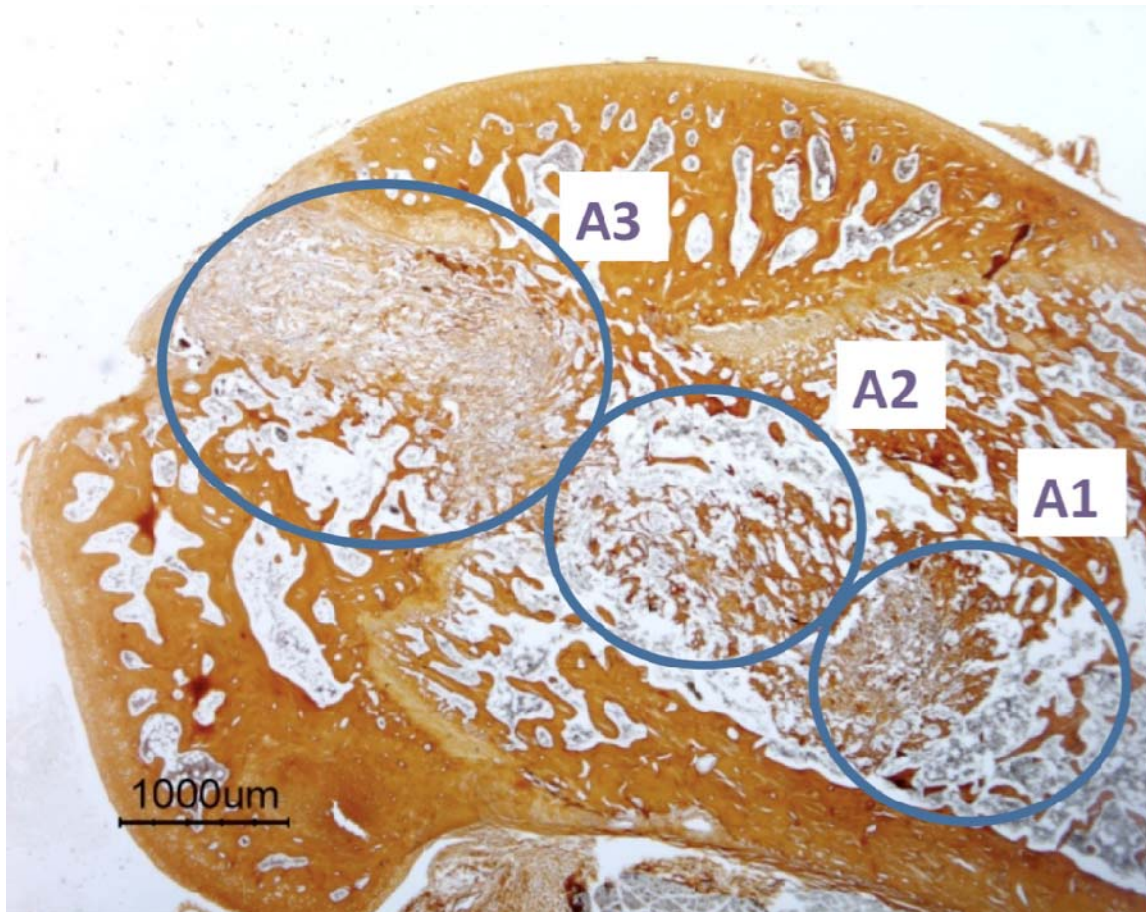


Figure 9. Mineralized sample (col I/III+nanoHA) implanted intraosseously after 45 days. There are three regions of the wounds: a deeper area where osteoclasts and osteoblasts coexist in large quantities, with osteoblasts depositing calcium phosphates on collagen fibers (A1), an intermediate area (A2) where the same process is occurring but delayed, and a top area where the inflammation reaction is severe, suggesting that the regeneration process has not yet occurred (A3)(RET)

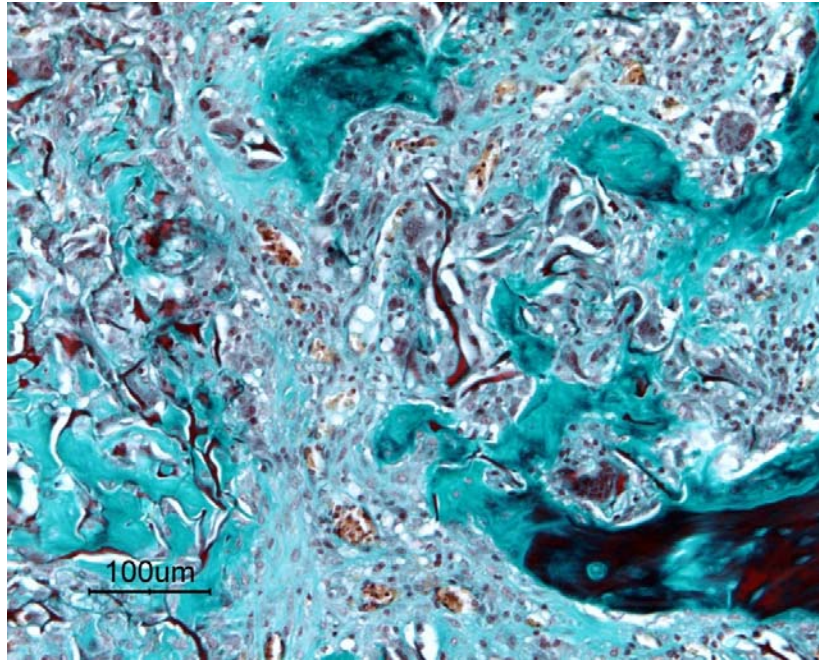


Figure 10. Typical new bone formation for uncrosslinked mineralized samples implanted intraosseously for 45 days. the presence of osteoclasts and osteoblasts in well-organized structures, divided by fibrosis trabecula (TC) is observed.

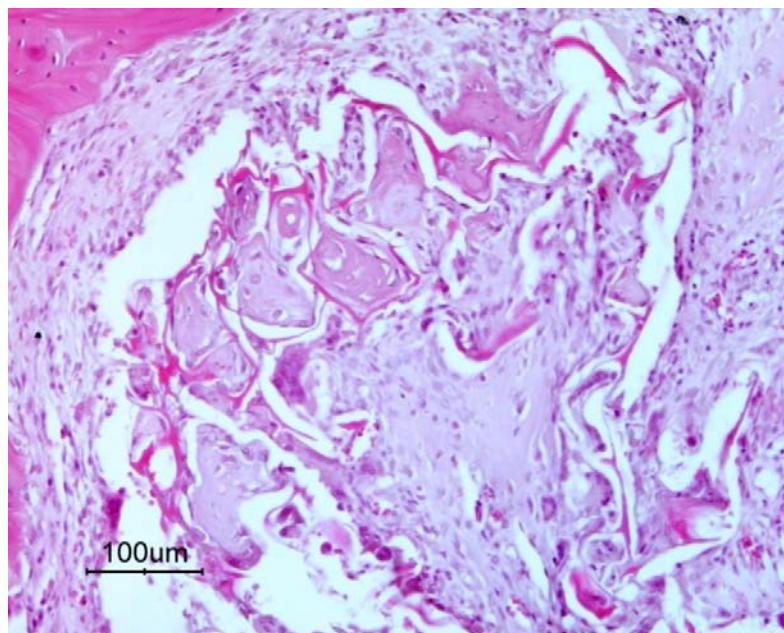


Figure 11. Structured bone between fibers with new blood vessels for uncrosslinked mineralized samples implanted intraosseously for 45 days (H&E).

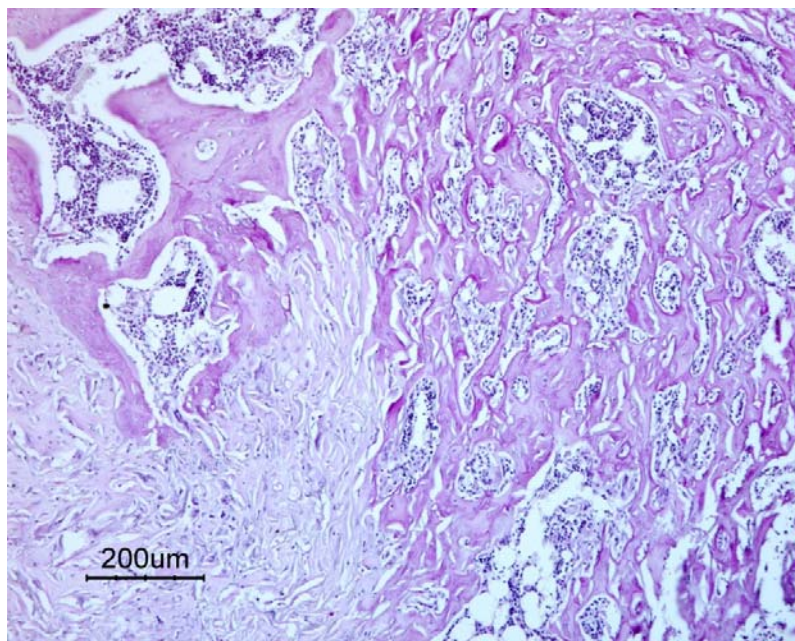


Figure 12. Old bone in the presence of increasing osseous medulla and newly organized bone and newly formed collagen fibers for uncrosslinked mineralized samples implanted intraosseously for 45 days. (H&E).

#### 4. Discussion

In the present study we have shown that mineralized scaffolds based on col I/III, SPARC and hydroxyapatite in the nanometric range are biodegradable, biocompatible, and present osteoconductive ability. However, osteoinductivity was not established, as there was no evidence of new bone formation next to the scaffolds implanted in soft tissue, even though, future *in vivo* studies with longer endpoints will need to confirm the absence of osteoinductivity of this proposed biomaterial. By now, we will focus our discussion on the aspects of *in vivo* bone-induction.

##### *In vivo bone-induction*

This study propose a biomaterial potentially interesting for bone regeneration, based on nanoparticles of hydroxyapatite supported on a type I and III collagen matrix and bridged by SPARC (secreted protein, acidic and rich in cysteine). Its behavior *in vivo* may be explained based on the understanding of its structure.

Hydroxyapatite is an attractive biomedical material owing to its excellent biocompatibility and non-toxicity of its chemical components in specific applications such as bone reconstruction and replacement as bone defect-filling, drug carriers and as coating of metal prostheses. However, its roll in this composite biomaterial is reinforced by its nanometric crystals size (nanoHA) providing a crystal structure similar to that of native bone and teeth minerals. In addition, nanoHA elicits a favorable biological response and forms a bond with the surrounding tissues.

It is important to emphasize that besides collagen, SPARC has a great affinity for calcium phosphates, due to its calcium-binding domain and EF-hand calcium-binding motifs, playing the role of biochemical bridge between nanoHA and the collagen matrix, thus being more than just calcium aggregates added to the surface of a collagen scaffold.

On the other hand, SPARC, also named osteonectin, is a calcium-binding protein found in many tissues undergoing morphogenesis or remodeling. SPARC has the capability of modulating the extracellular matrix (ECM) synthesis and turnover through its effects on collagen and extracellular proteases; and also, in mature bone, SPARC is found in the pericellular matrix surrounding osteoblasts and osteocytes.

Collagen constitutes the 3D network that supports the above materials uniformly anchored, accompanied by an adequate interconnective macroporosity to facilitate the cellular proliferation and colonization, thus favoring the nutrients transportation. Needless to say, that type I and III collagen have been used in biomaterials scaffolding in a variety of applications because of a number of useful properties, including low antigenicity and appropriate mechanical characteristics.

In addition, in order to determine other species conforming the biomaterial based on collagen I/III, nanoHA and SPARC present in raw collagen, PAS and Alcian blue staining were used and both tested positively for the presence of mucoproteins. Although both SPARC and mucoproteins derive from the same glycoproteins family, mucoproteins are proteins conjugates in which the prosthetic group is a polysaccharide consisting of hexoses and acetylated acids or sulfonated acids that can be found in different places where lubrication is needed such as the synovial fluid of the knees. So far, the exact origin of the mucoproteins contained in the original collagen has not been determined, even their composition or their amount present in the biomaterial as a whole, because it departs from the objective of this study, but it will undoubtedly be an interesting topic addressed in upcoming experiments, so as to quantify their influence on the *in vivo* performance of the biomaterial.

According to the above, this nanoHA/collagen/SPARC scaffold is a promising alternative for bone regeneration due to its biomedical features and its capability to mimic bone at nano and micro scale, been an adequate scaffold for colonization, proliferation, and remodelling by bone cells.

Current evidence further indicates that this biomaterial was very successful in terms of bone regeneration when it was implanted intraosseously, whereas subcutaneous implants produced a severe foreign body reaction. These antagonistic behaviors should require further studies.

The fact that bone regeneration was found after 15, 30 and 45 days following intraosseous implantation suggests that early endpoints must be analysed to find the time in which bone would start to regenerate by the action exerted by the biomaterial. The bone remodelling began by a blood vessels generation followed by a production of osteoclasts and osteoblasts inside the scaffold, continuing with calcium phosphate deposition along the thicker fibers of collagen.

The presence of cartilage and cartilage/bone tissue suggest the action of chondrocytes that was induced by the bone regeneration process, being a good sign, as it is an expected fact in natural trabecular bone formation.

Even though the inflammation reaction is still severe suggesting after 45 days, meaning that regeneration process has not occurred yet but it indicates that this process is progressing from the deepest area, and that there was not time enough for the process to be completed.

In further studies would be appropriate investigate this new biomaterial as a macromolecule delivery system and the possibility of enhancing bone ingrowth and regeneration in the treatment of bone.

## 5. Conclusions

The nanoHA/Col/SPARC biomaterial obtained with a similar nanostructure to bone in which hydroxyapatite nanocrystals 80X20 nm in size which aggregates in some micro size aggregates, were well dispersed and bound to a collagen matrix means SPARC.

The composite was incorporated into the remodeling process of bone, resorbed by osteoclastic cells, and new bone was formed by osteoblasts after the resorption, as if the composite were grafted autologous bone. This high-performance bioactivity of the present composite might result from its similarity of composition and nanostructure to bone. The nanoHA/Col/SPARC biomaterial can be applied for higher bioactive bone graftmaterials.

## Acknowledgements

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## GENERAL CONCLUSIONS

It may be concluded that new nanoHA based materials are certainly among the most promising challenges in bioactive ceramics for the near future, and consequently, the research effort put in their development shall continue.

Being bone engineering one of the areas where nanoHA play an important role, others such as pharmaceutical, cosmetics, catalysis and instrumentation, are industrial sectors eventually benefitting from the new technology applied in the nanoHA processing.

Therefore, it can be conclude that among bioactive ceramics, the hydroxyapatite and its processing into particles in the 100 nanometer range, have poured on this well-known ceramic, interesting new abilities in several areas. Specifically in biomedical engineering new opportunities are brought into scaffolding, drug delivery system and even DNA transportation.

A basic strategy in bone tissue engineering employs a three-dimensional porous scaffold to deliver bone-inducing factors or osteogenic cells to the defective site. Such bioengineering constructs of scaffolds with cells and/or factors are expected to substitute autogenous bone graft by providing similar properties. Several materials have been studied such as hydroxyapatite nanosized (nanoHA) and collagen due to their attractive compositional analogy to bone matrix. Moreover, and in particular in this study, a secreted protein acidic and rich in cysteine (SPARC) has been added to the porous matrix based on collagen type I, nanoparticles of HA and calcium phosphates (CaP).

SPARC was selected to be incorporated to the matrix because of its interesting role in bone biology such as morphogenesis, remodeling and cellular proliferation; and the results obtained during this study explicitly indicated its paramount roll in the calcium ions binding due to its high affinity of calcium ions, increasing notably the mechanical performance; producing less diameter contraction during cell culture; and increasing cellular viability of bone marrow stem cells after two weeks. However, additional work is needed to elucidate how the SPARC attachment to the components of the porous matrix occurs.

This new biomaterial may represent a novel approach to bone tissue engineering, as a strategy applying principles and materials that nature employs to develop its biological processes, and to be used for biomedical applications such as bone defect repair and biomimetic synthesis.

## ERRATA

| PAGE | LINE | WHERE IS WRITTEN                                                                                           | SHOULD BE                                                                                                  |
|------|------|------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------|
| 3    | 14   | This method was selected after a thorough study of other nanotechnology techniques for                     | This method was selected after a thorough study of other nanotechnology techniques for                     |
| 3    | 37   | Finally, <i>in vivo</i> experiments in rats were conducted to evaluate osteoinductive and                  | Finally, <i>in vivo</i> experiments in rats were conducted to evaluate osteoinductive and                  |
| 6    | 9    | Por fim foram realizadas experiências <i>in vivo</i> , em ratos, para avaliar o potencial de               | Por fim foram realizadas experiências <i>in vivo</i> , em ratos, para avaliar o potencial de               |
| 23   | 10   | of biological function imposes serious risk in patients.}                                                  | of biological function imposes serious risk in patients.                                                   |
| 23   | 20   | hydroxyapatite nanoparticles and microspheres, while chapters three and four are good examples             | hydroxyapatite nanoparticles and microspheres, while chapters three to five are good examples              |
| 23   | 24   | nanoHA was evaluated in a rat model, as reported in chapter VII.                                           | nanoHA was evaluated in goat marrow stem cells and a rat model, as reported in chapters VI and VII.        |
| 29   | 30   | There are three main types of bone [21] (See Fig. 3, [22]): osteoblasts, responsible for bone              | There are three main types of bone [21] (See Fig. 2, [22]): osteoblasts, responsible for bone              |
| 31   | 5    | or trabecular bone is formed by a network of bone lamellae, delimiting areolar cavities where the          | or trabecular bone is formed by a network of bone lamellae, delimiting alveolar cavities where the         |
| 32   | 28   | of cell types. Cell types that MSCs have been show to differentiate into <i>in vitro</i> or <i>in vivo</i> | of cell types. Cell types that MSCs have been show to differentiate into <i>in vitro</i> or <i>in vivo</i> |
| 51   | 9    | The Ewing Sarcoma Family of Tumors (ESFTs), which usually occur in bone but may also arise in soft         | The Ewing Sarcoma Family of Tumors (ESFTs) that usually occur in bone, may also arise in soft              |
| 54   | 14   | Their relative inertness to he body fluids, high compressive strength, and aesthetically pleasing          | Their relative inertness to the body fluids, high compressive strength, and aesthetically pleasing         |
| 54   | 37   | in the food in                                                                                             | in the food industry as a raising agent in the cheese production; as well as in a variety of dental        |
| 120  | 30   | The resident oral microflora is diverse and consist of a wide range of species of microorganisms.          | The resident oral microflora is diverse and consists of a wide range of species of microorganisms.         |
| 122  | 9    | transmission electron microscopy (TEM), inverted stand optical microscope analysis and mercury             | transmission electron microscopy (TEM), inverted stand optical microscope analysis, and mercury            |
| 131  | 17   | between day 6 and day 10 as well as between different antibiotic concentration were less obvious.          | between day 6 and day 10 as well as between different antibiotic concentrations were less obvious.         |
| 155  | 36   | phosphates, nanoparticulate HA, and SPARC for bone engineering, using the osteoconductive and              | phosphates, nanoparticulated HA, and SPARC for bone engineering, using the osteoconductive and             |
| 158  | 18   | unconfined cell employing 20 N load at a crosshead speed of 0.5 mm/s until 75% of sample                   | unconfined cell employing 20 N of load at a crosshead speed of 0.5 mm/s until 75% of sample                |
| 180  | 31   | they begin the process of endochondral ossification {Sato, 1998 #81}; in fracture healing, SPARC           | they begin the process of endochondral ossification [29]; in fracture healing, SPARC                       |
| 180  | 40   | useful alternatives to be used bone regeneration scaffolds. In vivo experiments using a rat model,         | useful alternative to be used bone regeneration scaffolds. In vivo experiments using a rat model,          |