Development and optimisation of calcium phosphate silica-based ceramics for medical applications

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To my parents Tinka and Valentin

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PREFACE

The calcium phosphate materials are of considerable interest to scientists from many fileds such as biology, mineralogy, chemistry and medicine. The reason for this interest is very clear since hydroxyapatite (as a member of calcium phosphate family) forms the mineral component of bones and teeth. It was found that hydroxyapatite is involved in the mineralization process, which opened new research attention on bone graft substutute materials using calcium phosphates. As a contribution to the this field of research, we have introduced a research project which focus on a recently developed manufacturing process, known as the sol-gel method. Using this method we believe we can form the ceramics in new shapes with well expressed porosity, which is an important property of the bone graft substitute material.

This work is a small attempt for the world`s contribution to the development of bone graft substitute material. The main mission of our research project was to synthezise a resorbable material based on calcium phosphate ceramics: hydroxyapatite silica-based ceramic and hydroxyapatite/tricalcium phosphate (known as biphasic) calcium phosphate silica-based ceramic. Our study introduces in the first chapter a literature review beginning in 1800's to the present day on biomaterials, its problems in replacing parts of the human body, and the classification of ceramic materials. This section also includes a discussion about medical usage of hydroxyapatite and tricalcium phosphate ceramics and reasons for investigating biphasic calcium phosphate ceramic. Next, we provide background information on bone structure, its composition and the processes of bone formation and remodelling, and classification of bone graft materials. In chapter three, we discuss the existing methods for synthesis of calcium phosphate powders as well as the problems related to the manufacturing of these ceramics. In addition, chapter three briefly indicates the chemistry of hydroxyapatite and tricalcium phosphate.

We continue in the following chapter with an explaination of the manufacturing process of calcium phosphate silica-based ceramics and the results of their related properties which include: viscosity, thermal stability, porosity and shrinkage, and mechanical testing. In the last two chapters, results of the *in vitro* and *in vivo* performances of our ceramic samples are presented as well as their relationship to the main application of these materials - for

biomedical purposes. Lastly, we discuss the obtained results of the use of these calcium phosphate ceramics in non-loading and load-bearing implantation sites in mini pigs.

Since the early 1890s calcium phosphates have been used medically in bone repair, because it was thought that these materials might stimulate osteogenesis (i.e. the formation and development of bony tissue). Years later calcium phosphate materials have not come to fashion as bone substitute materials, for the attention was paid mostly to metals and polymer materials. A great progress in the mediacal use of calcium phosphates was made between 1970s and 1980s with the discovery that these materials are osteoconductive, i.e. they can guide bone formation on their surfaces and can facilitate a chemical bonding with the newly formed bone.

The osteoconductivity was found when calcium phosphates were introduced into bioglasses and glass-ceramics, or were applied as hydroxyapatite ceramic, tricalcium phosphate ceramic and biphasic calcium phosphate ceramics. Since then, a series of calcium phosphate-based biomaterials including bioglass, glas-ceramics, different calcium phosphate ceramics, calcium phosphate cements, calcium phosphate coatings and bioactive composites with calcium phosphates have been developed. The areas of main medical applications of the above mentioned biomaterials are in dentistry, maxillofacial sugery and spinal surgery.

Twenty-first century is believed to be the golden age for synthetic bone grafts and substitutes. Therefore the attention of many scientist is dedicted to the manufacturing of ideal bone graft substitute material, which can give the patiens superior outcome and can eliminate the donor-associated problems, since almost all bone grafts used currently by thousand of surgeones are autogenous (taken from the same patient). Hydroxyapatite is one of the few materials that are classed as bioactive, meaning that it forms bond with the surrounding bone, unlike other materials such as alumina and zirconia, which are identified as foreign materials and become encapsulated by fibrous tissue when implanted. Tricalcium phosphate is another material which is considered as biomaterial. Its usage in biomedical fields has overtaken the use of hydroxyapatite for many years, nevertheless it is thought that tricalcium phosphate due to its high solubility cannot facilitate new bone formation.

As a result of the trends in the research field on bone graft materials, we have focused our attention on bioceramic using both hydroxyapatite and tricalcium phosphate powders. We believe that the combination of highly soluble tricalcium phosphate and the more favourable for bone ingrowth hydroxyapatite together with a certain amount of silica, will provide a better solution to the overwhelming demand for bone graft substitute materials.

CHAPTER ONE

Introduction

1.1 Literature review

The use of certain materials as constituents of surgical implants is nothing new. Substitutions of bone parts for repairing seriously damaged portions of the human body are documented since the pre-Christian era. Bronze or copper were then chiefly utilised, probably in the circumstances requiring the assembly of fractured bone parts. It was well known, for instance, that in the Inca civilisation some operations were carried out, even delicate ones, where bone fragments removed during such operations were subsequently replaced in their original position. Unfortunately, the technology in which materials other then bronze and copper were considered as substances suitable for implantations was not developed until the mid-nineteenth century.

Prostheses were therefore, as a rule, only external. Nevertheless, medical science had already made progress as to anticipate serious attempts to repair body parts by means of foreign materials. Following a logical structure in terms of compatibility, based on the analogy between the composition of ivory and of bone, in 1889 Gluck applied an ivory prostheses by using a colophony-based cement for anchorage. In the following years, around 1902 Jones interposed a gold capsule between the articular heads, which is a remarkable achievement that had long-term success. This concept had emerged that in order to obtain a successful result, a chemically more inert and consequently more stable material was needed.

By 1915, it was already possible, contrary to what is commonly believed, to carry out studies on substrata of flame-sprayed vitreous melts (Norf, 1915). This track was subsequently abandoned owing to difficulties in finding devices capable of spraying melted glass at that time. In 1923 Smith-Petersen developed a well-organised program directed

towards achieving a practical and stable athroplasty. At first he employed glass capsules, which however, proved brittle. Then, he turned to a type of celluloid, which is no longer on the market today, and produced an excessive tissue reaction. At last he discovered the **Vitallium alloy** which, compared with all the materials employed until then, displayed such qualities of mechanical resistance and chemical inertia that it became the material of choice. The first metal prostheses made of Vitallium alloy were produced by Bives-Wills in 1938 and then by Bursch in 1939. The latter used self-polymerizing methyl methacrylate for their fixation.

Until 1960, the situation remained substantially unchanged, though it was realized that the metal to metal contact was deleterious on account of ensuing corrosion. By the late 1960s there was widespread use of polyethylene, which had been already introduced and used since the early 1950s (Scaglietti, 1951). Unfortunately Lavor-Gnin found as a result of tests conducted on guinea pigs that plastics too, including high-density polyethylene, had a carcinogenic power which might have cast some doubts on the long-term tolerability of some materials commonly used in orthopaedic surgery. Therefore, Boutin (1972) therefore decided to turn to materials presenting no particular biological drawback and directed his attention to substances such as alumina, zirconia and calcium aluminate. From dense sintered Al_2O_3, prostheses were prepared, that produced such favourable results in many applications that their usage is practically everlasting.

Nicolini (1973) started some promising experiments with glass ceramics, which had many characteristics that proved better then those of the numerous materials already utilized. As a result, bioactive-glass-producing technology was started in 1971 by the Hench and Broemer groups. They used compositions, which are still being adjusted, to serve as coatings for metal prostheses to achieve simulated natural anchorage without renouncing the positive mechanical properties of the metal body. The incidence of orthopaedic interventions has increased enormously among the population since man entered the industrial era. The use of self-operating machines and the necessity for manual intervention are the main causes of the steady increasing number of accidents as production rhythm intensifies and interactions multiply. Furthermore, the moral awareness of everybody`s right to existence, which has improved life conditions and led to progress in the fields of social and medical security, also implies an attempt to rehabilitate those who are subject to congenital malformations, who in the past would have led a life of much lower quality.

For this reason the interventions aimed at diminishing, alleviating damaged or replacing missing limb parts as reliable as possible. Intervening in the human body has always

been regarded as a factor disturbing a natural unity, and consequently has sometimes been subjected to a sort of sacred interdict. When a foreign substance is introduced into a tissue, between the tissue and the substance is a physiological interaction which is mediated through a biochemical chain, often unknown and long, which in many cases tries to assimilate the substance.

1.2 Problems in replacing parts of the human body

Throughout the human history, surgery had found widespread support because it has always complied with the philosophical principle, which is universally accepted since the ancient times, that individual life must be safeguarded. As man increasingly improved his knowledge of nature, curiosity and necessity brought him to learn to control his own nature by applying those same criteria that have proved valid for the knowledge of the external world. When a part of a machine breaks, wears, or becomes useless, recourse is generally made to a new substitutive piece capable of restoring the original function; this concept can be also extended to the human body. In this case, however, it is important to be morally aware that this body belongs to a human being and is not merely a special kind of device. For this reason the guidelines involved in organ substitution must be extremely clear: one aspect is the nature of the substance from which the substitute piece is made, and another is the quality of functional simulation exhibited.

In general, a substance constituting an object able to substitute an original living part of the body is called a **"biomaterial"**. A comprehensive definition of this term was enunciated in 1982 at the NIH Consensus Development Conference (Galletti and Boretos, 1983) on the clinical Applications of Biomaterials as follows: **"any substance, other then a drug, or combination of substances, synthetic or natural in origin, which can be used for any period of time, as a whole or as a part of a system, which treats, augments or replaces any tissue, organ or function of the body"**. At the European Society for Biomaterials Consensus Conference held in Chester, UK, in 1986 there was significant disagreement with this definition and the following new simple definition was agreed: **"Biomaterial – a non-viable material, used in a medical device intended to interact with biological systems"** (William, 1987b). A synonymous term is **"biomedical material"**,

which indicates a substance, generally in solid consistency, useful for manufacturing an object. The object with its shape and structure, may consist of either living or non-living materials. The living materials are cells, cell-colonies, and tissues taken from one or more individuals, generally soon after death, including skin, bone, cornea and even whole organs such as liver and heart. Devices made from non-living materials and placed in the body are called **" implants"**, or **"implantable devices"** (1).

The expression "surgical implant" is frequently used to denote an implant placed into its desired location by means of a surgical procedure. Important in surgical implants is the host response to the biomaterial given by surrounding tissue, which is the reaction of a living system to the presence of this implanted material. For this purpose, the following restricted definition of biomaterial might be useful: **"A non-viable material used for medical purposes and interacting non-adversely with the living system"**. When no interaction occurs, the material is called "inert" or "bioinert". However, the implanted materials behaving in this manner do not actually in practice exist, inert or bioinert has quite often give rise to extremely weak interactions. Some important types of interaction, whereby the material constituting the implanted device is subject to modifications, are (1):

- **Biodegradation**, that is, gradual breakdown of a material mediated by specific biological and/or biochemical activity
- **Bioresorption**, that is, the removal process through cell activity and/or through dissolution by continuos ionic diffusion of the material constituting the device body when placed in a biological environment, and
- **Bioactivity**, that is, the behaviour of a material (called a bioactive material) designed to induce a specific biological activity.

1.3 Classification of ceramic biomaterials

A strong interest in the use of ceramics for biomedical engineering applications developed in the late 1960`s, exemplified by the work of Hulbert and co-workers (Hulbert et.al 1982-1983). Although, the interest reached a plateau during the late 1970`s and early 1980`s, there is now an increased pace of activity in the field of bioceramics. In the Consensus Conference of the European Society for Biomaterials held in Chester, 1986 there was an agreement for the definition of bioceramics: **"a bioceramic, is a ceramic, used as a biomaterial"**. The development of ceramic material applications in biomedicine has concentrated mostly in orthopaedics and dentistry. Orthopaedic bioceramics provide the advantage of chemical similarity to natural skeletal materials. As with orthopaedic materials, dental applications for ceramics are attractive due to chemical similarity between engineered ceramics and natural dental materials.

J. F. Shakelford (2) has classified the conventional bioceramics by their primary chemical constituents:

- ***Alumina-based ceramics*** - This is the first bioceramic widely used clinically. It has good strength, modest fracture toughness, high wear resistance, good biocompatibility and excellent corrosion resistance. The clinical applications of alumina-based ceramic include: hip replacement surgery, knee prostheses, bone segment replacements, bone screw, middle ear bone substitutes, corneal replacements. In dentistry alumina has been used in various dental implants, including blade, screw, jaw bone reconstruction and post configurations.
- ***Zirconia-based ceramics*** - Zirconia has become a popular alternative to alumina as a structural ceramic because of its substantially higher fracture toughness. Zirconia, in fact, has the largest value fracture toughness of any monolithic ceramic. The main application filed of zirconia-based ceramic is in the load-bearing surgery.
- ***Mixed ceramic oxides*** - A variety of simple mixed ceramic oxides were evaluated in the pioneering studies of bioceramics in the 70`s. CaO. Al_2O_3, CaO. TiO_2 (Perovskite) and

CaO. ZrO_2 as both porous and non-porous implants were used in soft and connective tissue for up to 9 months. Although such studies proved to be reasonably successful and helped to establish the basic understanding of the utility of ceramics in biomedicine and the role of porosity in their function, simple alumina ceramics generally performed better in implantation studies.

- ***Silicate ceramics and glasses*** - Silicates represent the dominant category of the traditional ceramics and glass industries. These materials are economical due to the abundant availability of raw materials and they provide adequate mechanical, thermal and optical properties for a wide range of traditional and advanced materials applications. With the development of **Bioglass** by Hench and co-workers the biomedical applications of noncrystalline silicate glasses has increased. Bioglass implants based on composition labeled 45S5 (containing 45 wt% SiO_2, 24,5 wt% CaO, 24,5 wt% Na_2O and 6 wt% P_2O_5) have been successfully applied in a variety of dental and medical applications: ear bones surgery, denture wearers, maintenance of jaw bone, restoration of bone next to teeth etc.
- ***Glass-ceramics*** - Glass-ceramics are usually called the "sophisticated form" of crystalline ceramics. Both stages glass and ceramic give excellent advantages: due to the glass stage the material can be formed into a complex shape economically and precisely by conventional glass-forming technology, due to the ceramic stage is the crystallization, which gives fine-grained microstructure with little or no residual porosity. Such a microstructure tends to provide optimal mechanical performance in a ceramic. Glass-ceramic products typically have good resistance to mechanical shock due to the elimination of stress-concentrating pores. Conventional glass-ceramics are based on composition systems such as Li_2O-Al_2O_3-SiO_2, which produce crystalline phases with exceptionally low thermal expansion coefficients and resistance to thermal shock. Glass-ceramics for biomedical applications are more typically based on compositions similar to **Bioglass** system. **Ceravital** (low-alkali silica glass-ceramic) has been used as implants in middle-ear surgery, Apatite-Wollastonite glass-ceramic has been developed in Japan and been used successfully in hundreds of patients in replacing part of the pelvic bone and in vertebral surgery. An easy-to-machine silica-phosphate glass-ceramic has been produced in Germany, which contains phlogopite (a type of mica) and apatite crystals.
- ***Ceramic-matrix composites*** - Ceramic-matrix composites are proving to have even higher values of fracture toughness, comparable to that in some common structural metal alloys. As with silicate ceramics and glasses, the more common ceramic-matrix composite used in industry are not necessarily appropriate for biomedical applications. In addition to the

advantage of improved fracture toughness, design goals in developing ceramic-matrix composites for biomedicine have focused on increasing flexural strength and strain to failure – a good example is an Apatite-Wollastonite glass-ceramic containing a dispersion of tetragonal zirconia which has a bend strength of 703 MPa and a fracture toughness of 4 $MPa.m^{-2}$.

- ***Hydroxyapatite*** - For many years hydroxyapatite did not come to fashion as a biomaterial, nevertheless its obvious chemical similarity to bones. Hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, is the primary mineral content of bone representing 43% by weight. It has the distinct physiochemical advantages of stability, inertness and biocompatibility. The relatively low strength and toughness of hydroxyapatite, however, produced little interest among researchers when the focus of attention was on bulk structural samples. Some of the applications of hydroxyapatite ceramics are summarised in Figure 1.1.
- ***Other calcium salts*** – tricalcium phosphate (TCP) with a chemical formula $Ca_3(PO_4)_2$ has been used as a bioceramic as early as 1920. This ceramic salt tends to stimulate the formation of immature bone but before used to be described as an example of unsuccessful bioceramic (Hulbert et al., 1982-83). Today, TCP remains as a useful bioceramic and a good example of the "resorbable" category of bioceramics. Most of the applications of TCP ceramics are in conjunction with HA ceramic.

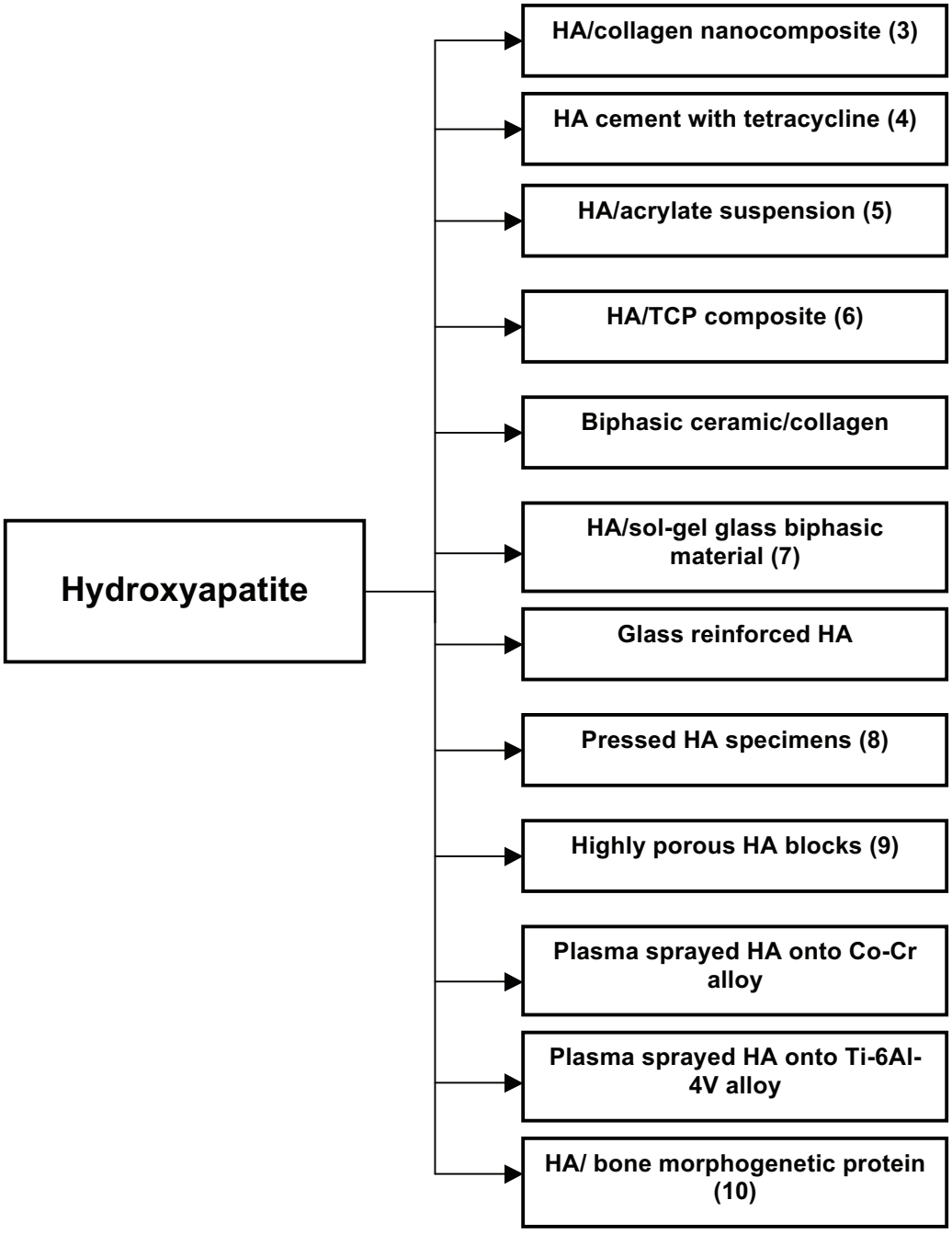


Figure 1.1: *Variety in the applications of HA ceramic*

1.4 A short overview of the medical usage of hydroxyapatite and tricalcium phosphate ceramics

Calcium hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ is the principal calcium phosphate used for biomedical application. Several investigators have shown that the crystalline planes of hydroxyapatite in the bioceramic matched those of hydroxyapatite in the mineral phase of bone and that biological apatite was deposited directly on the ceramic surface. Bulk HA has been used with success in a variety of applications (Fig.1.1, p.8).

Tricalcium phosphates $Ca_3(PO_4)_2$, are probably the most bioactive calcium phosphate ceramics. Their high degree of solubility compared with HA has indicated their use in areas in which a high degree of bioactivity is desired. This may include the coating of dental and orthopedic prostheses as well as in bulk form for use in dental, orthopaedic, and plastic reconstructive applications. Unfortunately the high degree of bioactivity may cause the material to be completely resorbed without a concomitant deposition of bone. This is a problem in certain dental applications, where resorption of the material leads to a clinical failure. Cutright *et al.* first investigated the use of tricalcium phosphate ceramics in the tibias of 48 rats. They observed that bone was deposited directly on the ceramics, and that no adverse tissue reaction was observed. The high resorption rate was evident in these early studies. The authors estimated that biodegradation of the ceramic was 95 % complete at 48 days after implantation (11).

Le Geros reviews the uses and clinical results of β-tricalcium phosphate in dentistry. In some cases, such as a periodontal osseous defects, TCP was reported to have a little benefit in repair of the defect. Metsger, Driskell, and Paulsrud reported that only 50 % of human patients had complete bone fill when the material was used to fill bony defects (11). Since dental uses of TCP did not show much promise, orthopaedic uses were investigated. In many of the studies in which TCP was used to fill segmental defects in dogs, filling of the defect with bone occurred whether TCP was used or not. Investigation of β-tricalcium phosphate granules for filling defects after tumor removal in humans was reported by Bucholz, Carlton and Holmes (11). They showed that these materials were effective in this application and that subsequent fracture did not occur. Betha-tricalcium phosphate granules were also implanted

with orthopaedic prostheses in hopes that they would stabilize the prostheses and encourage bone formation. Berry *et al.* reported that the interface bond strength between the implant and host bone was not increased by the addition of TCP. Fitzgerald *et al.* reported that the ceramic granules formed a barrier to bony ingrowth of the prostheses (11). Due to problems with resorption of TCP without associated deposition of bone, TCP is rarely used clinically. It is useful in applications in which bioactive materials are used as a drug delivery system.

1.5 Reasons for investigating biphasic calcium phosphate ceramics

Biphasic calcium phosphate ceramics consist of both β-tricalcium phosphate and hydroxyapatite. A material with ideal biodegradibility would be replaced by bone as is degraded. However, when used as biomaterial, the rate of biodegradation of TCP has been shown to be too fast. To slow the rate of biodegradation, biphasic calcium phosphate ceramics, consisting of HA and TCP have been investigated. Hubbard (11) was the first to investigate ratios of HA/βTCP for the use as a bone graft substitute in 1974. These composite ceramics offer several distinct advantages over either phase as described above. HA has been characterized as relatively bioinert compared with tricalcium phosphate ceramic. Although tricalcium phosphate ceramic phase is more bioactive, it may be quickly resorbed without subsequent bone formation. The biphasic material has the ability to provide a scaffold for new bone ingrowth due to the presence of the HA phase, as well as the ability to promote osteogenesis due to the presence of the bioactive β-tricalcium phosphate phase.

Recent *in vivo* studies indicate that the biodegradation rate of BCP ceramics depends on the ratio of TCP to HA in the compound (12). The calcium phosphate ceramics containing a greater amount of TCP phase showed greater biodegradation. Along with the biodegradation, the HA crystals with Ca/P ratio similar to that of bone-apatite precipitated around the calcium phosphate ceramics containing the TCP phase. Sintered HA ceramics revealed no noticeable biodegradation after implantation. Osteoconductibility was not significantly different between BCP, TCP, and HA. In addition, an *in vitro* dissolution study on commercilaly available calcium phosphate materials revealed that the BCP ceramics which contained a higher amount of TCP component dissolved more rapidly. In static physiological

solutions (which do not change during the immersion period), calcium and phosphate ions in the solutions may reprecipitate on to the calcium phosphate ceramics. Due to its greater stability, the HA in BCP acts as a seed material in physiological solutions.

Some studies on the effect of TCP materials (some tests were made with TCP as bulk materials, or granules, or most recently as a phosphocalcic hydraulic cement) on osteoblasts, which have been investigated in vitro and in vivo, showed controversial results, depending on study design and tested material. Some studies indicate a good bonding between tricalcium phosphate and bone, whereas other studies confirm soft tissue interpositions. J. Handschel *et al.* (13) have reported that no direct bone contact was found around the TCP particles, when the study was taken in a non-loaded bones – calvaria of Wistar rats. In their study they concluded that TCP is hardly resorbed and not osteoconductive in a non-loading model. The phase composition of biphasic calcium phosphates may affect their solubility, as reported by Daculsi (14) - BCP solubility depends on HA/βTCP ratio, the lower the ratio, the higher the solubility.

In the light of the current literature, we have decided that using hydroxyapatite silica-based ceramic and comparing it with biphasic calcium phosphate silica-based ceramic would be useful in order to better understand the behaviour of these ceramics *in vivo* when implanted in Goettinger mini pigs animal model.

CHAPTER TWO

Structure and composition of bone. Bone formation (Osteogenesis) and remodelling. Bone grafts

The goal of this chapter is first to define specific what are bones and its bone mineral. Next, an overview of previous research projects and their findings are presented to better understand the bone formation process, where a discussion over its three main steps is included. Lastly, this chapter contains a brief presentation of the bone remodelling process, its five distinct phases, and a brief overview of the bone grafs.

2.1 Bone structure

In the literature, the bone material is defined in several ways by scientist and accepted in the scientific community. For example, Pritchard defined bone as follows: the bone is a type of specialized cementious tissue, characterized by cells with long branches (osteocytes), hosting cavities (lacunae) and thin channels (canaliculi), and the bone has a dense and hard matrix consisting of groups of collagen fibres within an amorphous boundary substance (cement) impregnated with calcium phosphatic complex (1). The bone is not only a unique mineralized connective tissue that provides the structural support for the body, but it is also a storehouse for essential ions. The skeleton is a repository for 99 % of the body´s calcium, 80 % of the phosphate, and a major proportion of the body`s stores of magnesium, sodium and carbonate.

The bones of adult skeleton consists of cortical (or compact) and cancellous (or trabecular) bone. Cortical bone is dense or compact bone. It constitutes 85% of the total bone in the body, and is relatively most abundant in the long bone shafts of the appendicular

skeleton. Loss of cortical bone is the major factor for fractures that occur in the hip. Although cancellous bone constitutes only 15 % of the skeleton, the changes that occur in this type of bone determine whether the clinical features of osteoporosis will occur. The adult skeleton is steadily moving in a dynamic state, being continually broken down and reformed by the coordinated actions of osteoclasts and osteoblasts. This turnover or remodelling of bone occurs in focal and discrete packets throughout the skeleton. There are sequence of cellular events responsible for remodelling, which are locally controlled possibly by an autoregulatory mechanism. These sequences are always the same: osteoclastic bone resorption followed by osteoblastic bone formation to repair the defect. The bone formation is an extremely complex process, which is not well understood.

One of the most unique and important aspects of the bone formation process is the biological mineralization of the bone proteins to form a mineralized matrix. In 1987, a research by Posner has provided a recent review of the nature of bone mineral and the process of mineralization, which is still an area of controversy. The long-standing body of scientific knowledge suggest that mineral is initially deposited as amorphous calcium phosphate, which is the initial solid phase formed at neutral pH. This phase is randomly and poorly ordered. When following deposition of amorphous calcium phosphate, a series of solid-phase transformation steps occur that led to poorly crystalline hydroxyapatite as the final stable solid phase. The initiation of mineralization is probably caused by heterogeneous nucleation, that are actively binding calcium, phosphate and calcium phosphate complexes at the nucleation site in the matrix, rather then simple precipitation, which would result in a random organization of the crystals. Thus, it is possible that poorly crystalline calcium carbonate-containing hydroxyapatite is deposited in mature bones, rather than an amorphous calcium phosphate or hydroxyapatite (15).

2.2 Organic and mineral composition of bone

Another researchers such as Ravaglioli (Ravaglioli, 1992), proposed that there are mainly two salts formed and dispersed within the organic fraction of bone – calcium triphosphate and hydroxyapatite. The crystallized mineral fraction is practically

hydroxyapatite, in a crystalline form, that constitutes the framework providing the principal mechanical characteristics of bone. Approximately, one-third from the weight of the organic portion of compact bones is composed of dry free fats, while the remainder are divided into collagen, cementing substances, and cells. This remaining organic weight comprises 90-95 % of collagen protein fibres. The collagen protein fibres are sclero-proteins of jelly-like consistency, that are the main constituent of the organic fibrils found in such cartilaginous sections as those of the skin and of connective tissues. Collagen is chemically characterized by a high content of aromatic amino acids. These collagen fibrils are considered the elementary units of fibres and can be observed only by means of electron microscope techniques (Fig. 2.1). Many closely connected aggregates of such fibrils build fibre, which can be identified under the light of an optical microscope. Under polarized light one can see thin sections of bone disclose a definite relationship between the orientation of the apatite crystallites and the direction of collagen fibres. When the crystallites are placed lengthways parallel to collagen, their length corresponds to approximately one-third of 640 Å, which is the average fundamental unit of repetition of collagen fibrils. In addition, it has been observed that HA crystallites locate themselves also around and between fibres.

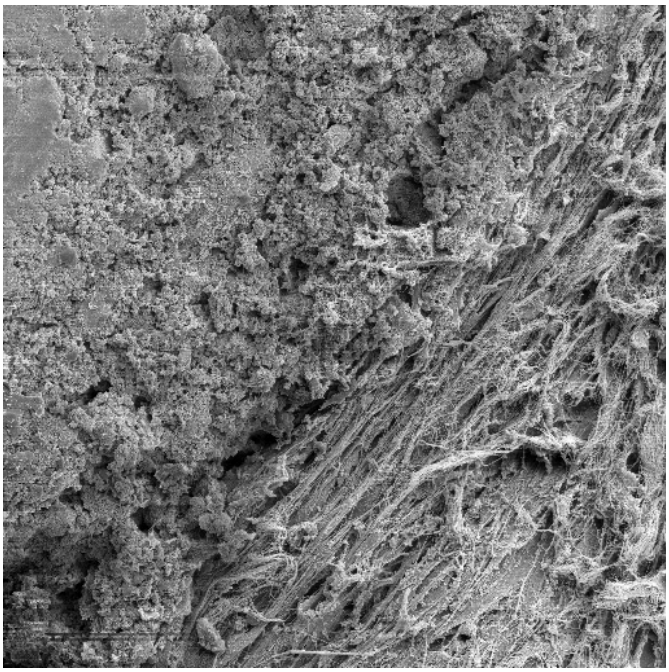


Figure 2.1: *Network of collagen fibers running closely to the ceramic surface, scanning electron micrograph, x3000*

Collagen fibres are surrounded by bone with a supporting substance referred to as “cement”. This organic cement, along which bone mineralizes, fills the spaces between fibrils. Such natural or organic cement is highly homogenous and amorphous, since it appears non-structured even at the highest resolution limit of electron microscopes. Although the chemical composition of cement is not definitely known, its nature largely consists of protein complexes.

Moreover, when evaluating the properties of bone, one should not miss the characteristics of its composition, especially those relative to its mineral part and particularly

in a view of the possible application of implants made of theoretically suitable materials. From this point of view, apatites and the compounds that may be associated with them are of fundamental importance, primarily in relation to the physical chemistry of hard tissues. The mineral components of these tissues are in the first place calcium phosphates, among which hydroxyapatite or HA with chemical formula $Ca_{10}(PO_4)_6(OH)_2$ - which is known as the most important compound. Bones, in fact, contain HA crystallites about 40 μm in length within tissue. Each elementary cells contains two stoichiometric units; the Ca^{2+} ion occupies two non-equivalent lattice positions. In the first one, with multiplicity 4 it co-ordinates 9 oxygens belonging to PO_4^{3-} tetrahedral groups, and these co-ordination polyhedrons are joined together so as to form chains along the direction of the c-axis (Fig. 2.2). Halogens and hydroxyl appear to be located at the top of the cell in the projection normal to c_o, but their co-ordinate with respect to this axis is still uncertain.

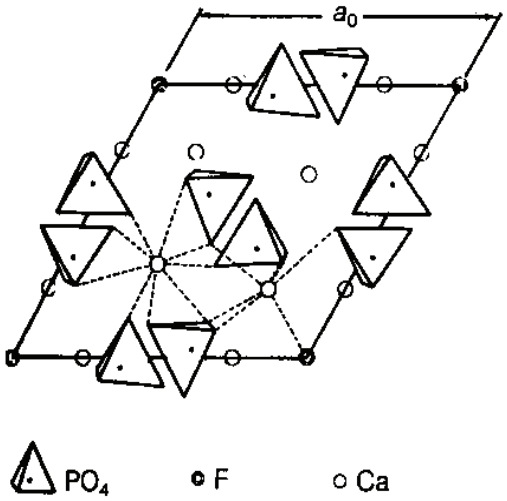


Figure 2.2: *Diagram of the apatitic structure (from Ref.1)*

The arrangement of the –OH groups into unit cell packing is of biological significance, since the structural difference between better and worse arranged OH^- groups. These arranged OH^- groups produce the stacking forms, that are the main cause of the stability to increase in the direction of fluorapatite. As a result, this means that pure hydroxyapatite does not have a good structural order, but rather disorder. Bones, containing apatites richer in F^- grow more rapidly, and have crystals of greater size compared with corresponding less fluoride-rich specimens. The chemistry of hydroxyapatite will be explained in the next chapter.

It is increasingly evident that HA may undergo a variety of modifications. Accurate transmission electron microscopy tests have displayed an ordered arrangement of the apatite mineral of bone, with slightly lower computed unit-cell values. Such tests have suggested that at the surfaces of HA crystallites the more developed exposed planes are those [223] and not the fundamental ones. This can be due to the easier mechanism of attraction in the interaction with the enzymatic carriers of the PO_4^{3-}, OH^-, and Ca^{2+} ions, for example alkaline

phosphatase, calmoduline, and others known and unknown. On the other hand, further factors may be involved that may interfere with the crystallographic arrangements of bony HA. For example, traces of elements such as Si, Al, Fe, have been found in the macropores of implanted pieces of pure HA ceramic, and the mechanism of their accumulation is unknown. These elements are also involved in the biochemical accumulation for the equilibrium of blood iron. One further replacement is that resulting from the interaction with CO_2. It can be admitted a substitution mechanism for the PO_4^{3-} group rather than for F^-, OH^-, Cl^- ions with regard to the space volume.

Yet another researcher Foresti (Foresti et al., 1980) found that biological apatite exhibits some marked characteristics of liability to alteration. The results of many other researchers indicate a significant difference between biological apatites and mineral hydroxyapatites. It was in fact suggested that of all substituted ions, the Mg^{2+} is the one inducing the formation of β-TCP ($Ca_3(PO_4)_2$) (Blumental et al. 1975). X-ray diffractions of HA preparations containing 4 to 6% carbonate indicate a behaviour very similar to that of mineral part of bone. Moreover, the heating of this preparation to beyond 700°C shows HA to be associated to β-TCP. The X-ray diffraction of HA containing Mg^{2+} conversely demonstrates that such preparations are crystalline to a very small extent, and that their heating gives rise to a full transformation into β-TCP, with Ca^{2+} partially replaced by Mg^{2+} (then called β-TCMP, betha tricalcium-magnesium phosphate).

Another further phosphatic component of hard tissues is calcium orthophosphate, or OCP, also called monophasic tetracalcium triphosphate - $Ca_4H(PO_4)_3$ $2.5H_2O$. There is a discussion as to whether it is present in bone as a distinct crystal phase or as “defective apatite” hydrated through a complex substitution of hydronium ions (H_3O^+) for Ca^{2+} in the stoichiometric formula. Optical, structural, and chemical properties prove OCP to be structurally correlated with HA, even though the two salts are not isostructural. This characteristic proves that epitaxial growths of one compound on the other are very easy to obtain by means of a sort of solid solution between the two compounds, which consist of planes (each corresponding more closely to either of the two pure compounds) bound by layers of water molecules. It is in fact commonly assumed that osteoblasts start their bony mineralization at pH ≥7.5 by an excretive process that leads to the production of OCP as a precursive substance that spontaneously converts itself into HA, which is more stable at those pH values. The equilibrium between HA and OCP is twofolded and is determined by the possibility of coexistence between crystals possessing a different habitus and undergoing two

different HA-forming processes. Crystals and steps of HA result from an either partial or complete OCP hydrolysis, whereas a direct precipitation will produce HA needles.

Some apatites found in nature exhibit a composition very similar to that of bone, as well as properties and crystallographic structure that correspond to those of the components present in bone. The ease with which adventitious substances and other foreign elements are incorporated into bone matrix has made their exact determination difficult, for these substances are simply adsorbed at the surface of crystals and are chemically combined in the crystal structure of calcium phosphate.

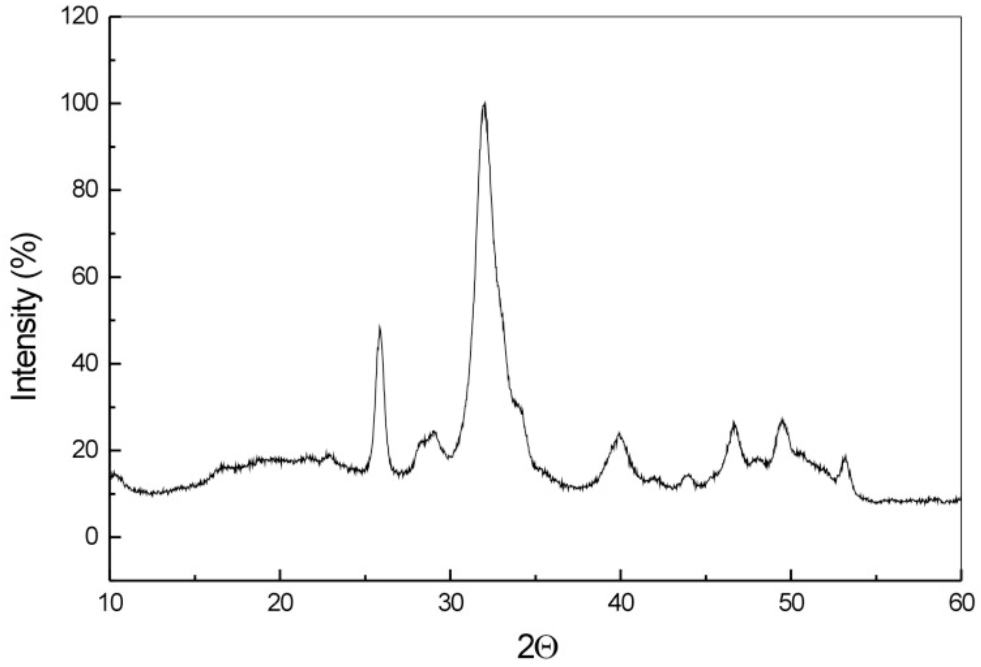


Figure 2.3: *X-ray powder diffraction pattern of natural bone (note the difference with Fig. 3.2 from Chapter 3)*

The size and shape of bone apatite crystals have been measured by direct observation and by indirect calculations employing X-ray diffraction line broadening. The literature on this subject is confusing for several reasons. Size and shape of the mineral particles change with species, age and disease, and a single specimen contains a range of particle sizes and shapes (Fig. 2.3). In addition, different measuring techniques may yield different average values of polydisperse samples. A representative study of bone from femurs of man, ox and rabbit, using X-ray diffraction and dark field electron microscopy, concluded that mineral particles are probably plate shaped. The particles were 50-60 Å in the smallest dimension and

the largest had a mean value of 320-360 Å, a standard deviation of 130-190 Å, and ranged up to about 1100 Å. In a given species the average crystal size is smallest at formation, increasing to maturity, and then has a levelling of this growth process. Concomitant with increase in crystal size is an improvement in chemical perfection. The earliest bone crystal is Ca- and OH-deficient and H-containing. With ripening, the bone crystal aproches, but never reaches, the perfect hydroxyapatite formula. When heated to the appropriate temperature bone apatite produces pyrophosphate groups and β-$Ca_3(PO_4)_2$, which is an evidence that bone mineral is a Ca-deficient, CO_3-containing, submicroscopic hydroxyapatite (1).

In the formation and maturation of bone mineral there is a considerable evidence that, as noted earlier, an unstable amorphous calcium phosphate is the first mineral deposited. It was concluded that only a portion of this amorphous precursor phase transformed to a poorly crystallized, CO_3-containing, Ca-deficient bone apatite, while the remainder appeared to persist as a separate amorphous phase even in mature bone. In a more recent study (16), the amount of amorphous mineral remaining in mature bone was estimated by comparing the X-ray radial distribution function (RDF) of mature rabbit cortical bone with that of mixture of amorphous calcium phosphate and CO_3-containing apatite. Considering the accuracy to which these RDF`s can be determined, is was estimated that in such a 2-phase model, mature bone could contain at most 10 wt % of an amorphous phase. Thus, amorphous mineral could exist in significant quantities in bone, either as a transient intermediary, or as a stable phase and would be undetectable by radial distribution function method.

2.3 Bone formation and bone remodelling

The process of bone formation (osteogenesis) involves three main steps:

1. The production of the extracellular organic matrix (osteoid)
2. Mineralization of the matrix to form bone
3. Bone remodelling by resorption and reformation

Firstly, the cellular activities of osteoblasts, osteocytes, and osteoclasts are essential to the process of bone formation. Osteoblasts synthesize the collagenous precursors of bone

matrix and also regulate its mineralization. As the process of bone formation progresses, the osteoblasts come to lie in tiny spaces (lacunae) within the surrounding mineralized matrix and are then called osteocytes. The cell processes of osteocytes occupy minute canals (canaliculi) which permit the circulation of tissue fluids. To meet the requirements of skeletal growth and mechanical function, bone undergoes dynamic remodelling by a coupled process of bone resorption by osteoclasts and reformation by osteoblasts.

Bone formation can occur via three related to each other processes. The process of endochondral bone formation, as occurs in embriogenesis, begins when mesenchymal stem cells (MSCs), progenitor cells that can differentiate into bone or cartilage-forming cells, start to differentiate into chondrocytes and secrete a cartilaginous matrix. During this time the cells continue to divide. As they pass through various lineage states, they secrete different molecules and eventually lose the capability to profilerate. Shortly after the appearance of the first bone, the chondrocytes surrounded by the periosteum enter the final stage of development and become hypertrophic chondrocytes, which produce proteins that are important in calcification of the matrix. At this time, phagocytic cells degrade some of the bony periosteum and resorb some of the interior matrix. To this point, the tissue is completely avascular, but following matrix resorption, the first capillares begin to appear. The cells that migrate into the hypertrophic cartilage work to create a highly vascularized marrow cavity. At this point, new set of MSCs begin to differentiate into osteoblasts that proliferate and form a bone matrix on the calcified cartilage. The bone produced by this process is immature woven bone. This type of bone is rich in osteocytes (mature bone cells) and has small collagen fibrils, which are oriented randomly. The spaces around blood vessels in woven bone are extensive. This bone will be remodelled to form lamellar bone, which is more organized and has superior mechanical properties.

Endochondral bone formation produces both long and short bones in the body. Flat bones usually develop via intramembranous ossification, which differs from the previous process in that no cartilaginous model is formed. Instead, MSCs differentiate into layers and then begin to produce a matrix that includes blood vessels and more MSCs. These MSCs differentiate into osteoblasts and begin to secrete bone matrix.

A third type of bone development, called appositional formation, occurs during the enlargement of bones and during remodelling. In this case, osteoblasts attach to existing bone and secrete matrix, often in layers. All these three types of formation occur constantly and a particular bone can be formed through any combination of them. As any type of bone formation occurs, the predominant collagen matrix secreted by the osteoblasts undergoes

calcification. As osteoblasts are separated by calcifying matrix, they are entrapped in spaces called lacunae. These entrapped osteoblasts are now called osteocytes and they gradually lose the ability to produce matrix.

These cells communicate with other osteocytes via long processes (canaliculi), which are organized before calcification. Mineralization of woven bone occur 24-74h after creation of the matrix. There are two steps to matrix mineralization: including nucleation of calcium phosphate crystals, followed by crystal growth. Nucleation can occur either heterogeneously or homogeneously. Homogeneous nucleation is the formation of crystals due to supersaturation of the local environment with the appropriate ions. Heterogeneous nucleation occurs only at surfaces where the interaction between the surface and the ions lowers the interfacial energy requirement so that nucleation can proceed at concentrations less than that of supersaturation. The main example of this in bone calcification is found with collagen-mediated deposition. After nucleation, amorphous calcium phosphate may be the first precipitate, which is then converted to octacalcium phosphate and finally to hydroxyapatite. After either type of nucleation forms the first small crystals, they quickly grow from the whole regions in the collagen molecules to the overlap regions, where they are aligned along the fiber axis.

As woven bone is formed and calcified, it is remodelled to form mature lamellar bone. On a larger scale, both woven and lamellar bone can be found in either cancellous or cortical bone. Lamellar bone is generated more slowly than woven bone and is less mineralized. Collagen fibers are thicker and have a preferential orientation, which alternates between layers and lamellae. Collagen fibrils extend between lamellae, thus increasing the bone`s strength. A final stage of remodelling converts some concentric lamellar bone to Harvesian systems (secondary osteons). The lamellae closest to the blood vessel are removed and new bone is added concentrically. At the interface between the new and the old bone is the cement line, a sheath of calcified mucopolysaccharides. Due to overlapping of areas of bone with differently oriented collagen fibers, lamellar/Harvesian bone is less flexible than woven bone. In concentric lamellar bone, the orientation of the osteons provides strength in tension and compression. When a lamellar bone does break, it usually occurs along cement lines rather than across osteons. Summarizing all the events that happen throughout bone remodelling, five distinct phases of remodelling process can be distinguished. These are:

***1. Resting state*:** the surface of the bone is lined within active cells. Former osteoblasts are trapped at osteocytes within the mineralized matrix.

***2. Activation*:** Hormonal or physical stimuli signal mononuclear monocytes and macrophages to migrate to the remodelling site and differentiate into osteoclasts.

***3. Resorption*:** Osteoclasts begin to remove the organic and mineral components of bone and form a cavity of characteristic shape and dimensions called Howship`s lacuna in trabecular bone and a cutting cone in cortical bone. When the cavity reaches a depth of about 60 μm from the surface in trabecular bone and about 100 μm in cortical bone, resorption at that location ceases.

***4. Reversal*:** Osteoclasts disappear and mononuclear macrophage-like cells smooth the resorbed surace by depositing a cement-like substance that will bind new bone to old. Pre-osteoblasts begin to appear.

***5. Formation*:** Differentiated osteoblasts fill in the resorption cavity and begin forming new osteon in a two-stage process. First, they deposit osteoid (mostly collagen type 1). The rate of matrix apposition is initially very rapid and the osteoblasts are columnar and densely packed. Mineralization of the osteoid begins when the cavity has been filled to 20 μm. With the onset of mineral apposition, the rate of mineralization exceeds the rate of matrix apposition and continues, with a substantially lower rate, even after the termination of matrix synthesis, until the bone surface returns to its original resting state (17).

2.4 Bone grafts

Bone is the second most frequently transplanted tissue in humans. In the United States alone, it is estimated that over 200,000 operations per year are performed that requires bone grafts (Statistic from 1986 according to Ref. 11). Surgical uses of bone grafting materials include surgical intervention of ossesous nonunions, restoration of the structural integrity of bone after trauma, and filling defects following bone tumor removal. Different grafting materials and graft substitutes are available for use as bone grafts. Here the comparisons between several bone graft materials in use are given (11):

- ***Autograft*** - Autograft bone is bone taken from the patient who will receive it. This material may be obtained from the iliac crest, rib, fibula, or tibia (Fig. 2.4). Autogenous cancellous bone graft is currently the preferred graft materials for several reasons. First, it

is biocompatible and nonimmunogenic since it comes from the same person. Second, it will not transmit a disease to the recipient. Third, it has osteogenic potential since cell on the trabecular surface of the graft become active after transplantation. Although autogenous bone grafts offer several advantages over other bone grafts, they also have some distinct disadvantages. In the first place, autogenous bone grafts can only be taken in limited amounts from the iliac crests or ribs. Even when a small amount is taken, morbidity at the donor site due to the surgical procedure to remove the graft may be significant. Furthermore patients requiring autogenous bone grafts are often in groups found to be at greatest risk of complication, due to the metabolic disease, and offering poor quality bone for reconstruction.

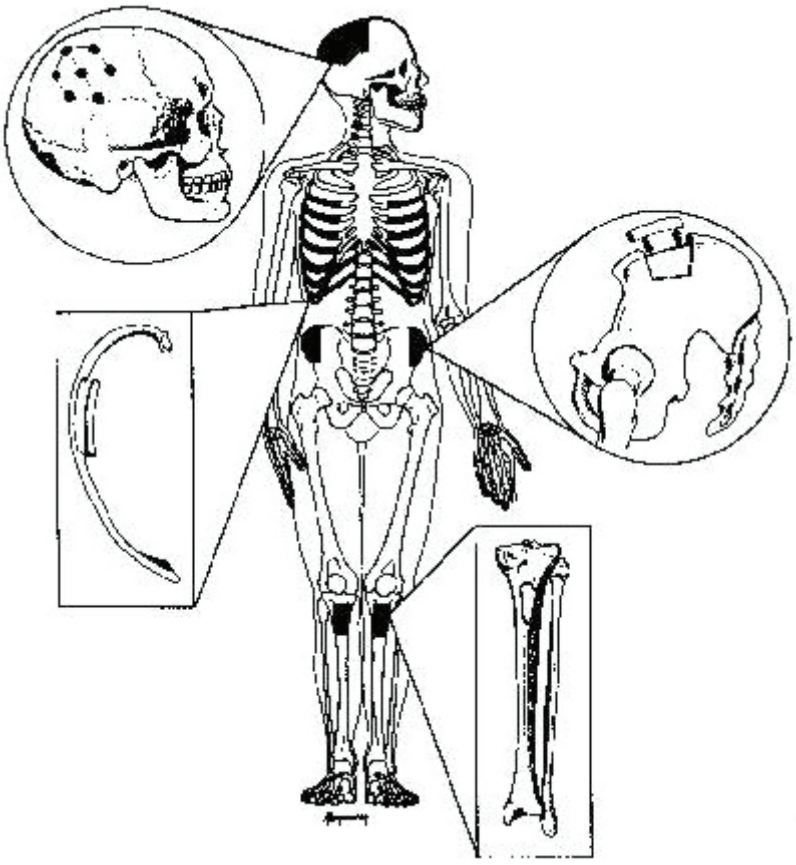


Figure 2.4: *Bone graft donor sites (from Ref. 18)*

- ***Allograft*** - Another graft material, which is the second most frequently used in surgeries requiring a bone graft, is allograft. Allogeneic bone is defined as bone taken from a donor of the same species. Allograft bone has several advantages over autogenous bone. First,

the materials is available in an unlimited supply. Massive reconstructive surgeries, such as hip athroplasty revisions, are often performed with allograft bone. Second, there is currently no other graft material or graft substitute that can restore structural integrity to the site as satisfactorily as massive allografts. Allogeneic bone grafts have also some disadvantages. First, the materials do not have the osteogenic potential of autogenous bone grafts. Allografts may also elicit immunological response when implanted. This reaction depends on the degree of mismatch between the transplanted tissue and the host tissue. The problems with immunogenic response and concomitant resorption are suspected as the major cause for fracture of the allografts, which has been reported to occur in roughly 10% of the cases. Allograft bone may provide initial stability as a bone graft, but inherent problems may cause graft rejection, resorption, and loss of biomechanical stability at the graft site. Further, the ossibility of transmission of disease makes the use of allografts precarious.

- ***Xenograft*** – Xenografts are explored as bone grafts to compensate the limitations of autografts and allografts as mentioned above. They can be prepared including frozen calf bone, freeze-dried calf bone, decalcified ox bone, deproteinized bone, and anorganic bone. The main disadvantage of xenografts is that they are antigenic and nonosteoinductive. To facilitate osteogenesis, many researchers have added components of autograft or autologous marrow to the xenograft.

CHAPTER THREE

Methods for synthesis of calcium phosphate powders

Several methods currently exist for the preparation of synthetic calcium phosphate powders, which are used in the fabrication of bulk ceramics. The techniques used in each of these methods have advantages and disadvantages in the fabrication of bulk ceramics. For instance, the properties of these powders, such as impurities, particle size, and particle morphology effect the ability of the powders to become bonded in sintering process. Therefore, the realization of ideal compressive strengths of the ceramics for many loading applications depends directly on the properties of the starting calcium phosphate powders. Secondly, the properties of the final ceramic product such as phase present (purity) and calcium solubility are influenced by the properties of the initial calcium phosphate powders (11). This chapther will focus firstly on the following methods for preparation of calcium phosphate powders: hydrothermal method, solid-state reactions, precipitaion method and sol-gel method. Secondly, discussed are the problems related to the fabricaion of calcium phosphate ceramics and how the sintering and pore size influence the medical application of these ceramics. Very briefly we have mentioned about the chemical nature of hydroxyapatite and tricalcium phosphate. Lastly, we tried to give here an explanation why do we investigate biphasic calcium phosphate ceramics.

3.1 Hydrothermal conversion of corals (calcium carbonate)

One technique that can be used to produce the synthetic calcium phosphate materials is known as the hydrothermal conversion of coral skeleton which forms predominantly hydroxyapatite. The use of corals from the group of *Goniopora, Porites, Favites* and

Lobophyllia has been investigated. These coral specimens consist of 99 % calcium carbonate in the form of aragonite and 1% organic material. After being ultrasonically cleaned to remove the organic material, this material is heated at elevated temperature and pressure in the presence of aqueous phosphate solutions. This causes a replacement of phosphate ions for the carbonate ions and changed the crystal structure to calcium hydroxyapatite.

The porosity of these corals has advantages and disadvantages for implantation purposes. One significant advantage was pointed out early in the investigation of the corals for use as bone graft substitutes. In 1977, Chiroff *et al.* (11) observed interconnected pores and uniformity of pore size. Further studies showed that certain corals have uniform porosity in the range of 500 to 600 μm in diameter, which is known to be advantageous for osseointegration. One disadvantage of corals is that the porosity varies only slightly from 46% to 48%. The fixed character of porosity can be biologically undesirable in certain instances. When converted and unconverted corals are incorporated into the graft site they are slowly resorped. The difficulty in obtaining sufficient starting material from commercial sources is a disadvantage for this method. Another disadvantage of the substituted corals is the variability in the final chemical composition of the ceramic. It is known that ions such as magnesium, sodium, chloride, and fluoride, which are present in the seawater and become incorporated into the coral, affect sintering parameters of these calcium phosphate implants and may have an effect on the biological response.

3.2 Preparation by solid-state reactions

Another technique for the preparation of synthetic calcium phosphate powder is the preparation by solid-state reactions, where the final product is formed by calcination. *Calcination* is the process of heating a substance to a high temperature, but below its melting point, to cause a loss of moisture, reduction or oxidation. Some authors have investigated calcining of commercially available dibasic calcium phosphates such as calcium hydrogen orthophosphate ($CaHPO_4$, known as monetite) or calcium hydrogen orthophosphate dihydrate ($CaHPO_4.2H_2O$, known as brushite) to obtain the alpha and beta phases of calcium pyrophosphate. Using these same techniques, commercially available tribasic calcium

phosphate, $Ca_3(PO_4)_2$, powders can be calcined to obtain hydroxyapatite, the alpha and beta phases of tricalcium phosphate (TCP), and ratios of these two.

Furthermore, the extreme variability in the original composition of these commercially available powders has frequently led to results that are not reproducible. The method has a major disadvantage in that the final product depends on the composition of the starting material, which may vary from batch to batch. Thus, the final phase can range from β-tricalcium phosphate to hydroxyapatite, even though the starting powders appear identical by X-ray diffraction. In many instances, the commercially available powder is amorphous initially, which cannot be detected by X-ray diffraction. It is hypothesized that the nonstoichiometry of commercially available powders, as well as the presence or absence of water in apatite structure, results in the formation of different phases upon calcining. Impurities in the staring powders obtained commercially may hinder the ability of these powders to become bonded in the sintering process, thus causing a significant decrease in compressive strength, and may affect the biological response to the materials. Because of the need for a controlled, predictable, and reproducible biological response to these materials, solid-state reactions are rarely used to fabricate calcium phosphate ceramics for biomedical applications.

Recently a new method, called mechanosynthesis for obtaining HA and HA/TCP mixtures was developed. Under conditions of mechanical grinding different reactants are crushed between a balls or ball and wall whereby they absorb the part of energy provided by the collisions, which allows their reaction. Variations of the starting materials, reaction time and sintering temperature lead to formation of required calcium phosphate (19).

3.3 Precipitation methods

A third method for obtaining different calcium phosphate powders is via precipitation reactions. Calcium orthophosphates, calcium pyrophosphates, tricalcium phosphate, calcium hydroxyapatite, and ratios of hydroxyapatite and β-tricalcium phosphate can be prepared directly or indirectly using precipitation techniques. That is, these phases are precipitated directly, or powders obtained from the precipitation reactions can be calcined to yield the

desired phases. HA and ratios of HA/βTCP can be precipitated at a basic pH using calcium nitrate, $Ca(NO_3)_2.4H_2O$, and ammonium phosphate, $(NH_4)_2HPO_4$, as starting materials.

This technique offers the ability to vary pH of the reaction by adding different amounts of concentrated ammonium hydroxide (NH_4OH), which results in different precipitants. Also, the temperature at which the reaction is carried out can be varied, thus affecting the size of the resultant precipitate particles. A range of the size of these precipitates may be beneficial in sintering since particle size is one of the parameters that affects sintering. In addition, variation of the time at which the precipitate is harvested will greatly affect the resultant phase obtained. The use of reagent-grade starting material with minimum amounts of impurities allows the production of pure starting powders. Impurities in the starting powders, as mentioned before, inhibit grain boundary bonding and result in poorly sintered ceramics with low compressive strength values. Impurities may adversely affect the ceramic resorption or other biological response by effecting changes in solubility. It is known that highly substituted apatites elicit a different biological response than calcium hydroxyapatite when implanted. Due to the ability to vary reaction parameters and to control the purity of the starting powders, precipitation techniques are invaluable and preferred in preparing calcium phosphate phases for a variety of biomedical applications.

Relatively new method, called liquid mix technique, based on Pechini patent was developed for synthesis of hydroxyapatite. This Liquid Mix Technique gives a single phase hydroxapatite, which is thermally stable in temperature range between 1000°C and 1300°C. The method, as described by its inventors allows massive, reproducible and low cost production of hydroxyapatite with precise homogenity (20).

3.4 Sol-gel method

One very novel and advantageous method for preparing calcium phosphate powders and ceramics is sol-gel method. In the past 20-30 years this method for synthesis has reached great popularity due to its low cost, lower temperatures of making than the traditional processing methods and the ability of controlling the surface chemistry of the required material. The chemically based process is used for producing not only ceramics, as well as glasses, glass-ceramics and composites. Many authors have described the history, theory,

processing details and applications of sol-gel processing (Iler, Brinker and Scherer, Hench and West). The sol-gel method was used by Jarcho, and many others subsequently, to make hydroxyapatite ceramics. New generation of bioactive glass-ceramics have been made using the sol-gel method by Li *et al.* (21).

Sol-gel synthesis of HA ceramics has recently attracted much attention. The sol-gel method offers a molecular-level mixing of the calcium and phosphorous precursors, which is capable of improving chemical homogeneity of the resulting HA to a significant extend, in comparison with conventional methods such as solid state reactions, wet precipitation, and hydrothermal synthesis. The synthesis of HA requires a correct molar ratio of 1.67 between Ca and P in the final product. A number of combinations between calcium and phosphorous precursors were employed for sol-gel synthesis. Besides the difference in chemical activity of the precursors, such as hydrolysis, polycondensation etc., it appears that the temperature that is required to form the apatitic structure depends largely on the chemical nature of the precursors. Table 3.1 gives a summerized description of the different research groups who synthesised HA via sol-gel route and the variety of precursors used (where T_f is the temperature of formation e.g. crystalization of HA phase).

Once the amorphous or poorly crystalline powders are obtained from the precipitations and dried, they can be calcined to yield a calcium phosphate phase that depends on the composition of the precipitated powder. Calcining, as mentioned before is a heating below the melting temperature, by which the present water and some organic residues are removed. Isostatic pressing is another method for compacting the powders. After applying a hydraulic isostatic pressure of 25 000 pounds per square inch (psi) for example, the resulted powders have an increased compressive strength and decreased porosity due to the packing of the loose powders together, which is beneficial for sintering.

RESEARCH GROUP	PRECURSORS	T_F OF HA PHASE	AGING TIME
Gross *et al.*	Calcium dietoxide-$Ca(OEt)_2$; Triethyl phosphate- $PO(OEt)_3$	Above 600°C – monophasic HA	Not longer than 24 hours
Jillavenkatesa *et al.*	Calcium acetate-$Ca(C_2H_3O_2)_2$; Triethyl phosphate - $PO(OEt)_3$	775°C – mixture of HA and CaO	Further HCl is required to eliminate CaO
Brendel *et al.*	Calcium nitrate-$Ca(NO_3)_2.4H_2O$; Phenyldichlorophosphite- $C_6H_5PCl_2$	Below 400°C – low purity and crystallinity of HA powder; above 900°C – pure, well-crystallized HA phase	
Takahashi *et al.*	Calcium nitrate- $Ca(NO_3)_2.4H_2O$; Phosphonoacetic acid- $HOOCCH_2PO(OH)_2$	700°C– pure HA powder with increasing the crystallinity to 1100°C	
Chai *et al.*	Calcium diethoxide- $Ca(OEt)_2$; Triethyl phosphate- $PO(OEt)_3$	No formation of HA	
Chai *et al.*	Calcium propionate; Triethyl phosphate- $PO(OEt)_3$	500°C – appearnace of HA phase	
Qiu *et al.*	Calcium nitrate- $Ca(NO_3)_2.4H_2O$; Ammonium dihydrogen phosphate– $NH_4H_2PO_4$	400-1100°C – increasing of crystallinity with T	
Haddow *et al.*	Calcium acetate- $Ca(C_2H_3O_2)_2$; phosphoric acid-H_3PO_4, phosphorous pentoxide-P_2O_5, triethyl phosphite	Higher than 600°C for triethyl phosphite and calcium acetate precursors	
Lopatin *et al.*	Calcium nitrate $Ca(C_2H_3O_2)_2$ in 2-methoxyethanol; N-butil acid phosphate	300°C- poor crystallinity, which increased above 500°C	Impurities of CaO and $\beta Ca_3(PO_4)_2$
Weng *et al.*	Mixed ethanol sol. of calcium nitrate $Ca(NO_3)_2.4H_2O$ and phosphorous pentoxide- P_2O_5	500°C – highly crystallized HA coating	Heat treatment for 12-24 hours

Table 3.1: *Sol-gel processing of HA powder using different precursors (from Ref.22)*

3.5 The effect of pore size and sintering on the biomedical application of calcium phosphate ceramics

To improve osseointegration of calcium phosphate ceramics in the bony tissue, pores are created in the ceramics using different methods. It is well known that pores in range of 150 to 500 μm are optimally beneficial for bony ingrowth. One of the ways for creating pores is so called Hubbard method. This method entails mixing the calcium phosphate powders with sized naphthalene. When naphthalene is sublimed out, pores are left behind that retain the size of the naphthalene particles. Standard sieves can be used to obtain sized naphthalene particles

in the desired porosity range. In the fabricating of porous calcium phosphates during the second thermal treatment (i.e. sintering) a shrinkage in porosity size occurs, which must be taken into account. The first stage in thermal treatment is to sublime out the naphthalene at about 400°C. By adjusting the amount and the particle size of naphthalene, the resultant porosity of calcium phosphate ceramics can be varied from 0 % to 70 % (11).

The second stage of thermal treatment is to effect bonding of the calcium phosphate particles. Sintering is a process by which particles become denser (decrease in porosity) when subjected to high temperatures, below the melting temperature of the material. Sintering parameters for calcium phosphates vary from temperatures of 1000°C to 1300°C and times of 1 to 24 hours. After the sintering some microporosity in order of a few microns could remain in the final material due to the gaps between the sintered particles. According to some authors microporosity is not an important factor for osseointegration (e.g. for bone ingrowth), but in Chapther 4 we will discuss the different types of porosity of our ceramic samples and how is porosity connected with bone ingrowth and blood vessels circulation. Sintering of calcium phosphates increases their density and strength. A wide variety of mechanical properties may be obtained due to the variability in the shape, size, and purity of the starting powders, as well as the sintering time, temperature and heating/cooling rate for each of the calcium phosphate phases. As is typical for many ceramics, the compressive strength of the calcium phosphates is quite good. Unfortunately, low values for tensile strength and fracture toughness limit the use of calcium phosphates for load-bearing applications.

Although dense calcium phosphates have superior mechanical properties compared with porous calcium phosphates, the lack of porosity does not allow for bony ingrowth or osseointegration of the implant. It is also known that mechanical properties decrease as microporosity and macroporosity of calcium phosphates increase. Since calcium phosphates are being investigated for use as bone graft substitutes for limited load-bearing situations, it is important to consider the mechanical properties necessary for each graft site. Due to the ability to tailor-make the calcium phosphates with various sizes and amounts of porosity to foster osseointegration, it follows that the mechanical properties will also vary as the porosity changes.

3.6 Problems related to the manufacturing of calcium phosphate ceramics

Researchers in many fields (e.g. calcified tissue research, medicine and dentistry, chromatography) involving apatites have a frequent use for synthetic hydroxyapatite as a starting, reagent or reference material. However, the differences among preparations reported to be "hydroxyapatite" might lead one to wonder whether there really is such a thing as pure, stoichiometric hydroxyapatite. Variously prepared hydroxyapatites are widely known to vary in many ways. As McDowell *et al.* (23) commented, "it is well known that hydroxyapatites have variable indices of refraction, unit cell dimensions, impurity contents, degrees of disorder in the OH position, and may possibly vary in their Ca and OH contents". Arends and Jongebloed (23) have particularly commented on some of the properties of hydroxyapatite, which make difficult the preparation of pure stoichiometric HA. These include strong tendencies:

- To become nonstoiciometric (e.g., by sustaining Ca or OH vacancies)
- To take up impurities in the structure, e.g. F, CO_3 and H (as HPO_4), as well as a long list of other elements
- To form surface complexes, and
- To be formed in aqueous solutions, via precursors rather then directly.

3.6.1 Ionic substitutions in the hydroxyapatite lattice

One potential method for improving the bioactivity of hydroxyapatite is the incorporation of silicon (or silicate groups) into the hydroxyapatite lattice. However, it is important that the ionic substitution of silicon does not result in thermal instability of the silicon-substituted hydroxyapatite as this effect upon sintering would result in the decomposition of Si-HA to undesirable second phases. In a study by Gibson *et al.* (24) is described the preparation of a new hydroxyapatite-based material by an aqueous precipitation route, which contains small level of silica (0.4 wt %) and which retains the HA structure on

sintering. Further in this work is shown that the silica substitution gives very small changes in the chemical composition and the crystal structure, such as unit cell parameters, which are the result of the incorporation of a slightly larger silicate ion in the place of a phosphate ion and the loss of some of the OH^- groups.

E. M. Carlisle had examined in early 70`s approximately 5000 electron probes quantitatively for silicon, calcium and phosphorous on 50 specimens of normal tibia from mice and rats. The findings from her study had shown that silica was localized in active calcification sites in young mouse and rat bone. Thus most probably silica is a possible factor in bone mineralization (25). Micromolar concentrations of aluminium and silicon ions have been reported to stimulate osteoblast proliferation and differentiation; however, high concentrations of aluminium have been demonstrated to suppress bone mineral formation (26).

After comparing the results of previously made hydroxyapatite preparations from different researchers, we have decided in order to avoid any complications like impurities, second phases in the powders, variation in Ca/P ratio etc., to use a commercially supplied hydroxyapatite powder which should have constant parameters. We have chosen hydroxyapatite powder supplied from Aldrich (Germany) and tricalcium phosphate supplied from Fluka (Germany). As it was already pointed out, the main aim of this study is to check the bioresorbability of hydroxyapatite silica-based ceramic and to compare it with bioresorbability of biphasic calcium phosphate silica-based ceramic, when both are implanted in Goettinger mini pigs animal model.

3.7 General chemistry of hydroxyapatite

Apatites have the formula **$Ca_5(PO_4)_3X$** , where X can be ab F^- ion (fluorapatite FAp), OH^- ion (hydoxyapatite, HA, or OHAp), or a Cl^- ion (chlorapatite, ClAp) for example. Gerhard reported in 1786 (27),

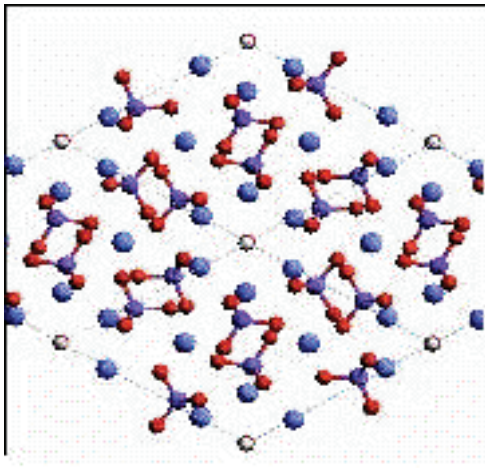


Figure 3.1: C*rystal structure of HA in the a,b-plane viewed down the c-axis. The OH^- lie in the channel formed by Ca^{2+} at the intersection of the four unit cells shown. (H- white, O_2-red, Ca-blue, P – purple)*

that the name apatite had been coined by AG Werner from the Greek απαταω, to deceive, because it was frequently confused with other minerals such as aquamarine, olivine, amethyst, fluorite, schorl, etc. The apatite structure is very tolerable to ionic substitutions, and for example, Ca^{2+} ions can be partly or completely replaced by Ba^{2+}, Sr^{2+} or Pb^{2+} ions, and PO_4^{3-} by AsO_4^{3-} ions. Thus there are lead minerals with the apatite structure which include $Pb_5(PO_4)_3Cl$ (pyromorphite), $Pb_5(VO_4)_3Cl$ (vanadinite) and $Pb_5(AsO_4)_3Cl$ (mimetite). Solid solitions of halide and OH^- are of frequent occurrence.

Coupled substitutions frequently occur in apatites. In these, one ion is replaced by another of the same sign, but different charge, and neutrality is maintained by substitutions of ions with dissimilar charged or vacancies elsewhere. The CO_3Aps (carbonate apatites) are an important and complex example that have been the subject of much controversy. These carbonate-substituted Aps include the minerals francolite, dahllite and the rock-phosphates, and the biological apatites. Nonstoichiometry, with vacant lattice sites, occurs in the biological apatites and frequently in synthetic apatites, and considerably complicates their crystal chemistry. Apatites (mostly FAp and CO_3Fap) form an important series of minerals. They occur as a minor constituent of many igneous rocks, in most metamorphic rocks, especially crystalline limestones. The basic apatite structure is hexagonal with space group $P6_3/m$ and approximate lattice parameters a=9.4 and c= 6.9 Å with two formula units ($Ca_5(PO_4)_3X$ or $Ca_{10}(PO_4)_6X_2$) per unit cell. The values of lattice parameters and refractive

indices often depend slightly on the mode of preparation because of frequent nonstoichiometry (Fig. 3.1).

The large number of different synthetic routes and published preparations demonstrates the difficulty of making stoichiometric HA (s-HA). Most of synthetic methods have to rely on the assumption that s-HA is the equilibrium phase under the preparation conditions used. Although, this may be approximately true, there is no thermodinamic reason why an apatitic equilibrium should be exactly stoichiometric. The stoichiometry has been determined experimentally by accurate chemical analyses, by calculated cell contents from the lattice parameters and by density measurements. What evidence there is, suggests that the equilibrium phase is not generally exactly stoichiometric.

One method that has been used to prepare s-HA is to use accurately stoichiometric quantities for the synthetic reaction, and attempt to ensure formation of a single phase without unwanted gain or loss of chemical species. Another approach is to use the aqueous system $Ca(OH)_2$-H_3PO_4-H_2O at a composition in which HA (assumned to be stoivhiometric) is the only stable phase, and to attempt to ensure that the system is in equilibrium. Yet another technique is to synthesise a nonstoichiometric HA (ns-HA) under noequilibrium conditions, and to process this to give a stoichiometric composition. For example, solid state reactions have been used in which samples of nearly stoichiometric compositions are heated to a high temperature so that s-HA is formed as the major phase, together with a minor phase (CaO or β-TCP, depending on whether the Ca/P molar ratio is greater or less than 1.667 respectively) in an amount dependent on the departure of the overall composition from s-HA. The minor phase is then extracted from the product. A further method is to rely on the easier synthesis of a related stoichiometric apatite (s-ClAp or s- BrAp) that can be converted to s-HA. In the beginning of this chapter a detailed description of the variation in methods for synthesising HA was reported.

Hydroxyapatite and related materials (e.g. biological apatites) are of great interest because of their similarity with the inorganic component of teeth and bone. Typical compositions of the inorganic component of enamel, dentine and bone are given in Table 3.2.

Component	Enamel (wt %)	Dentine (wt %)	Bone (wt %)
Ca	37.6	40.3	36.6
P	18.3	18.6	17.1
CO_2	3.0	4.8	4.8
Na	0.7	0.1	1.0
K	0.05	0.07	0.07
Mg	0.2	1.1	0.6
Sr	0.03	0.04	0.05
Cl	0.4	0.27	0.1
F	0.01	0.07	0.1
Ca/P molar ratio	1.59	1.67	1.65

Table 3.2: *Average composition of the mineral in specimens of human calcified tissues (from Ref. 27)*

It is obvious that CO_2 contents of dentine and bone are similar and significantly higher than that of enamel, the magnesium contents of bone and dentine are higher than that of enamel, but there is almost twice as much magnesium in dentine as in bone, the Ca/P molar ratio of enamel is significantly lower than the ratio for HA (1.667) whilst the Ca/P ratio for dentine and bone is approximately equal to the ratio for HA (and can be even higher). As would be expected for biological material, the composition of the inorganic component of teeth and bones is rather variable, depending on the part of tissue sampled. There are often also changes during formation and maturation, and with age and disease. The composition can also be species dependent. Figure 3.2 presents the typical XRD spectra of commercially supplied hydroxyapatite powder (Aldrich, Germany) heated at 1200°C, which we used as a starting material in our experiments. The powder has a well expressed crystallinity and does not show decomposition upon sintering.

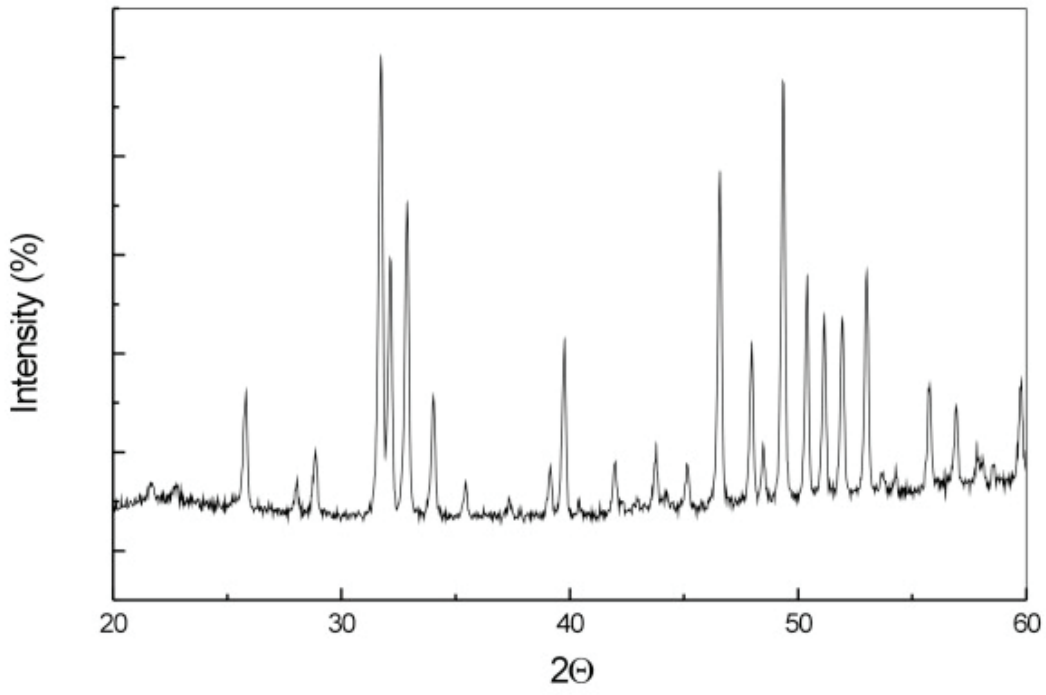


Figure 3.2: *X-ray diffraction pattern of commercially supplied hydroxyapatite powder, heated at 1200°C*

3.8 General chemistry of tricalcium phosphate

Tricalcium phosphate (whitlockite) was first described in 1941 as a hydrothermal product from a granite pegmatite and named after the mineralogist Herbert P. Whitlock. The mineral was reported with a supposed formula of $Ca_3(PO_4)_2$. As a result the terms whitlockite and β-TCP have been used interchangeably, but there are subtle structural differences between these substances. Tricalcium phosphate occurs in two forms: α-TCP, which is the higher temperature phase, and β-TCP. The β-TCP to α-TCP transition temperature is 1125°C, however, lately another high temperature phase of α-TCP was dicovered, which is stable above 1430°C and is called super- α-TCP (27).

Betha-TCP has the rhombohedral space group R3c with unit cell a=10.439, c=37.375 Å (hexagonal setting) with 21 formula units per hexagonal unit cell. Figure 3.3 presents the typical X-ray diffraction pattern of TCP powder, which was commercially supplied from Fluka (Germany) for the purposes of our study.

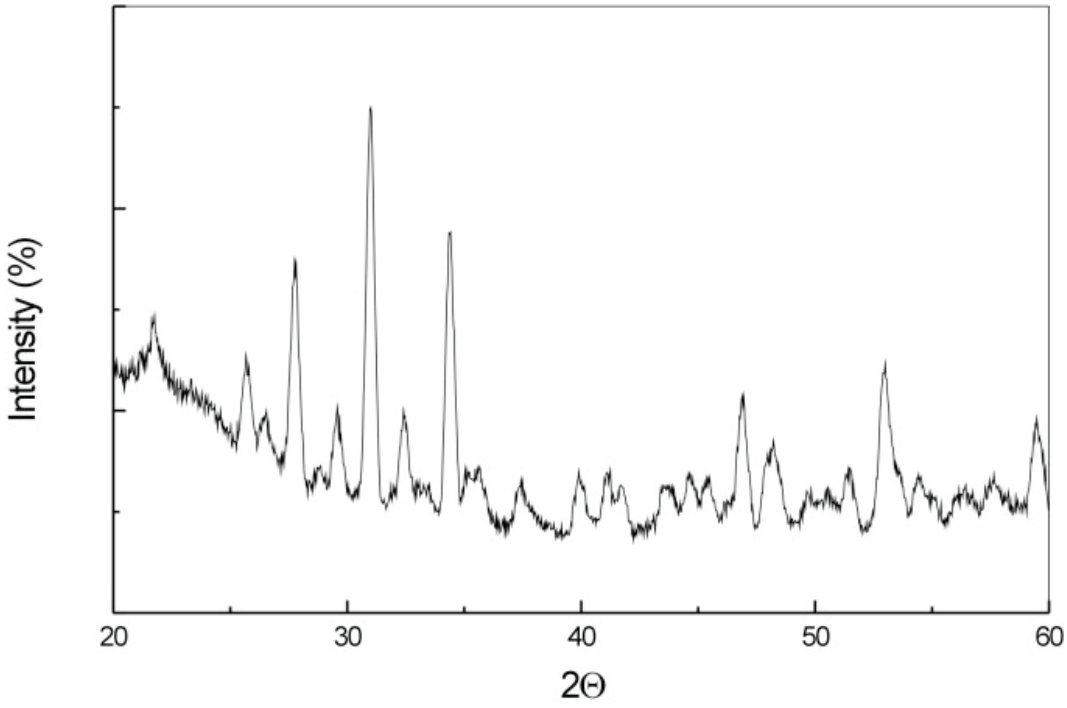


Figure 3.3: *X-ray diffraction pattern of commercially supplied tricalcium phosphate powder*

The β-TCP structure is a distortion of the parent lattice $Ba_3(VO_4)_2$, with layers perpendicular to the c-axis. Because of the size of Ca^{2+} ion, there is reduction in the number of PO_4 tetrahedra in the structure compared to that of the parent lattice and a reduction in the number of formula units within its hexagonal unit cell. Two types of Ca sites exist within the β-TCP unit cell: those known as Ca(5) are fully occupied, while a particular set of cation sites known as Ca(4) are only half occupied (28).

CHAPTER FOUR

Manufacturing and results from properties examination of calcium phosphate silica-based ceramics

After giving a sufficient background regarding the history of bioceramics, about the different methods for synthesis of calcium phosphate powders including the specific advantages and disadvantages, the main aim of this chapter is to describe the manufacturing process of calcium phosphate silica-based ceramics, which we made in our laboratory. Secondly, a detailed discussion of the viscosity behaviour, the thermal stability and the mechanical properties of the ceramic materials is included. Lastly, we will analyse the results from the porosity measurements and the thermo-gravimetry measurements.

4.1 Preparation of calcium phosphate ceramic samples

For the purpose of our study we have prepared the samples using the following procedure: Tetraethyl orthosilicate (TEOS, which is used as a precursor for silica) was mixed with water, ethanol (used as solvent), and hydrochloric acid (HCl, used as a catalyst). The molar ratio r_w (H_2O/Si) was 4. The process is called sol-gel and can be described by the following reactions (29):

hydrolisis

$$\equiv Si - OR + H_2O \leftrightarrow \equiv Si - OH + ROH \quad (1)$$

esterification

alcohol condensation

$$\equiv Si - OR + HO - Si \equiv \leftrightarrow \equiv Si - O - Si \equiv + ROH \quad (2)$$

alcoholysis

water condensation

$$\equiv Si - OH + HO - Si \equiv \leftrightarrow \equiv Si - O - Si \equiv + H_2O \quad (3)$$

hydrolysis

where R is an alkyl group, C_xH_{2x+1}. The hydrolysis reaction (Eq.1) replaces alkoxide groups (OR) with hydroxyl groups (OH). Subsequent condensation reactions involving the silanol groups produce siloxane bonds (Si–O–Si) plus the by-product alcohol (ROH) (Eq.2) or water (Eq.3).

Since water and alkoxysilanes are immiscible, a solvent such as alcohol is normally used as a homogenizing agent. However, gels can be prepared from silicon alkoxide-water mixtures without added solvent, since alcohol produced as the by-product of the hydrolysis reaction is sufficient to homogenize the initially phase separated system. In our system, we added additionally ethanol, because ethanol improves the homogeneity of the gel, and increases the gelling time (30). The molar ratio H_2O:Si (r_w) in Eq.1 can vary from less than one to over 50, as well as the concentrations of acids or bases can vary from less than 0.01 to 7M, depending on the desired product. The following net reaction can be shown:

$$nSi(OR)_4 + 2nH_2O \rightarrow nSiO_2 + 4nROH \quad (4)$$

where is believed that an r_w value of 2 is theoretically sufficient for complete hydrolysis and condensation to anhydrous silica.

In the experiments we made, the r_w had value of 4, but even with the excess of water, the reaction of hydrolysis and condensation does not reach completion. Therefore, we consider that hydrolysis is finished after the change in solution colour – from translucent to transparent, and when the temperature of the reaction (which is exothermal) stopped

increasing. It seems that variations in the synthesis conditions (e.g. value of r_w, the catalyst type and concentration, the solvent, the temperature, and pressure) cause modifications in structure and properties of the silicate products. More information about the synthesis, properties, structure and variety in applications of sol-gel materials can be found in Ref. 29.

Once hydrolysis reaction is completed as mentioned above, we immersed the solution in cold water to allow the temperature to cool down (around 20°C) and then we added the calcium phosphate powders. The cooling of the sol is a necessary step for the production cycle of our ceramic samples, because higher temperatures can lead to increasing in the gelling rate itself. Furthermore, the aggregation of silica particles is proportional to the activation energy and involves a kinetic phenomenon. When the gellation occurs before mixing the sol with calcium phosphate powders, the final product will be only silica gel, which is not a priority for the purpose of our experiments. The pH value of the sol after hydrolysis is 1.8-2.0, and after mixing it with calcium phosphates it arises to 4.8-5.2, which is the optimum pH value of the sol, when slow gellation of the system is needed. According to the pH/gelation time dependence we can control gel formation with variations in pH, but for our system the pH value is naturally controlled by the addition of calcium phosphate powders. As mentioned by Iler (31) “the main step in gel formation is the collision of two silica particles with sufficiently low charge on the surface that they come into contact so that siloxane bonds are formed, holding the particles together”. This process is guided by the catalytic action of hydroxyl ions, or by dehydration of the surface of particles at higher pH. This is evidenced by the fact that the rate of gel formation in the pH range 3-5 increases with pH and its proportional to the hydroxyl ion concentration. Above pH 6, insufficiency of hydroxyl ions no longer guides the rate of gelling. Instead, the rate of aggregation decreases because of fewer collisions between particles owing to the increasing charge on the particles, and thus decreases with higher pH. The net result of these two effects is a maximum in rate of gelling at around pH 6 and stability of sols in the pH range 8-10 (Fig.4.1).

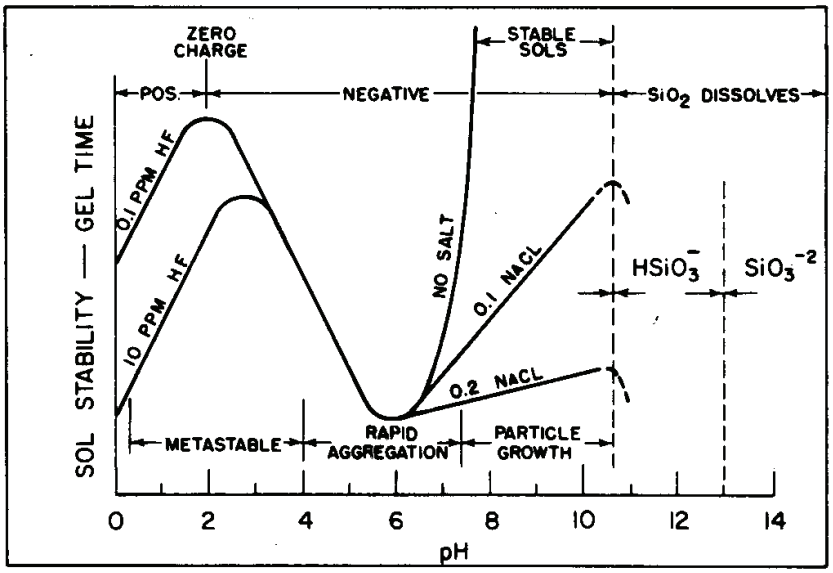


Figure 4.1: *Effects of pH on gelation time of silica sol (from Ref. 31)*

Silica sol and calcium phosphate powders were mixed using ultrasonic bath, for better homogenization and for preventing the formation of particles from unmixed powder. After mixing the samples, they were left to dry in ethanol atmosphere for 2-3 days. This is a necessary step in manufacturing process, since contact between air and as-prepared samples leads to faster drying and cracks on their surfaces. As a result one can get completely destroyed materials, which cannot be used for further investigations (i.e. for medical application). A consequent heating of the materials for two hours in an air atmosphere was performed, using an electrical oven with a temperature of 200°C. Figure 4.2 is a flowchart that presents the processing steps followed until the final product was obtained.

One of the main requirements for bone graft material is to have a certain porosity and sufficient pore size to allow bone ingrowth. Following this requirement we completed the heating only up to 200°C, because sintering the samples to higher temperatures would lead to decreasing of pore volume and pore size. Some investigators have found that sintering up to 700°C of hydroxyapatite ceramic produces a foreign body reaction after implantation and affects the solubility of Ca and P ions (32).

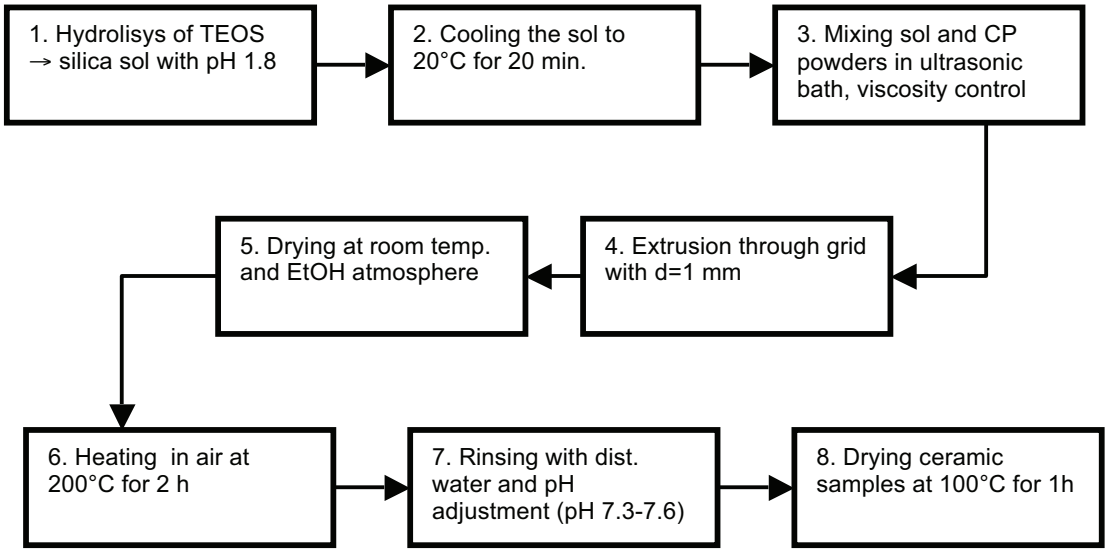


Figure 4.2: *Preparation diagram of calcium phosphate silica-based ceramic*

Although, our ceramic samples were not heated to 700 °C (or even more) they did not show any foreign body reaction when used *in vivo* (see discussions about *in vivo* behaviour in Chapter 6). In this study (32) the authors claimed that hydroxyapatite ceramic sintered to 1100°C has demonstrated superior osteogenetic activity than one sintered at 900°C. Later we will come to the discussion about the heat treatment of our ceramic samples, where we will provide an useful conclusions regarding sintering and thermal decomposition of HA and BCP ceramics.

Figure 4.3: *View from the ready for implantation material (BCP silica-based ceramic) in form of "spaghetti"*

We tested two different bioceramics mixed with silica sol – hydroxyapatite ceramic and biphasic calcium phosphate ceramic (Fig. 4.3). The way of preparation of both ceramics is equal with the only difference that the rate of gel formation vary, which we found very sensitive factor to Si^{4+}/Ca^{2+} ratio, thus, time for extruding the ceramic to shape it in the desired form (like spaghetti lying in a bowl) is dissimilar. In hydroxyapatite ceramic the Si^{4+}/Ca^{2+} ratio is 2,6 and the total silica content 24 wt %. In biphasic calcium phosphate ceramic we have 1,3 Si^{4+}/Ca^{2+} ratio and 16 wt % silica (Table 4.1)

MATERIAL	SI^{4+}/CA^{2+} RATIO	SI CONTENT IN WT. %	R_W RATIO	PH OF SILICA SOL	PH AFTER ADDING CP
HA	2.6	24	4	1.8-2.0	5.17
BCP	1.3	16	4	1.8-2.0	4.85

Table 4.1: *Production characteristics for hydroxyapatite and biphasic calcium phosphate ceramics*

Recently, there has been a growing interest on the role of soluble silica in new bone formation. Many scientists have focused their research on the possible applications of silica gels as starting materials for preparation of bioactive glass and glass-ceramics. In our laboratory we designed a novel ceramic material which is suitable for bone substitutes and it is unique because of its special composition. The decision for choosing silica sol and consequent mixing it with calcium phosphate powders is due to the fact that firstly, silica plays an important role in biological systems (25), and secondly calcium phosphates (e.g. hydroxyapatite and tricalcium phosphate) are the most used ceramics for bone replacement. These ceramics have relatively poor mechanical strength. Therefore the combination between the silica matrix with higher mechanical strength and calcium phosphates with better biocompatibility should give greater result for material with promising future in biomedical applications.

4.2 Viscosity behaviour of calcium phosphate silica-based ceramic

In recent years, the sol-gel process for making ceramics, glasses and composites has received considerable attention; investigations on processing and physical properties are voluminous. However, rheological chararcterization of sol-gel materials is relatively limited. Qualitative rheological considerations are often used to define the point of gelation. For example, Sakka *et al.* defined the gelling point as the loss of fluidity, i.e. no flow of the sol as observed upon tilting the container. Yu *et al.* defined the gelling time as the time interval from the start of aging to time recorded when the surface of the sol remained steady while the container was tilted for 2 minutes (33). Unfortunately, the above definitions of the gelling point are uncertain and qualitative, making the comparison between different studies difficult. The gelling point is often defined as the aging time at which the viscosity of the sol increases abruptly. Viscosity measurements have also been used to identify point during the sol-gel transition when sols are suitable for various processing operations, e.g. fiber drawing, substrate coating, etc. Sacks *et al.* (33) reported that silica fiber formation was possible when the viscosity of the sol was ~ 1-100 Pa.s., and for thin film formation with viscosity between 1 and 10 Pa.s. Leroy *et al.* indicated that the optimum condition for ZrO_2 fiber drawing occurred when the viscosity of zirconium acetate solutions was ~ 20-50 Pa.s (33).

As it was mentioned in Paragraph 4.1, determining the viscosity of our material prior to extruding it in a shape of spaghetti is very important part from fabricating the ceramic into a certain form, especially in the case of extruding, where the process is dependent on the gellation time (e.g. viscosity). Rheological flow characteristics were determined using Brookfield viscometer with cone cylinder. Steady rotational flow curves (i.e. shear stress τ, vs. shear rate γ) were generated by increasing the shear rate from zero to the maximum desired value in 30 sec. Immediately decreasing the shear rate back to zero in the same time interval was performed. The viscosity was determined using the relation:

$$\eta = \tau / \gamma \qquad (5)$$

For the mixture of calcuim phosphates and silica sol, according to the rotational flow curves the type of flow behaviour can be specified as a pseudoplastic (the one which displays a

decreasing viscosity with increasing of the shear rate) with rheopexy flow (fluid`s viscosity increases with time as it is shared at a constant rate), or it is also called shear thinning flow behaviour. To examine that our calcium phosphate silica-based mixture has shear thinning flow behaviour, would be unnecessary to make viscosity measurements, because it is very obvious that the gelation is time dependent. Since this is a very sensitive process, one needs more detailed study about the viscosity/time dependence. Figure 4.4 shows the change in viscosity as a function of gelling time using share rate of the cone 1 rpm (rotations per minute) for biphasic calcium phosphate silica-based ceramic.

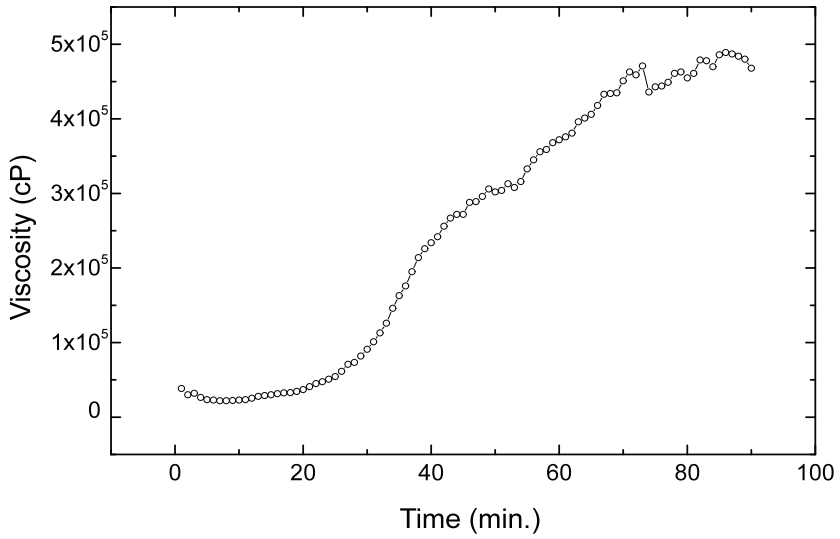


Figure 4.4: *Viscosity-time dependence for biphasiccalcium phosphate silica-based ceramic*

Until 40 minute from the beginning of the measurement the viscosity remains almost constant, the fluid has a Newtonian behaviour, which can be due to the low particle-particle interactions. Further with aging (respectively with gelling), agglomeration of polysilicate species is expected. As in a suspension with agglomerates, this leads to increase in the viscosity due to immobilised liquid within the interparticle void space, so the “effective” solids loading increases. In the early stages the polysilicate species/agglomerates have relatively small volume and the effective solids loading is low. Minimal particle-particle interactions exist in the sol mixture, so the flow behaviour remains Newtonian. As aging proceeds, large agglomerates (or microgel regions) form and the effective solids loading increases rapidly. At low shear rates these microgel regions result in high viscosities. As the

shear rate is increased, agglomerate breakdown occurs releasing immobilised liquid and resulting in low viscosities due to the decreased effective solids loading. This is reflected in the observation of shear thinning flow behaviour.

Viscosity data often functions as a “window” through which other characteristics of a material may be observed. Viscosity is more easily measured than some of the properties that affect it, making it valuable tool for material characterization. One of the most obvious factors that can have an effect on the rheological behaviour of a material is the temperature. Some materials are quite sensitive to temperature, and a relatively small variation will result in a significant change in viscosity. Fortunately, our ceramic material when subjected to viscosity measurements did not show any temperature sensitivity in the temperature interval 20-30°C. The condition of a material during measurement of its viscosity can have a considerable effect on the results of such measurement. Variables such as Viscometer model, spindle/speed combination, sample container size, sample temperature, sample preparation technique, etc., all affect not only the accuracy of the measurements, but the actual viscosity of the material. Another factor which may affect viscosity measurements is the homogeneity of the sample. Such a tendency of a material to separate into non-homogeneous layers for our sample is not observed, for the mixing between silica sol and calcium phosphate powdes was done in an ultrasonic bath, and therefore the mixture is very homogenous. Aging phenomena must be also considered for time-dependent materials. But changes in the viscosity of many materials can occur over time even though the material is not being shared.

Many materials could undergo changes in viscosity during the process of a chemical reaction, so that viscosity measurement made at one time in the reaction may differ significantly from another one made at another moment. Here is necessary to notice another detail, which might be also of an importance when discussing gellation-time dependence. This is the speed of mixing the calcium phosphate powders with the silica sol. As it has been shown by the practice, when the speed is too high (too much quantity of powder is mixed at once), the gellation occurs faster in comparison with the slower adding the powders into silica sol solution. This can be explained by the pH-time dependence, which was already mentioned in Paragraph 4.1, i.e. faster adding of calcium phosphate powders causes rapid increase in the pH value and dellation occurs in shorter time. The increase of pH value is especially sensitive in the interval between 1.8 (the pH of silica sol) and 5.8. It should be stated that viscosity measurements are strongly dependent on the volume of the mixture examined, as well as on the environmental conditions. If the system which has to be checked is open, the viscosity reaches its high values for shorter time due to evaporation. In closed-measured systems

gellation time is longer and respectively viscosity reaches its high value for many more hours. All the difficulties related to viscosity measuements, and respectively to the extrusion of our ceramic samples in spaghetti form, once again underlined the importance of materials characterization to obtain a better product.

4.3 Thermal stability of calcium phosphate silica-based ceramics

The decomposition of HA at high temperatures is frequently encountered during the fabrication of HA ceramics. This inhibits the sinterability of the apatite ceramics and partly or completely transforms HA into a mixture of tetracalcium phosphate (TeCP), $Ca_4(PO_4)_2O$, and tricalcium phosphate (TCP), $Ca_3(PO_4)_2$. If such reaction occurs in the bioceramic material under physiological conditions, the mechanical integrity and osteogenetic activity of the ceramics is damaged.

Hydroxyapatite can be prepared by various synthesis methods as it was already shown in Chapter 3. The results have shown that the thermal stability of a HA is intrinsically dependent on its stoichiometry and the crystal structure. These are, in turn, related to both the nature of precursor and synthesis conditions. The optimum thermal stability (up to 1370°C) was observed in HA samples prepared by the hydrothermal method and by hydrolysis of brushite (34). Before examining the thermal stability of our ceramics, we have tested the thermal stability of the starting powders (HA and TCP, respectively) used in the manufacturing process of the bioceramics. An XRD pattern of hydroxyapatite powder (supplied by Aldrich-Germany) heated at 1200°C shows no decomposition into TCP phase, proving that the powder is stoichiometric (Fig. 3.2).

It was reported by Fang *et al.* (34) that nonstoichiometric HA decomposes via dehydration according to the following reactions:

$$\mathbf{Ca_{10-x}(HPO_4)_x(PO_4)_{2-x}nH_2O \xrightarrow{\leq 700^\circ C} Ca_{10-x}(P_2O_7)_x(PO_4)_{6-2x}(OH)_2 + nH_2O(g)}$$

$$\mathbf{\xrightarrow{700^\circ\text{-}800^\circ C} (1\text{-}x)Ca_{10}(PO_4)_6(OH)_2 + 3Ca_3(PO_4)_2 + xH_2O(g)} \qquad (6)$$

The resultants after themal treatment are stoichiometric HA and TCP. At temperatures higher than 900°C, further partial dehydration of HA may take place as follows:

$$Ca_{10}(PO_4)_6(OH)_2 \xrightarrow{\geq 900°C} Ca_{10}(PO_4)_6(OH)_{2-2y}O_y + yH_2O(g) \qquad (7)$$

According to the above mentioned reactions one should observe a gradual wt. loss before 700°C, a sharp loss in 700°-800°C region, and a slight and gradual loss above 900°C in the TGA thermogram of a nonstoichiometric HA sample. There should not be any sharp loss for stoichiometric HA in its TGA curve between 700°C and 800°C. Thus, the TGA curve of our HA ceramic heated to 800°C shows once again that the powder which we used for the synthesis of the ceramics samples is stoichiometric hydroxyapatite (Fig. 4.5).

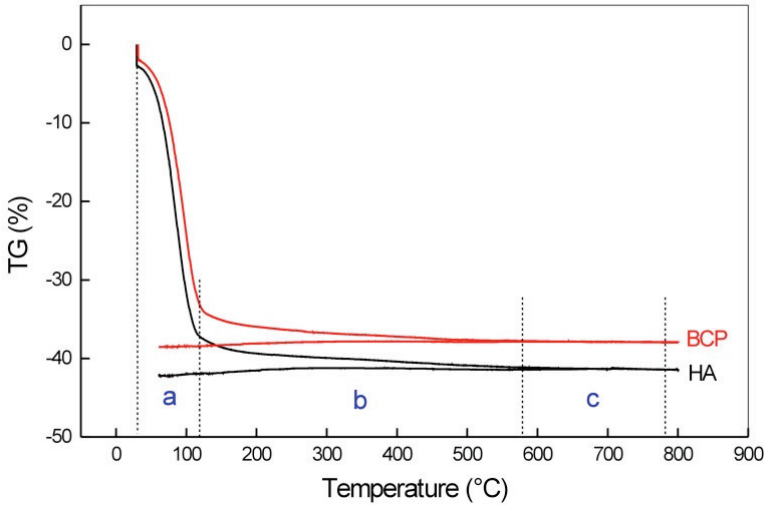


Figure 4.5: *Thermogravimery anlysis (TG) for HA and BCP siica-based ceramis performed up to 800°C.*

On Fig. 4.5 three regions can be recognized: in region a) up to 120°C a sharp sample mass loss was detected due to water loss. In region b) from 120-590°C further decrease in sample mass is visible involving the release of rest of the water and that of the organic parts (e.g. alcohol). The last temperature region c) from 590-800°C shows no changes in the sample mass. These results are in accordance with the DTA curves of our samples (Fig. 4.6). Note that both ceramic materials have similar behaviour when exposed to TG-TD analysis.

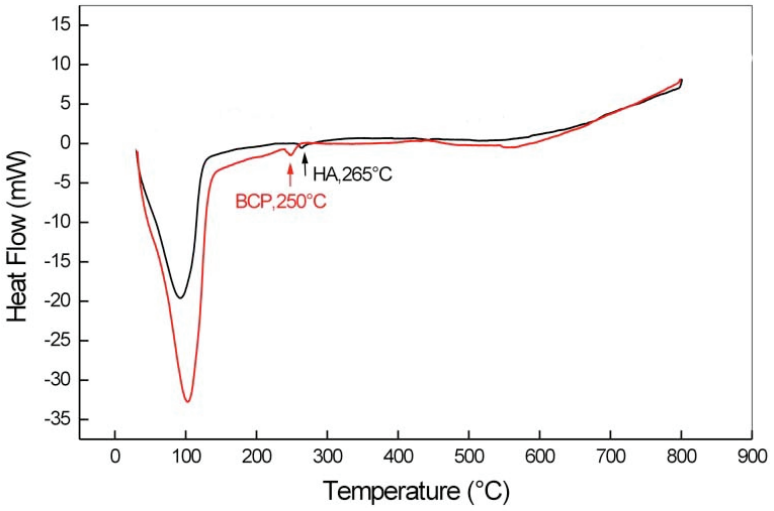


Figure 4.6: *Differential thermal analysis (DTA) for HA and BCP silica-based ceramis performed up to 800°C.*

Two exothermic peaks appeared on the TDA diagram. The first at 100°C is very sharp for both HA and BCP probes. Still there is a very small difference between the two ceramics, that is that the water release from BCP system requires more heat. The second exothermal peak shows again a slight difference between HA and BCP samples. In BCP ceramic the release of the organic matter and rest of water happened at 250°C, while for HA ceramic the peak position is at 265°C. Actually, since the dissimilarities from TG-TD analysis of HA and BCP ceramics are negligible, the results from both ceramics can be interpreted in the same manner.

Tricalcium phosphate undergoes also thermal decomposition, which can be expressed by the following reaction:

$$Ca_3(PO_4)_2.nH_2O \xrightarrow{680\text{-}720°C} \beta\text{-}Ca_3(PO_4)_2 \xrightarrow{1290°C} \alpha\text{-}Ca_3(PO_4)_2 \xrightarrow{1540°C} \alpha\text{-}Ca_3(PO_4)_2 \text{ superstructured} \quad (8)$$

Over the century the decomposition process of mineral tissues with time has been tested on human bones. When X-rayed, these bones revealed the presence of such compounds as $Ca_4P_2O_9$, α- $Ca_3(PO_4)_2$, β- $Ca_3(PO_4)_2$ (1). This suggests that the natural decomposition of bone develops according to the following reactions:

$$Ca_{10}(PO_4)_6(OH)_2 \rightarrow 2Ca_2P_2O_7 + Ca_3(PO_4)_2 + 3CaO + H_2O \quad (9)$$

$$Ca_{10}(PO_4)_6(OH)_2 \rightarrow 3\,Ca_3(PO_4)_2 + CaO + H_2O \quad (10)$$

$$2Ca_2P_2O_7 + 2CaO \rightarrow Ca_4P_2O_9 \quad (11)$$

The thermal stability of our mono- and biphasic ceramics were investigated by XRD analysis of the air-quenched samples after heating. The samples were heated at various temperatures (600°C, 750°C, 900°C, 1000°C and 1200°C) in air. The firing time for all samples at all temperatures was 1 h. After calcination the samples were left in the furnace and allowed to cool. XRD data were collected using CuK_α radiation (λ=1.541Å), over the 2θ range 10-110° with step size of 0.02°. The thermal behaiviour was characterized by the decomposition temperature of the HA and BCP ceramic. Since the HA decomposition is always accompanied by the appearance of TCP (either in β- or α-form), the minimum temperature at which HA or the BCP ceramics were heated and the TCP phase was detected from the sample, was recorded as the decomposition temperature of that ceramic. In the BCP ceramic where TCP phase exists in the starting material, the TCP amount was determined by changes in HA/TCP ratio compared to the one of the staring material.

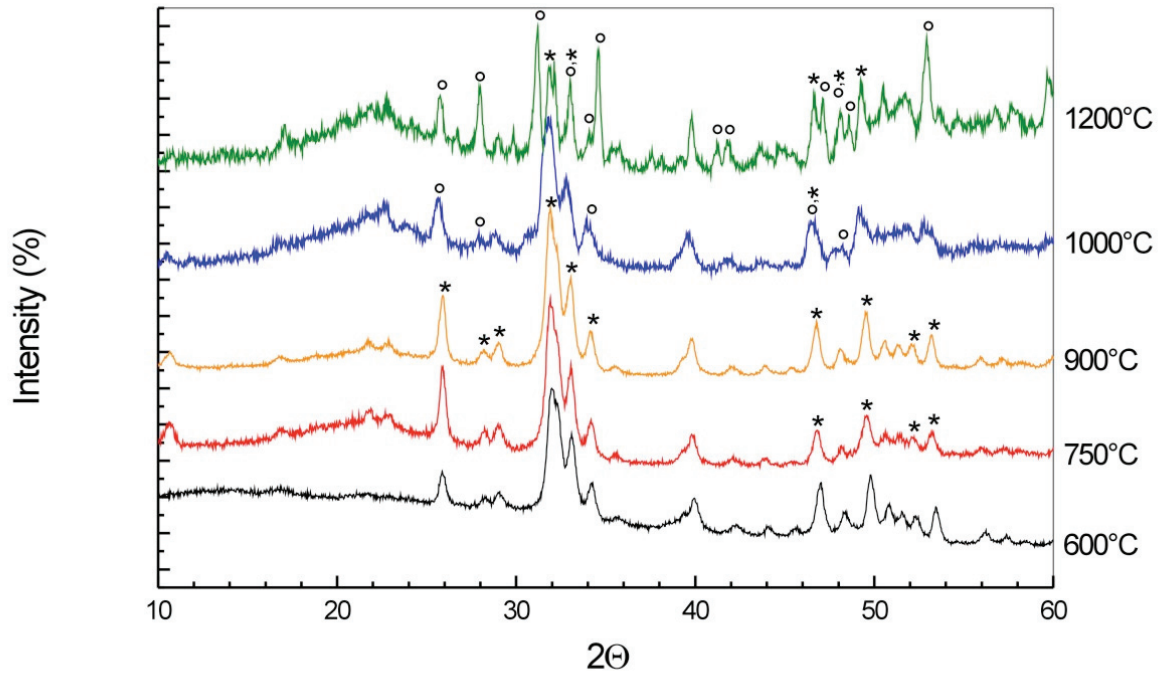


Figure 4.7: *XRD spectra of hydroxyapatite ceramics heated between 600°C and 1200°C, (*) HA phase; (°) βTCP phase*

Figure 4.7 shows the changes in XRD spectra of HA ceramics upon sintering. With exposing the ceramics at temperatures up to 900°C no changes in the XRD spectra can be detected. The ceramic consists of hydroxyapatite phase only. At 1000°C the first peaks that belong to the βTCP phase appeared, at this temperature we therefore have a material consisting of both HA and TCP phases, predominating the first. At 1200°C, the decomposition of HA phase into βTCP probably results into a higher percentage of the βTCP. According to these results the decomposition temperature of our hydroxyapatite silica-based ceramics is 1000°C. Further investigations of these ceramics for medical applications should not exceed 1000°C as sintering temperature due to these phase transformations, which could lead to completely different by its cmposition material.

Another proof of HA ceramics undergoing thermal decomposition is given by in-situ temperature-dependent X-ray diffraction spectra of HA silica-based ceramic made in DESY HASYLAB, Hamburg (Fig. 4.8). The measurement was done at the F2.1 beamline in energy dispersive mode. Figure 4.8 indicates the partial phase transition of the HA into βTCP phase, which is retained in the final spectra of the cooled sample at 50°C. The first βTCP diffraction peak appears at 900°C; in parallel the initial HA peaks which start to disappear; at 1200°C the pattern corresponds to βTCP phase.

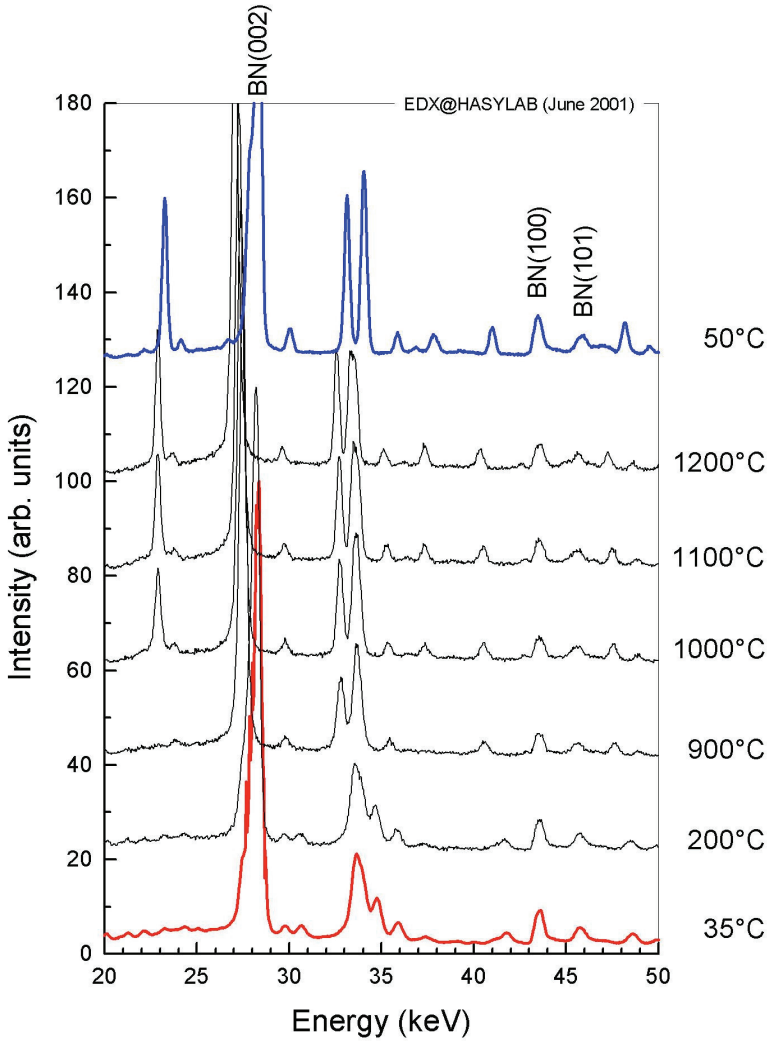


Figure 4.8: *Energy dispersive X-ray diffraction pattern of HA silica-based ceramic*

The picture of biphasic calcium phosphate ceramic undergoing phase transformations is more complicated, due to the fact that from the beginning both phases (HA and βTCP) are present in the X-ray diffraction spectra (Fig. 4.9). At 600°C the ratio between HA and TCP phases should be 60/40 wt. %, the same as the initial ratio of the prepared samples. Up to 750°C the X-ray diffraction patterns remain unchanged. With further sintering and increasing temperature more peaks form βTCP phase are observed in the spectra. When the sintering temperature has reached 1200°C very few of the characteristic peaks for HA can be detected, since most of this phase is transformed into the βTCP phase.

The thermal behaviour of hydroxyapatite silica-based ceramic differs from the thermal behaviour of biphasic calcium phosphate silica-based ceramic. In the first case we obtained

partly transformed hydroxyapatite into tricalcuim phosphate phase, but with the presence of HA phase in the spectra at 1200°C, while the spectra of BCP ceramic at 1200°C shows almost complete transformation of the HA phase into the tricalcuim phosphate phase.

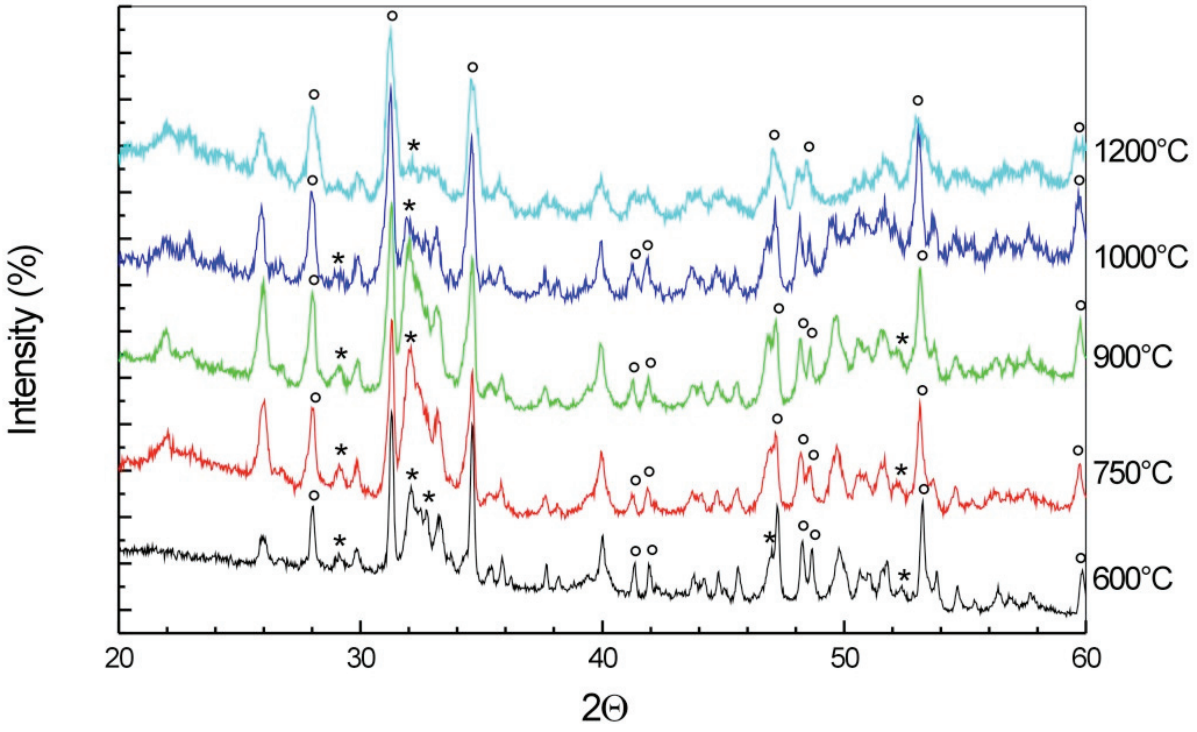


Figure 4.9: *XRD spectra of biphasic calcium phosphate ceramics heated between 600°C and 1200°C, (*) HA phase; (°) βTCP phase*

A number of recent studies have attempted to clarify the transformation of Ca-deficient hydroxyapatite to β-TCP. Changes in the size and morphology of the crystallites were observed with increasing calcination temperature by Yubao *et al.* (35). The temperature range at which the transformation occurred was established as 650-750°C. The disappearance of the HPO_4^{2-} bands in the FTIR spectra at this temperature was also observed. A study by Gibson *et al.* (35) indicates a decrease of the crystallite size of calcium-deficient hydroxyapatite over the temperature region 600-700°C, followed by a large increase of the grain size as the sintering process begins. These results suggest that after the loss of water and the transformation into β-TCP at 710-740°C, no further changes occur. Although no change in the symmetry of the β-TCP formed between 740 and 1100°C was observed, a very unusual change in the lattice parameters was observed as the calcination temperature was increased.

This change indicated that the structural changes that occur during the transformation from calcium-deficient hydroxyapatie to β-TCP are not complete at 740°C and further changes occur between 740 and 1100°C. The structural changes probably involve changes/distortions in the PO_4 tetrahedra, where three different types of crystallographically non-equivalent PO_4 groups in the β-TCP structure are reported. These changes are small enough not to affect the symmetry, but significant enough to result in changes of the lattice parameters.

Another research group working with hydroxyapatite/sol-gel glass biphasic materials (7) has shown, that by heating of their mixture to 1000°C leads to a decreasing surface area, which affects bioactivity, and led to the formation of new crystalline phases such as SiO_2, wollastonite, α- and β-TC. The presence of quarz and wollastonite can be explained as a result of crystallization from the gel-glassy matrix, while the last two phases result from thermal decomposition of hydroxyapatite. Since stoichiometric hydroxyapatite does not show any decomposition at this temperature according to (7), the reaction observed has to be related with the above mentioned interaction with the gel. Although in our hydroxyapatite ceramics we have 24 wt % silica, at 1200°C of hydroxyapatite X-ray diffraction pattern we did not detect the presence of SiO_2 as a single phase. The disagreement comes from the dissimilar way of preparation of our ceramic samples and the ones prepared according to (7).

Biological apatites have also shown decomposition when exposed to higher temperatures. Upon heating to 1000°C the bone mineral of most mammals shows significant transformation to tricalcium phosphate. In some cases there is no such transformation, and the discrepancy is ascribed to greater Ca/P ratios, and lower amount of HPO_4^- and Mg in human bone mineral (36).

The thermal behaviour of the designed in our laboratories calcium phosphate bioceramic was well documented and it is in accordance with the reported phase transformations of hydroxyapatite into tricalcium phosphate when heated (1, 7, 34, 35, 36). Every disagreement (i.e. phase transformation temperature, percentage of the phases present at a certain temperature) between our bioceramics and the materials mentioned in the literature is due only to different making procedure, different starting materials and conditions. Hydroxyapatite silica-based ceramic is thermally stable until 1000°C, after which temperature partly transformates into betha-tricalcium phosphate. Biphasic calcium phosphate silica-based ceramic is thermally stable as well at 1000°C. The decomposition of BCP silica-based ceramic at temperatures higher than 1000°C, indicates a higher presence of the tricalcium phosphate phase when compared with HA silica-based ceramic at the same temperature. Since the main aim for producing these ceramics is their biomedical application,

temperatures higher than 1000°C shoud be avoided in order to prevent the above stated phase transformations.

4.4 Porosity and shrinkage

Data on biodegradibility of the different calcium phosphate ceramics (hydroxyapatite, tricalcium phosphate, dense or porous) are conflicting. If one studies the experimental conditions as reported by many investigatiors, it is clear that not only have a variety of surgical procedures and animal models been used to investigate the resorption behaviour, crystal structure, and degree and types of porosity, but conditions of manufacture have often not been clearly defined. However, factors presumed to govern biodegradation that are most often mentioned are molar ratio Ca/P, crystallography and degree of porosity.

Provision of a suitable macroporous structure is important in order to obtain good implant incorporation through rapid vascularisation, bone ingtrowth, and where the implant is resorbable, possibly remodelling. Conventional ceramic processing techniques have been used to form hydroxyapatite bodies, and several methods of inducing porosity have been described. The simplest methods involve the incorporation of compounds that may be volatilized prior to sintering. Materials such as naphthalene and calcium stearate can be used. The degree of porosity, thus, is controlled by the amount of degradable material added. Porous calcium phosphates have been formed by the addition of hydrogen peroxide (H_2O_2) to the powder slurry (37). Both pore size and pore volume are controlled by the quantity of H_2O_2 added and the rate at which the slurry is dried. Such techniques can control the total amount of porosity, but the size of pores is scattered around the required mean.

In order to examine the change in porosity with sintering temperature, we have produced samples of hydroxyapatite silica-based ceramics and biphasic calcuim phosphate silica-based ceramics with rectangular shape (2.9 x 1,5 x 0.8mm) and heated them between 200°C and 1200°C. The relative density of the sintered materials was measured by the Archimedean method in water. The porosity was calculated according to the following equation:

$$\textbf{Porosity \%} = (\rho_0/\rho_1) \times 100 \qquad (12)$$

where ρ_0 is the density of the dried samples, and ρ_1 is the density of the wet samples. At 200°C BCP and HA samples posses different porosity – for HA is about 55% and for BCP is 65% (Fig. 4.10). The porosity of both ceramics remain unchanged until 800°C – the small increases at 400°C for HA samples, and at 500°C and 750°C for BCP samples are probably related to differences in block´s size between the starting ceramics. Although we have produced similar in shape samples there are variations in their size, thus, the changes before the sintering temperature can be detected. Starting from 800°C both ceramics showed fast decrease in porosity, due to sintering and decreasing of the free space between the crystals. The final porosity at 1200°C is different for both ceramics, and it is surprisingly that BCP which has higher porosity at 200°C shows lower porosity at 1200°C than HA ceramic. The value for BCP ceramic has dropped down to 25%, while the HA ceramic is slightly higher than 35% at 1200°C. One of the possible explanations for this result can be the higher amount of calcium phosphates in the starting material for BCP ceramic, which upon sintering shows tendency for aggregate formation (increase in grain size), therefore the porosity of biphasic ceramics depends on the proportions of the two phases – HA and TCP. Not only porosity, as well as microstructure and densification ratio of biphasic ceramics can be linked to the diffusion phenomena and to the particle coalescence that occur at lower temperature in comparison with densification of monophasic ceramic.

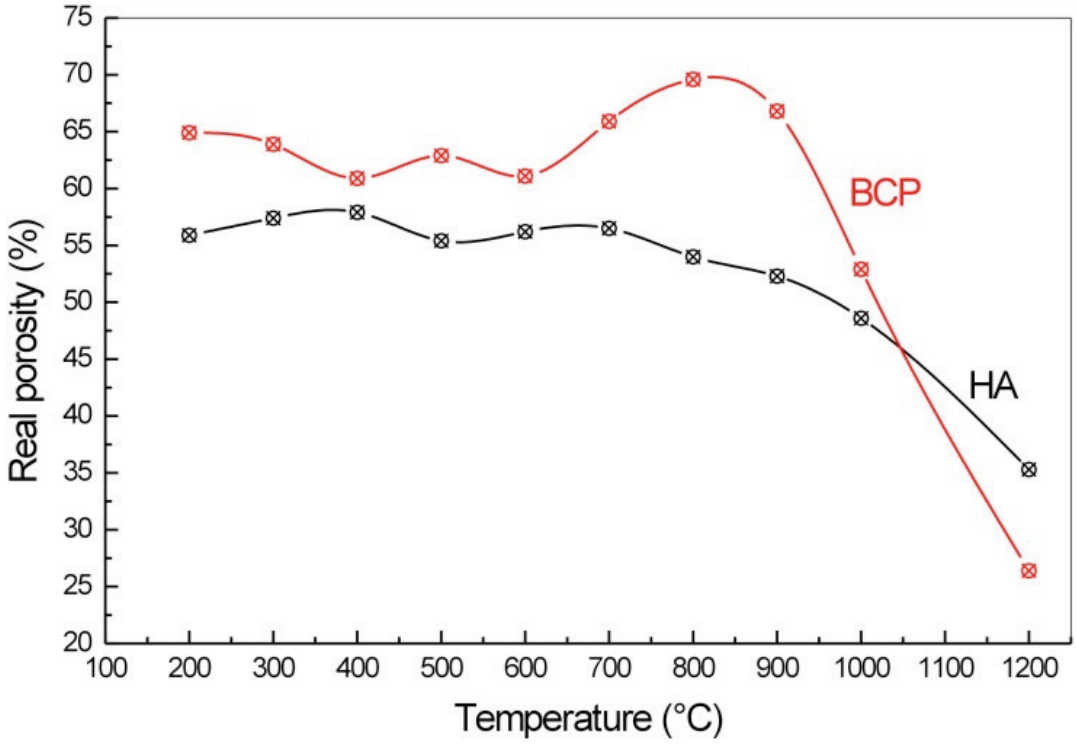
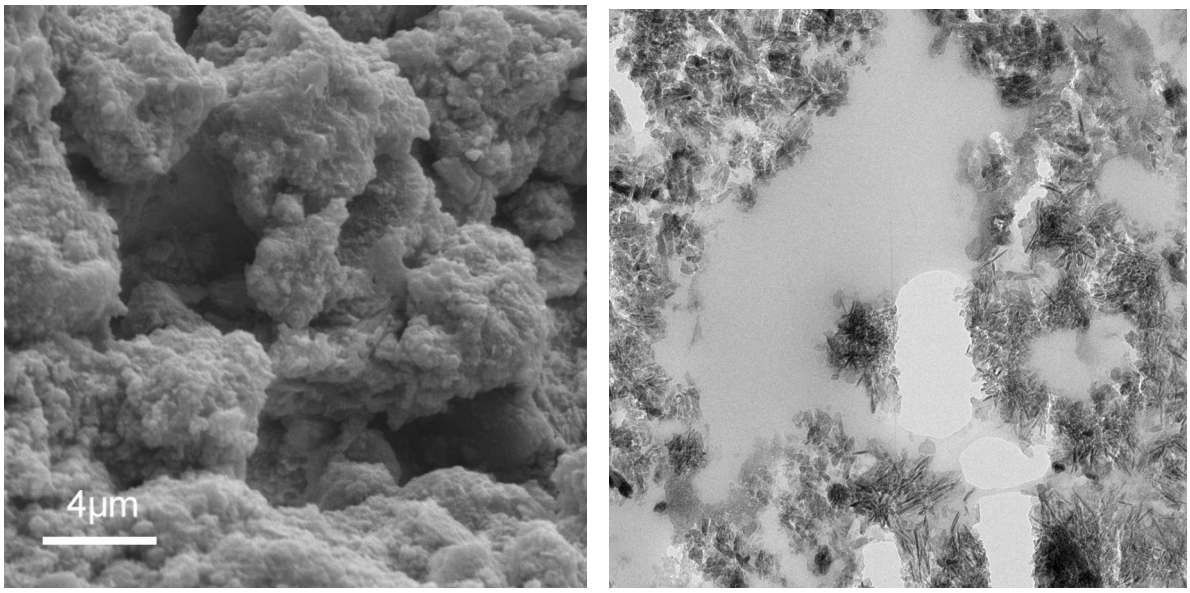


Figure 4.10: *Change in real porosity of HA and BCP ceramics with temperature*



a) b)

Figure 4.11: *Porosity in µm range (picture a, where scale bar represents 4µm, SEM) and in nanometer range (picture b, TEM), which shows the loose packing between the crystallites, wrapped by the SiO_2-matrix*

It is generally admitted that 80-100 μm is the minimal pore size for osteoconduction. A pore size of 50-400 μm is recommended for metallic implant fixation to provide better bone ingrowth than with larger pores of 400-800 μm. Some studies have reported notable bone ingrowth for pores smaller than 100 μm, but many authors consider that only pores larger than 100-150 μm facilitate ingrowth of mineralized bone (38). Therefore, the design of porous implant materials with pore size larger than 150-200 μm, appears to be an essential criterion for clinical purposes. However, the existence of pores, particularly the macropores (i.e. those pore have a few hundred micrometers in dimension), can be accompanied by a significant reduction in mechanical properties of HA and HA/TCP ceramics, and subsequently restricts their applications where sufficient strength is needed. Our ceramic samples showed well expressed microporosity in the range of 100 nm up to 10 μm, the first formed due to the SiO_2 gel matrix, and the latter formed naturally by the manufacturing process during the heating (Fig. 4.11). Macroporosity is as well nicely presented, which appears to be of a great importance for the biodegradation of our ceramics. While the ceramic samples were extruded in the spaghetti form, macropores in mm range were formed between the spaghetti (Fig. 4.12). The existence of micro- and macropores on the other side, weakens the mechanical properties of these ceramics.

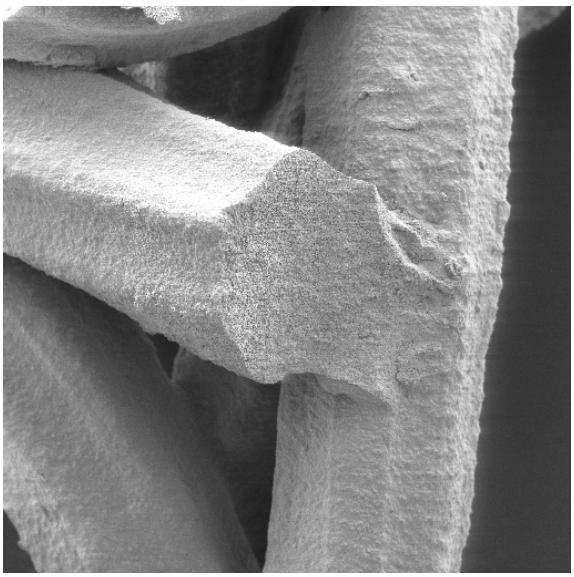


Figure 4.12 : *Scanning electron micrograph of spaghetti-shaped ceramic, which shows pores in macrorange, magnification x50*

Le Huec *et al.* (39) have demonstrated the influence of porosity on the compressive strength of the porous HA. They correlated the resulting compressive strength with both macroporosity and microporosity by a polynomal equation and indicated the possibility for designing a porous HA ceramic with optimized properties. It was reported by them that macropores have a greater influence upon the compressive strength than the micropores. At a given porosity, the compressive strength was found to decrease roughly linearly with macropore size as illustrated in Figure 4.13. The porosity-dependent strength

becomes insensitive to the porosity volume when the level of porosity reaches greater than 70 vol. %.

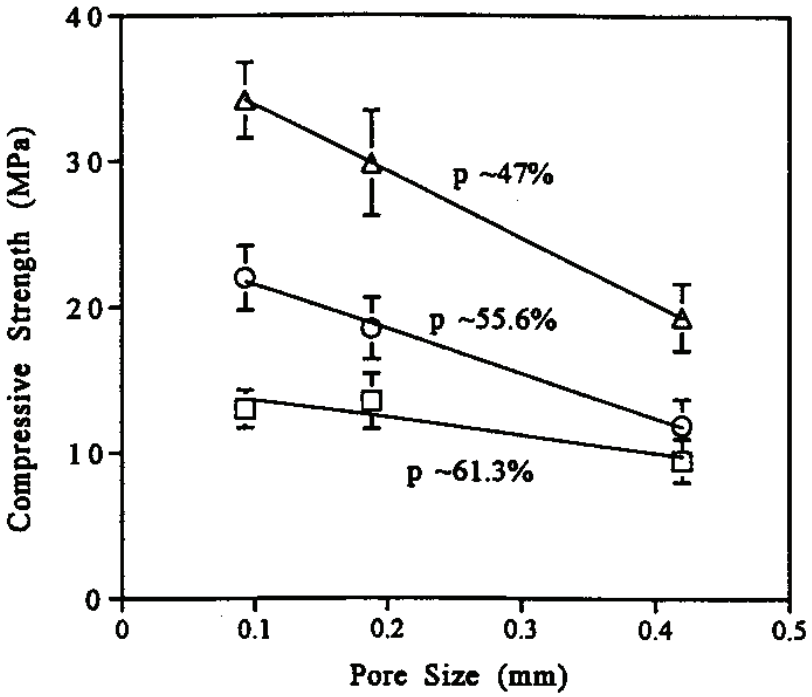


Figure 4.13: T*he compressive strength of HA ceramic of nearly identical porosity decreases linearly with increased macropore size (from Ref. 36)*

The influence of pore geometry on the compressive strength has currently been found to be important in producing porous HA or HA/TCP ceramics. The porous HA ceramic containing oblate pores is expected to have a lower strength than that with either ellipsoid or sphere pores, and the pores with spherical geometry exhibit better resistance to stress loading (39).

The shrinkage can be calculated using the following formula:

$$\textbf{Shrinkage \% = 100 - } (V_1/V_o \textbf{ x 100}) \qquad (13)$$

where V_o is the volume of the samples before the heat treatment, and V_1 is the volume after the heat treatment. The development of shrinkage/temperature dependence can be seen on Figure 4.14. From the starting temperature 200°C until 800°C no visible changes in the volume shrinkage of HA and BCP ceramics are observed. After this temperature both materials show an abrupt increase in their shrinkage; for HA ceramic it is from 7 % to 57 %, and for BCP ceramic is from 3,5 % to 50 %. Although biphasic calcium phosphate silica-

based ceramic has shown lower porosity when sintering temperature increased, when comparing the shrinkage of both materials, we should note that HA and BCP ceramics behave in similar way. The small difference in shrinkage between HA and BCP ceramics at high temperatures can be neglected, since both display a linear increase upon sintering. Nevertheless, the higher shrinkage in HA ceramic in comparison with BCP ceramic can be explained with the different amounts of calcium phosphate powders in the starting materals. Since in HA ceramic there is less calcium phosphate powder and a higher portion of silica gel, the latter upon sintering shrinks, which reflects to the shrinkage of the whole material. In contrary, in BCP ceramic the silica portion is less (in both ceramics the molar ratio Si^{4+}/Ca^{2+} differs) and with sintering its shrinkage does not reflect so greately the shrinkage of the whole body. Krajewski *et al*. (40) had reported that in the temperature interval between 1200-1250°C hydroxyapatite undergoes a sharp evolution and the shrinkage reaches its highest value – about 25% overall. Raynaud *et al*. (41) had explained the shrinkage phenomena as a presence of two domains in the shrinkage/temperature dependence. The first one they associated with calcination for temperatures up to 700°C, and the second is associated with sintering above that temperature, where densification mechanisms occur. Our resuls can also be interpreted by using the same explanation, since before 700°C no great changes in the shrinkage behaviour of HA and BCP ceramics occurred.

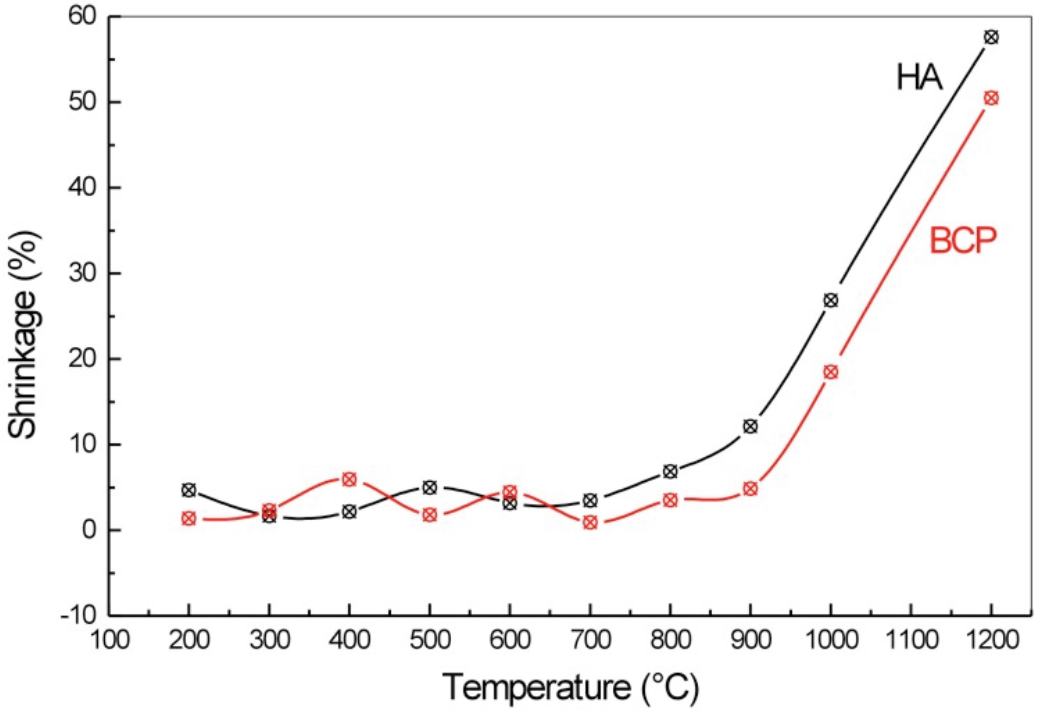


Figure 4.14: *Shrinkage-temperature dependence of HA and BCP ceramics*

It has been demonstrated that the implantation of orthopaedic implants modifies the physiological mechanical stress pattern of the surrounding tissue and that the mechanical environment is directly related to the elastic property (stiffness) of the implanted materials. To minimize the mismatch in stiffness difference between the implant materials and hard tissue, one of alternatives is to make porous implants. The introduced porosity allows the stiffness of the implant materials to be adjustable; however, deteriorates the strength. Liu *et al.* (39) had reported an exponential decrease of elastic constant with porosity of HA ceramic, over the porosity ranges of 18-50 %. Human bones have elastic constants generally in the range of 10-25 GPa, corresponding to the porous HA ceramic with a porosity of approximately 50 vol.%.

The ceramic samples which were used as implants in the mini pigs animal model, have shown a very good porosity level – between 60 and 80 %, with three types of pores in macro-, micro-, and nanorange. On one side this fact is very promising for biodegradibility of the graft substitute material, but on the other side is a limitation for the usage of these ceramics in load-bearing applications.

4.5 Mechanical testing of calcium phosphate silica-based ceramics

In weight bearing structures such as the human skeleton, each newly developed biomaterial needs to be mechanically tested to ensure its functionality and reliability. For metals, the test protocols are well known and standartized. For bioceramics, measurement of their multiaxial mechanical properties is more difficult, since their behaviour depends heavily on the microstructural defects associated with the manufacturing process. The mechanical properties of dense synthetic hydroxyapatite, as with all engineering ceramics, are highly dependent on the processing route, as a consequence of the effects of processing on the microstructure of the final product. This dependency results from mechanical sensitivity to parameters, such as variations in grain size, micropore size and micropore distribution.

Conventional methods of mechanical characterization such as tensile, biaxial and impact testing are unsuitable when applied to porous materials due to the difficulties encountered in machining and gripping test pieces without causing pre-damage. Compression testing has been successfully used by a number of authors in the characterization of cancellous bone and has also been adopted in the testing of porous and dense hydroxyapatite.

However, in addition with the difficulties resulting from the inherent variations in porous samples, testing conditions should be carefully controlled. Comprehensive investigations into a number of key test-piese parameters, such as specimen shape, aspect ratio and size, have demonstrated that cylindrical specimens with an aspect ratio or 2:1 (length-diameter) should be standardized for compression testing of highly porous materials (26).

The samples we used for mechanical testing were shaped as bars with rectangular (2,9 x 1,5 x 0,8mm) form. After drying they were exposed to heat treatment in electrical furnace in air atmosphere for 2 hours at 200°C, 300°C, 400°C, 500°C and 600°C. The mechanical tests were performed on a Shimadzu AG-50kNG instrument under computer control in DOT Company (Rostock, Germany) with 1 N load cell. The compressive strength of the bars was determined using a centric load applied to a spring-suspended steel bearing block and test speed of 1mm/min, until catastrophic brittle failure occurred. Figure 4.15 represents the results from compressive strength measurements for hydroxyapatite silica-based ceramic.

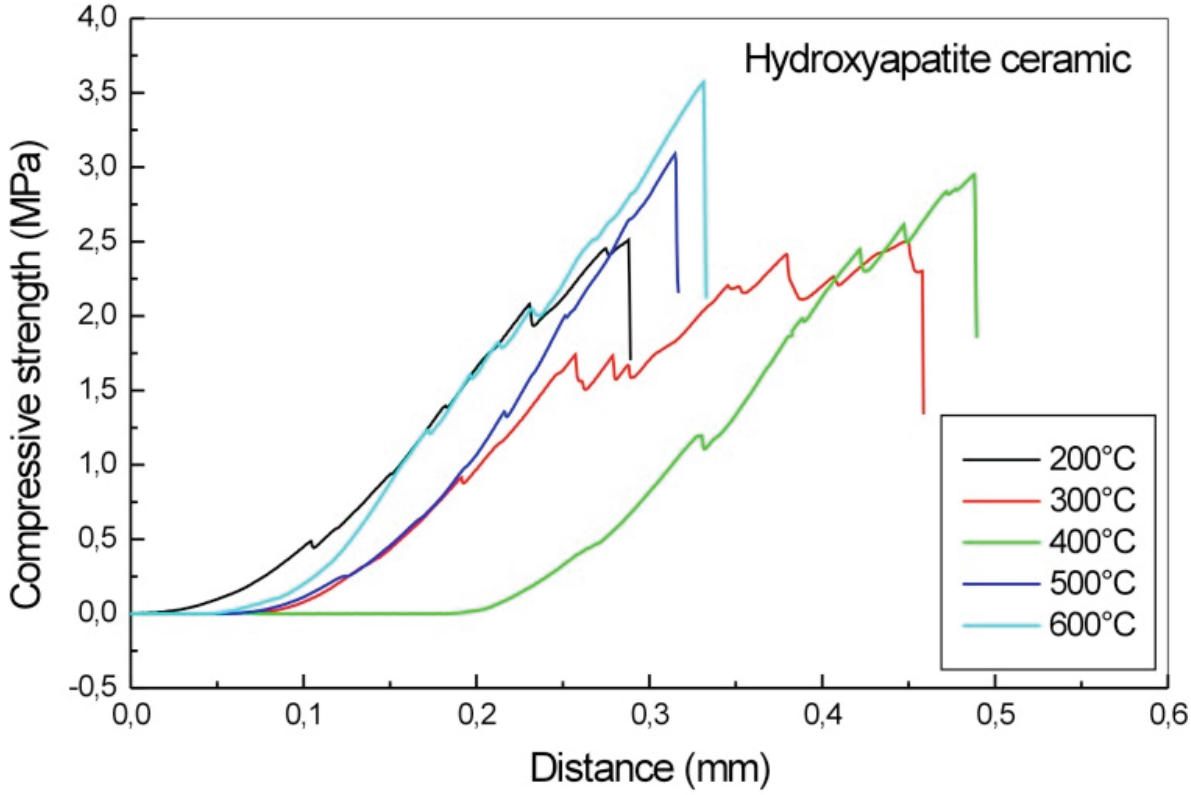


Figure 4.15: *Temperature dependence of compressive strength for HA ceramic*

It is prominent that almost no changes occur in mechanical strength with increasing the temperature from 200°C to 600°C. The highest strength is 3,5 MPa for samples heated at 600°C, which does not differ very much from the strength of the samples heated at 200 °C – 2.5 MPa. There is an explainable increase in compressive strength with increasing the temperature, but in the temperature interval between 200°C and 600°C this increase is almost negligible. The strength difference of our samples is more obvious when they were exposed to temperatures up to 1200°C – in this case we have found out that the compressive strength has increased more than 10 times for samples exposed to 1200°C in comparison with samples heated at 300°C. Figure 4.16 shows the compressive strengths for BCP ceramics, on which the specimen heated at 1200°C has 51 MPa compressive strength, and the one heated at 300°C has less than 5 MPa compressive strength. These results are impressive and seem to be very useful for further study, where mechanical strength would be a necessary requirement for load-bearing applications. There is one possible trouble to be worthy of, and this is the thermal decomposition of our ceramic samples upon sintering, which was already discussed.

The thermal behaviour of a biomaterial is as important for *in vivo* applications as the biodegradation and resorption. From material standpoint both HA and BCP ceramics have shown very promising mechanical properties, which can be successfully applied as load-bearing materials. But in order to escape from undesired changes and transformations during heating (i.e. sintering) we do not recommend higher heat treatment for HA and BCP silica-based ceramics than 1000°C (see also the explanations about the thermal decomposition in paragraph 4.3).

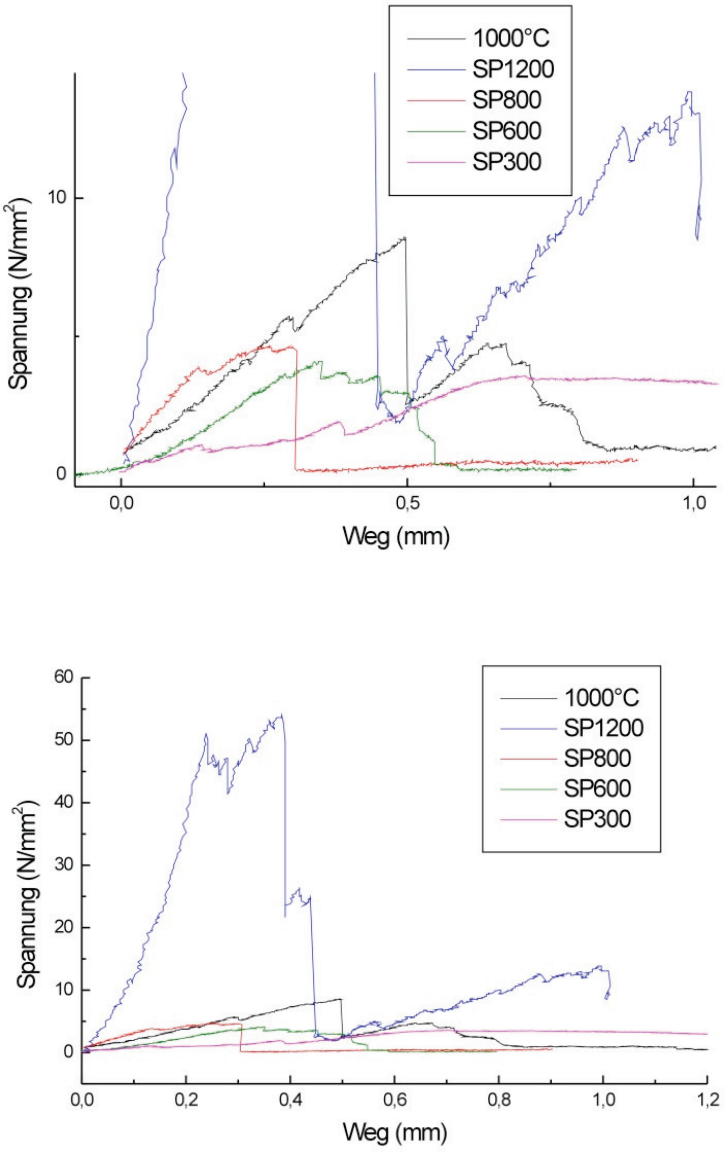


Figure 4.16: *Temperature dependence of compressive strength for BCP ceramics heated between 300°C and 1200°C (Spannung is strain in N/mm² or MPa, Weg is distance in mm)*

The mechanical tests obtained for BCP ceramics are very similar to those obtained for HA ceramic (Fig. 4.17). The escalating of temperature leads to an increasing of the mechanical strength, which at 600°C shows slightly higher value compared with HA ceramic heated at the same temperature (3,6 MPa for BCP and 3,5 MPa for HA cramics). This small difference can be neglected, but according to the molar ratio Si^{4+}/Ca^{2+} of the starting materials, which in the case of BCP ceramic is lower, one can expect that the more the amount of calcium phosphates in the specimen, the higher the mechanical strength. Perhaps this difference can be overtaken due to the almost no changes in compressive strength of both ceramics up to 600°C, but when increasing the heating temperature, it could be possible to obtain higher values of the mechanical strength for BCP than for HA ceramic.

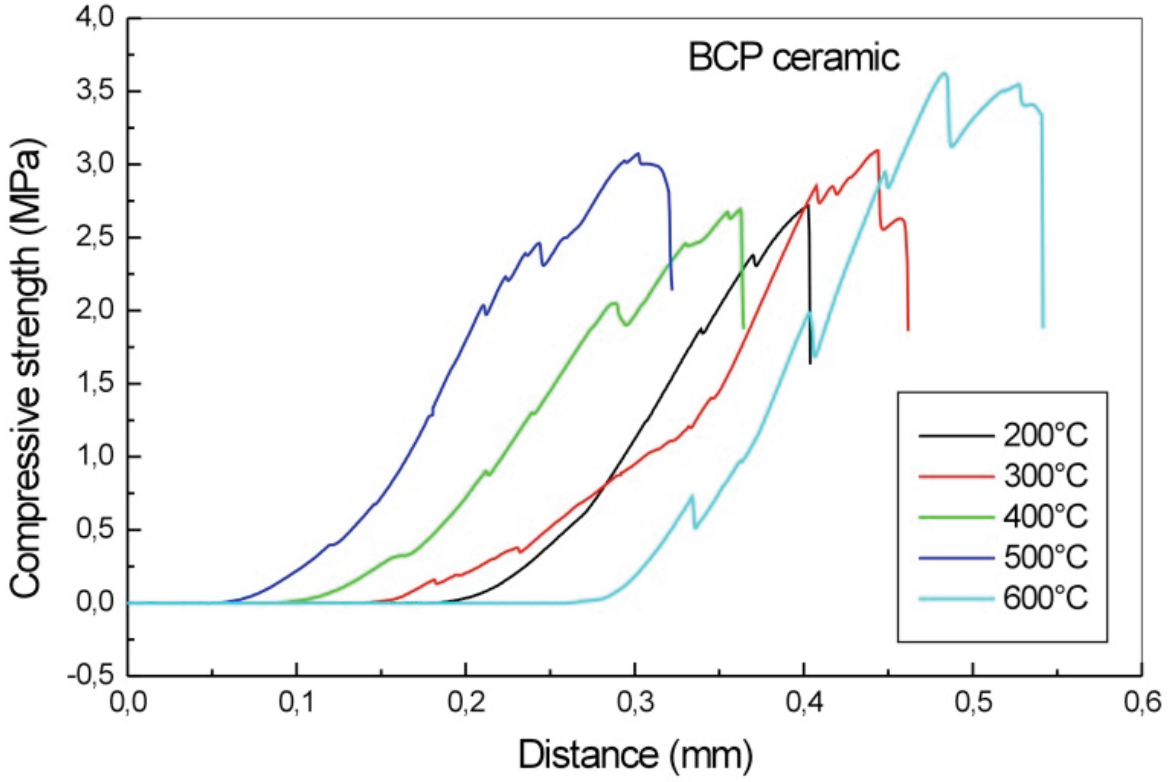


Figure 4.17: *Temperature dependence of compressive strength for BCP ceramics*

In their work Akao et al. had mentioned that the compressive strength of sintered hydroxyapatite, synthezised by them is approximately 3 to 6 times as strong as that of cortical (compact) bone. It should be mentioned that the strength of compact bone depends both on degree of calcification and on fibre orientation. The hydroxapatite precipitated and used by these researchers had shown very good and even superior mechanical properties than cortical bone, dentine and enamel and as they have suggested it can be used as bone and tooth implant as well as for load-bearing applications (42).

It must be noticed that the strength of samples which consist of biphasic calcium phosphate ceramic is the same as the strength of monophasic ceramic, where no particular changes in the structure of both ceramics up to 600 °C occur. The mean compressive strengths of our materials (heated only to 600 °C) are given in Table 4.2 in comparison with the strengths of enamel, dentin, compact bone, and cancellous bone (11).

Material	Compressive strength (MPa)	Tensile strength (MPa)	Modulus of elasticity (GPa)
HA ceramic	3,7 (our mat.)		
HA/TCP ceramic	3,45 (our mat.)		
Enamel	241(23); 384(11)	10,3(11)	82,4(11)
Dentin	138(23); 295(11)	51,7(11)	18,2(11)
Compact bone	167(23);163,8(11)	88,9-113,8(11)	3,88-11,7(11)
Cancellous bone	1,86		

Table 4.2: *Compressive and tensile strength, and modulus of elasticity of various hard tissues (Data for HA and HA/TCP ceramics are elevated after heating at 600 °C)*

As many investigators had pointed out (38) the mechanical properties of calcium phosphate ceramics improve rapidly *in vivo* due to bone ingrowth in macropores and reprecipitation of biological apatites in micropores. As it will be shown later when presenting the results from our *in vivo* experiments, the defect created in the right proximal tibia of Goettinger mini pigs was healed very well, and no complications regarding absence of mechanical strength were observed. From all of the tested animals (6 in number), only one has gotten a fracture, which could be related to different reasons, e.g. surgical conditions, acceptance of the implant, animal health status, etc. In our research we did not measure the compressive strength after the implantation, but many other authors have reported an increase in compressive strength for bone derived porous HA from 4 to 20 M Pa after 3 months *in vivo* (43). The same authors had stated 300% increase of the compressive strength within 5 weeks, despite complete osseointegration at this time. These results indicate, that with time, the mechanical behaviour of osseointegrated implants many become independent of the initial implant strength as a result of reinforcement by bone ingrowth. Therefore, to provide a good compromise between satisfactory bone ingrowth and acceptable mechanical resistance, many animal and clinical studies recommended calcium phosphate ceramics with approximately 50 % macroporosity. Our ceramics materials have a porosity between 60 % and 80 % and relatively low compressive strength, but during the implantation period the implants have shown a good biodegradibility and ability to promote bone substitution. We do not find it as a disadvantage that our ceramics do not posses a high mechanical resistance. Notwithstanding, the achievement of high porosity in combination with sufficient mechanical resistance should be one of the next priorities to investigate in our laboratories.

CHAPTER FIVE

In vitro study. Results and discussion

The evaluation of cell-material interactions is of main importance when developing new materials for biomedical applications. A biocompatibility study should be done in order to prove the biodegradibility of certain designed biomaterial, and its bone conducting behaviour. This chapter will present an osteoblast cell culture study, which have been used in order to determine the potential of calcium phosphate silica-based ceramics to elicit specific responses to bone cells *in vitro*.

5.1 Variety of in vitro study tests and tissue responses to a foreign body

Formation of a biologically equivalent apatitic surface, a common characteristic of bioactive materials, can be reproduced in vitro by immersion using a simulated physiologic solution that mimics the typical ion concentrations in body fluids, or cell containing media (44). Such experiments have shown that the materials with high solubility also readily induce the precipitation of a biologically equivalent apatite on their surface. Using this *in vitro* immersion methodology, considerable evidence has been obtained revealing the mechanisms of surface reactions. The reactions include dissolution, precipitation and ion exchange accompanied by absorption and incorporation of biological molecules.

Bone collagen is considered to be intimately involved in the nucleation of bone mineral, a hypothesis supported by electron microscope studies of developing bone in which a periodic deposition of new bone crystals is seen in relation to collagen fibrils (16). Since there is a broad range of materials available and only a very small percentage have been used in

biological environments, there has been a continual need for quick methods that can be used *in vitro*. These can be divided into two general classes:

- **Tissue culture methods**
- **Blood contact methods** - for blood contact applications in cardiovascular system

Within each of these classes there is a wide variety of tests and variations of test methods. Some variations are traditional for particular applications, while others are the practice of a particular laboratory.

Tissue culture refers collectively to the practice of maintaining portions of living tissue in a viable state in vitro. There are three generic methods:

1. **Cell culture:** The growth of initially natural matrix-free, disassociated cells. Cells may be grown in solution, or on agar, or other media substrates. Exposure to biomaterials may be through direct contact with bulk materials, diffusional contact through agar (or other gel) intermediate layer, or by inclusion of extracts or elutants from materials in the culture media.
2. **Tissue culture:** The growth of portions of intact tissue without prior cellular dissociation. Exposure to biomaterials is similar to that for true cell culture.
3. **Organ culture:** The growth of intact organs in vitro. This may vary from the use of fetal bone explants which can survive without external support systems to the use of whole, adult, perfused organs such as the kidney or heart. A variety of exposures to biomaterials is possible.

Cell types may vary in activity and properties between species, and, in species, between individuals, and within a specific cell line, between generations. Tissue culture techniques as a group may be used to study the cell survival (toxicity, membrane integrity), cell reproduction (growth inhibition), metabolic activity (energetics, synthesis, catabolism), effective activity (inhibition of locomotion, chemotaxis and phagocytosis, alteration of cellular shape and size) and cell damage (chromosomal aberration, mutagenicity and carcinogenicity) (45).

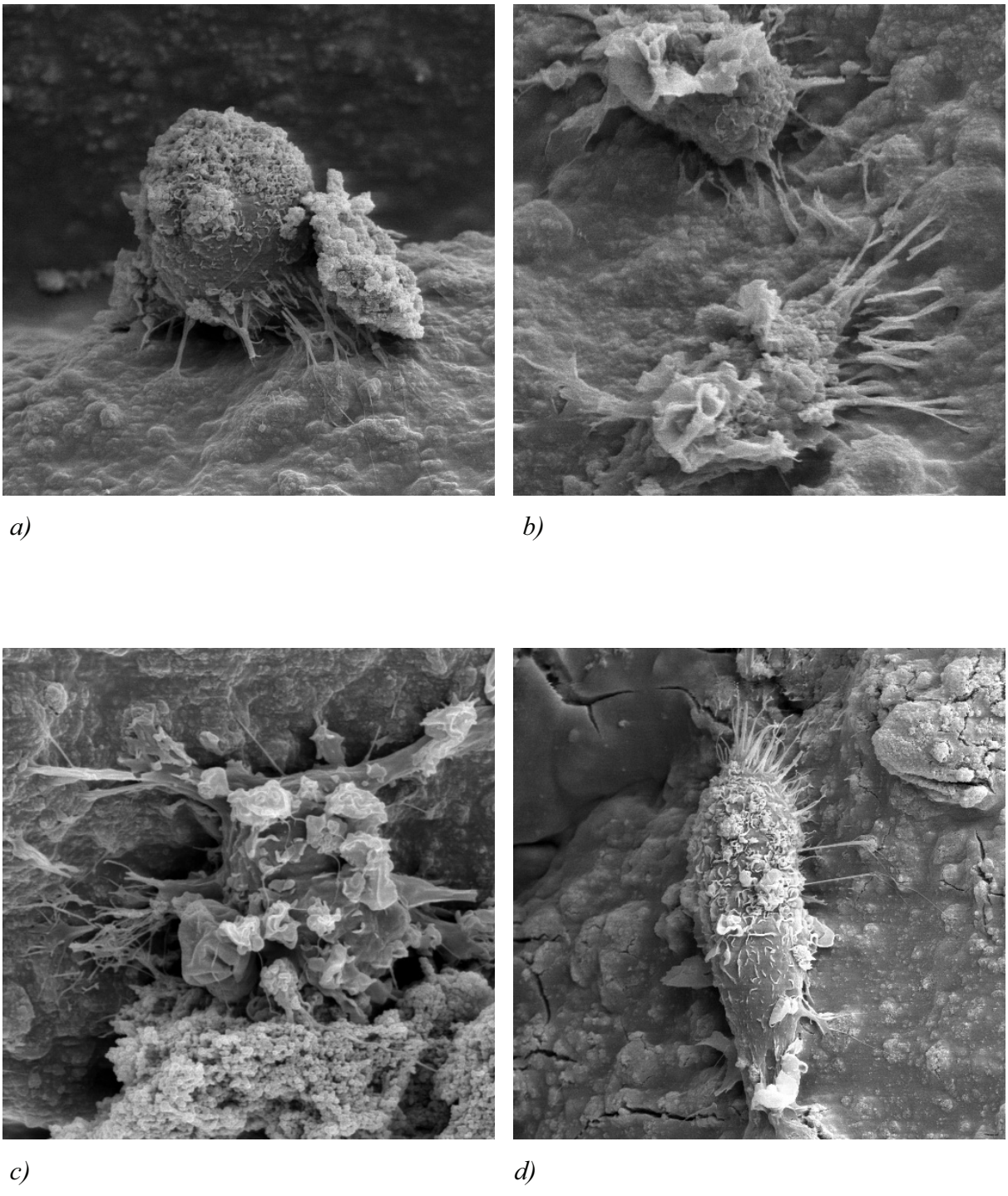
5.2 Cell isolation and culture

A cell culture system has been used in collaboration with our colleagues from biochemistry department to study the biocompatibility of tricalcium phosphate silica-based ceramic and hydroxyapatite silica-based ceramics with different silica/calcium ratios. Osteoblasts are cells that support the formation, secretion and mineralization of the extracellular matrix of the bone tissue (46). Therefore, some of the fundamental principles like cytoxicity, cell proliferation and differentiation, cell viability can be tested *in vitro* using osteoblast cultures. Many researchers have used lineage cells, because of their easier culture behaviour (46). According to a method used by Gerber *et al*. (47) for the *in vitro* test we have used osteoblasts, obtained from the hips of patients with primary coxarthrose. The cells isolation was done by the use of enzymatic treatment with dispase and collagenase of the bony specimens. The ceramic materials were seeded in the culture medium for 10 days. After the cultivation with osteoblasts the ceramic samples were fixed with 4% glutaraldehyde solution and were prepared for examination with raster electron microscopy.

5.3 Results and discussion

Figures 5.1 a-d showed the scanning electron micrographs of hydroxyapatite samples with different Si/Ca molar ratio: from 2.9 up to 6.5. On the surface of each material attached cells with round form can be seen and they seem to be active. Figure 5.2 represents the surface of hydroxyapatite ceramic without cell culture. The roughness of the ceramic is obvious, where isolated smooth islands of silica gel are visible. These areas have different thickness and small cracks, possibly due to heating of the samples prior to the preparation technique. The cells attach preferably to the ceramic surface with less (in thickness) silica gel. Therefore, the adhesion of cells which lie down directly on the ceramic surface is greater than the one on smooth surface, which consists mainly of silica gel. Figures 5.3 a-b are SEM photos of tricalcium phosphate silica-based ceramic which has been cultured for 10 days.

Here the activity of cells, which adhered to the ceramic surface is higher and they produced collagenous extracellular matrix, which spreads over the surface.



a) *b)*

c) *d)*

Figure 5.1 : *In vitro testing of hydroxyapatite silica-based ceramics with different Si/Ca molar ratios, where a) is 2.9 ,x2000; b) is 3.6, x2000 ; c) is 4.5, x3000, and d) is 6.5, x2000*

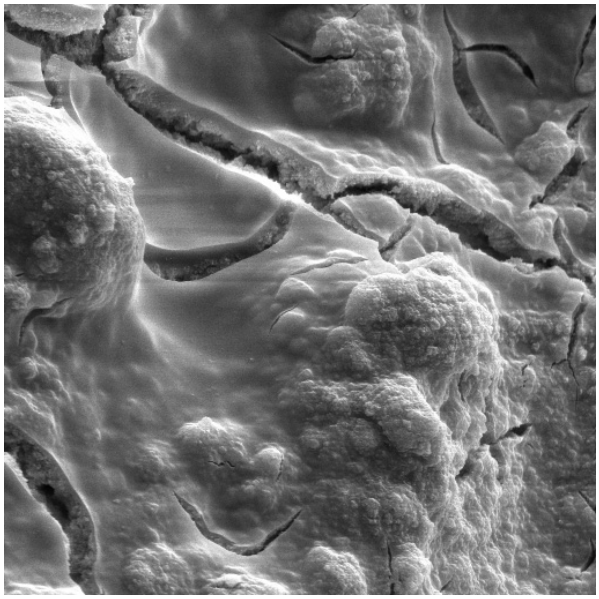


Figure 5.2 : *Scanning electron micrograph of hydroxyapatite ceramic surface, magnification x3000*

The surface roughness of materials has long been recognised as an important factor in the generation of cell and tissue responses to implants. Behling and Spector have reported an increased cell adhesion to rougher than to smoother surfaces (48). Our ceramics materials have shown to have surfaces which can be determined as mixed, rather than only smooth or rough because on one side there are smooth regions, which are not distributed uniquely over the whole surface area, and rough domains which are responsible for the cell attachment and further mineralization (Fig. 5.2). Similar results were confirmed by Gomi *et al*. (49) where statistical analysis had shown greater number of attached marrow cells on the rougher hydroxyapatite surface than on the smoother hydroxyapatite surface.

a) *b)*

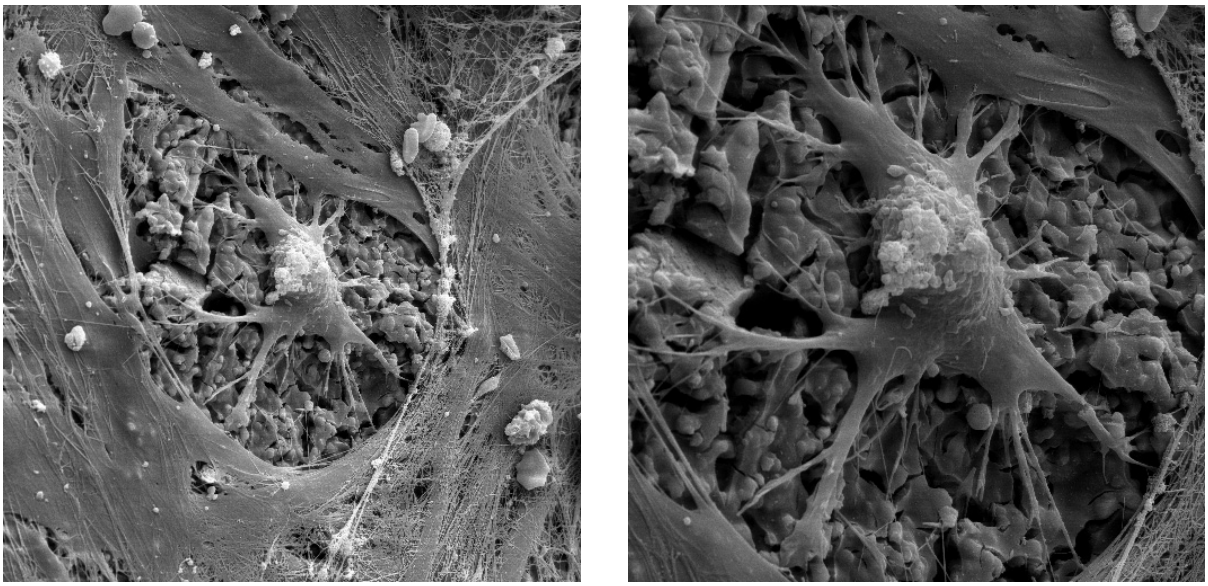


Figure 5.3: *In vitro testing of calcium phosphate silica-based ceramic. Note the formation of collagenous extracellular matrix on (a), x1000; (b) is a higher maginification of (a), x2000*

High-resolution transmission electron microscopy has revealed the two stages of physicochemical events occurring after the implantation of biphasic calcium phosphate ceramics, i.e. crystal dissolution and the precipitation of biological apatite characteristic of bone-bonding capacity (50). These dissolution/precipitation processes are not caused directly by cells but by fluid, even though they may be influenced by absorption of cell-derived proteins onto the ceramic surface. Yet it is reasonable to suppose that, apart from these physicochemical events, cellular events also occur *in vivo* circumstances in which numerous cells of various kinds are present.

The osteoclastic degradation of ceramic surfaces (i.e. formation of resorption lacunae) results from the total effects of ceramic properties (and especially solubility) and the action of osteoclasts on ceramics (resorption activity). It would appear that more soluble a calcium phosphate ceramic is, the more extensively it is resorbed by osteoclasts, which is in fact the case when ceramics solubility is within certain limits. The chemical properties of ceramics may influence osteoclast resoprtion activity. For example, a very soluble ceramic might not be suitable for active resorption by osteoclasts, since a lagre number of released calcium ions could inhibit osteoclast activity.

The authors (51) have compared 4 types of calcium phosphate ceramics: pure HA, pure βTCP, and BCP ceramics with two different molar ratios – one with 25/75 and another with 75/25 HA/βTCP molar ratio. Their findings suggest better osteoclast resorption on pure βTCP surface and on BCP ceramic with lower HA/βTCP ratio. It is well known that the solubility of pure βTCP is higher than that of BCP and HA ceramics, thus the osteoclast resorption is well expressed on the surfaces of these ceramics. However, an increased ceramic solubility is not always related to extensive osteoclast resorption, as mentioned before. Osteoclast resorption activity may be influenced by osteoblast activity or bone matrix proteins produced by osteoblasts. Using a two-stage culture system, de Bruijn *et al.* (52) showed that osteoclastic resorption of TCP occurred under an extracellular matrix layer formed by osteoblasts. They concluded that the extracellular matrix was able to increase the resorption activity of the osteoclast.

The physicochemical events occurring at the interface between calcium phosphate materials are thought to be responsible for chemical bonding of bioactive implants with bone. LeGeros *et al.* (53) summarized these events as:

1) Decrease in the pH in the environment of the calcium phosphate ceramics causing partial dissolution of the ceramic monocrystal;
2) increase in the concentration of calcium and phosphate ions in the microenvironment;
3) formation of CO^{3-} apatite crystals (by precipitation, by transformation from one Ca-P phase to another, or by seeded growth) incorporationg ions (e.g. Mg^{2+}, CO^{3-}) from the biological fluids;
4) association of the CO^{3-} apatite microcrystals with an organic matrix, and
5) incorporation of these microcrystals with the collagenous matrix during the formation of new bone at the interface.

In the present *in vitro* tests all calcium phosphate ceramic materials supported the expression of cell growth on their surfaces. This may actually suggest that these materials can enhance osteogenesis. As a result, these biomaterials can be considered excellent bone substitute materials. In order to understand this, a very good correlation between the *in vitro* data with the *in vivo* phenomena should be performed. Therefore, we have decided to continue with the animal tests in order to prove the ability of our ceramics to degrade *in vivo* and to act as a scafold for bone formation.

CHAPTER SIX

In vivo examinations of calcium phosphate silica-based ceramics. Results and discussion

With the current state of affairs in biomaterials science, only patients with relatively small bone defects can be effectively treated with reconstructive bone surgery. With larger bone defects it is always necessary to use either autologous (i.e. from the own patient) or donor bone. There are no suitable treatments available for major load bearing bone defects. This is because bone is a living, dynamic tissue with specific biological and mechanical properties that are not found in artificial materials. Bone uniquely combines elasticity and stiffness and is always capable of adapting itself to changing circumstances. This adaptability is primarily due to the combined action of bone forming and bone resorbing cells. In this chapter we will discuss the *in vivo* behaviour of the produced in our laboratory ceramic materials (hydroxyapatite calcium phosphate silica-based ceramic and biphysic calcium phosphate silica-based ceramic), and will try to give relevant explanations about mechanisms of bone mineralization and formation with the help of histological, polarized and electron microscopy, and X-ray diffraction observations.

6.1 Implantation procedures

In order to observe the *in vivo* behaviour of the designed in our labortory mono-and biphasic calcium phosphate silica-based ceramics, we used Goettinger mini pigs as an animal model. The implantation period was divided to 5 weeks (short-term test) and eight months (long-term test). The surgical procedure was made as follows: a total of 16, 1-year old, adult Goettinger mini pigs weighing between 25 and 30 kg were used for the study. In four pigs the

biphasic ceramic was introduced in their lower left jaw and these animals were left for 5 weeks, after which period they were sacrificed and the results were analyzed with electron microscopy and by histological methods. In the rest 12 pigs, the mono- and biphasic ceramics were separately introduced in the lower left jaw, where a defect of 5 cm^3 was created by removing the whole cortical bone (6 animals received monophasic ceramic and 6 animals received biphasic ceramic). Additionally a second operation in 8 of these pigs was made, in which another defect was created in the right proximal tibia of each pig and the monophasic (2 animals) and the biphasic (6 animals) ceramic materials were introduced (Table 6.1). All ceramic samples were sterilized before the operations in autoclave at 180°C. After the surgical procedure the animals were left in their natural surrounding and were watched daily for any complications. Eight months after the surgery the 12 pigs were sacrificed. Histological, X-ray and microscopical studies were accomplished. Four pigs were used as a control group with empty defect, in which no material was introduced.

Test period	Number of tested animals	Implanted material	Implantation site
Short term-five weeks	Total 6 animals	BCP	Lower left jaw
Long-term- eight months	Total 12 animals	BCP - 6 animals	Lower left jaw
		HA - 6 animals	
		BCP - 6 animals	Right proximal
		HA - 2 animals	tibia
Short term-control group	Total 4 animals	Empty defect	Lower left jaw

Table 6.1: *Distribution of the test animals related to the implantation periods*

6.2 Results from short-term in vivo study (five weeks)

The short-term in vivo test is important for observation of an early mineralization and bone formation and the mechanisms for occurring of the latter. As it was already mentioned in the beginning of this chapter, for the purpose of this examination period a total number of six mini pigs were used into which only biphasic calcium phosphate silica-based ceramic samples were implanted. The implantation site was the left lower jaw (mandible), where the spaghetti look-like samples were introduced (see also Fig.4.3). These defects had an overcritical size (5 cm^3), which means that usually no healing could occur by the natural remodelling and deposition of bone. In the mandible the defects were created by removing the cortical bone and thus leaving two openings from the inner and outer part of the bone. Figures 6.1 to 6.5 represent the operation procedure, performed by surgeons in the Department for Oral and Maxillofacial Surgery at Rostock University.

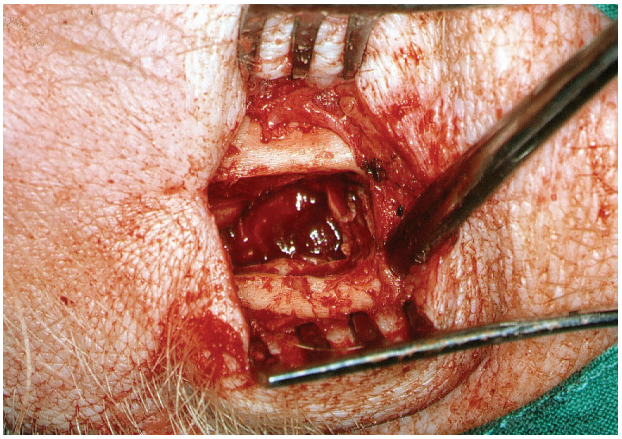


Figure 6.1: *Creation of the defect in the lower left jaw of Goettinger mini pig after drilling a holes in the inner and outer part of the manible*

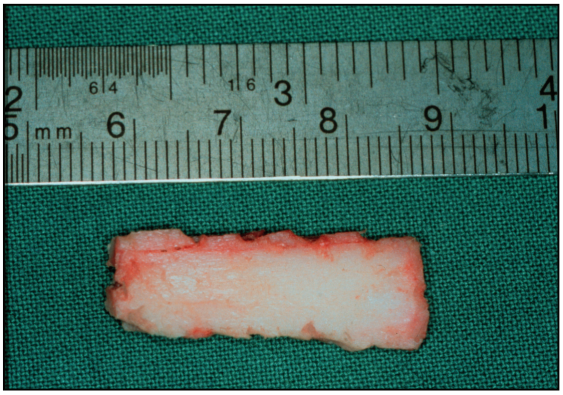


Figure 6.2: *View of the removed piece of bone from the mandible with an overcritical size – 5 cm^3*

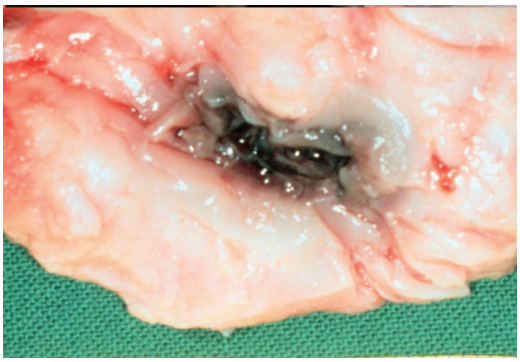


Figure 6.3: *Control group defect after 5 weeks. Note the formation of the callus, typical when no healing occurs due to bigger defect size*

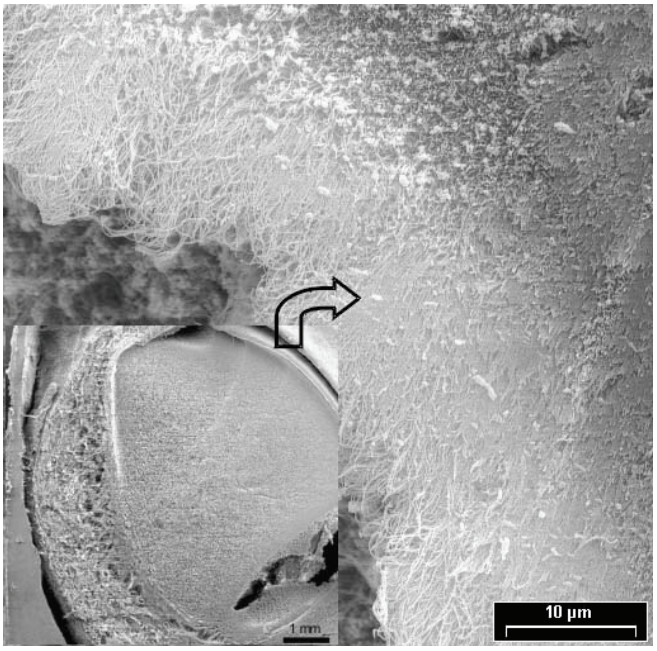


Figure 6.4: *Scanning electron micrograph after 5 weeks, showing the encalpuslation of the empty defect by a fibrous tissue, scale bar 10 µm*

By raster electron microscope one could observe the encapsulation around the empty defect by fibrous tissue (Fig. 6.4). In the photograph image of the control defect it is possible to see that the defect in five weeks is still not closed due to its overcritical size (Fig. 6.3). This could mean that the healing of the defect is continuing.

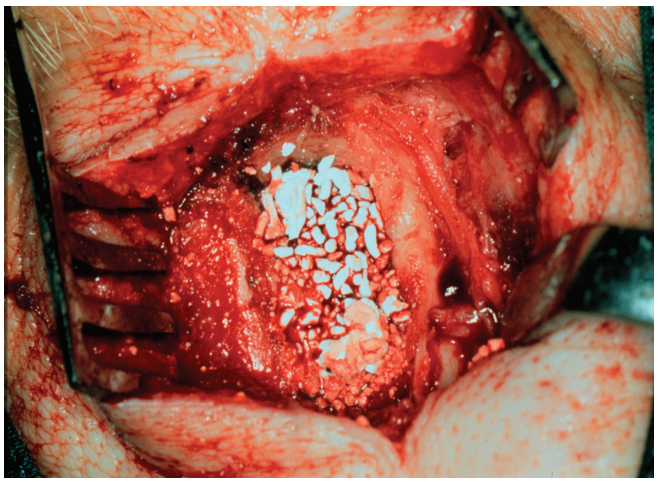


Figure 6.5: *Filling the defect with BCP ceramic and waiting for the material to be absorbed by the blood proteins, before closing the defect*

Before closing the defect, the surgeones had waited until the bioceramic has absorbed the blood proteins, as a necessary precaution against further complications. Figure 6.5 shows the process of filling the defect with the bioceramic particles, which after absorbing the blood proteins due to their high porosity became red.

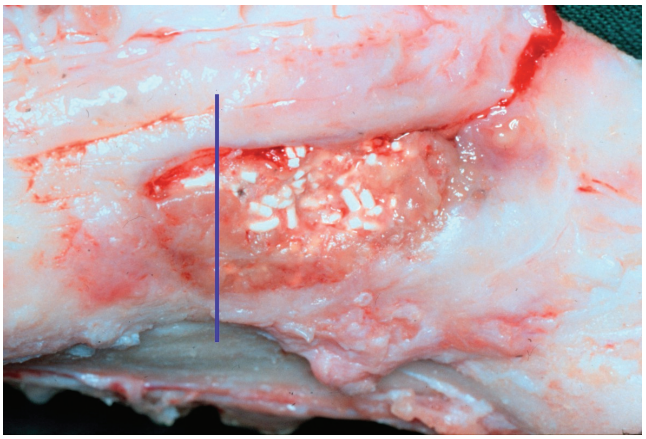


Figure 6.6: *Defect with BCP ceramic after 5 weeks. Note the presence of newly formed bone on the left and right site of the defect and still not resorped material in the middle. The blue rod indicates the place where thin histological sections were cut from defect*

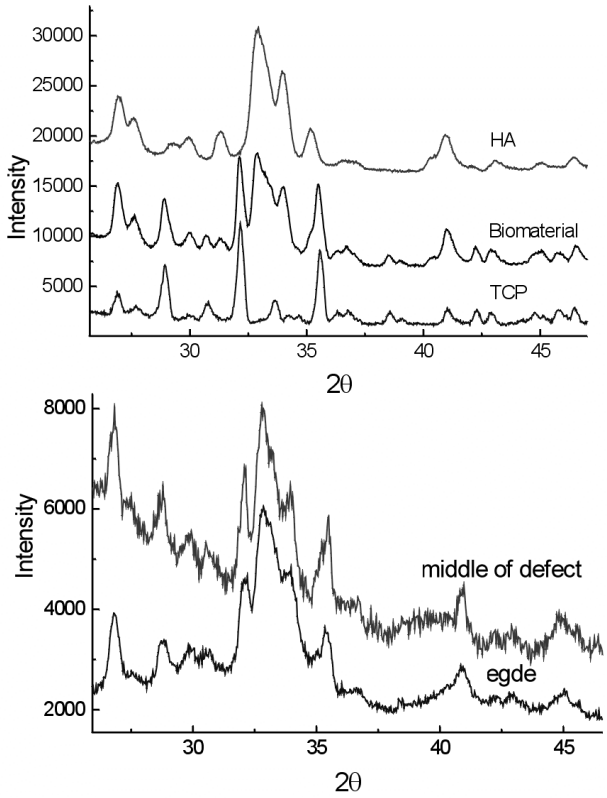


Figure 6.7: *X-ray diffraction pattern of the bioceramic before (upper part) and after5 weeks of implantation (lower part). Note the intensity of the correspoding peaks for TCP from the edge of the defect, which is lower compared with TCP peaks in the bioceramic. This is a result from fast ceramic resorption within five weeks*

Figure 6.6 presents the defect and the bioceramic, which was removed after five weeks. From the edge of the defect (blue rod on Fig. 6.6) thin sections were cut, which were afterwards used for the histological studies and for microscopical observations. An X-ray powder

diffraction taken from the implanted material after 5 weeks, shows almost 50 % decrease for TCP corresponding peaks, in comparison with their intensity from the bioceramic X-ray powder diffraction spectra (Fig. 6.7). The decrease of TCP peak is an effect of dissolution and it occured independently on the tissue ingrowth as can be seen on Fig. 6.7 (lower part), because there is very slight difference between the peak intensity taken fom the edge, where new tissue growth is faster and the one taken from the middle of the defect.

The histoligical cuts from the fifth week, registered the first results from the degradation of biphasic calcium phosphate ceramic and formation of new bone tissue. Figure 6.8 shows the resorption of our ceramic by employing Toluidine blue staining, which is a basic staining usually used in histological tests. The pieces of ceramic (A) are surrouned by bone (coloured in blue, B), which is in a very intimate contact with the material. The surface of the ceramic has a mouth eaten appearance, where the osteoclast activity is very high (C). Due to this osteoclast activity the ceramic material is undergoing resorption and consequent degradation. Figures 6.10 and 6.15 support as well the evidence for high osteoclast activity. Parallely to this, another change in materials´s state had occurred and this is its degradation (D), which is caused by material composition, and by the biological fluids which surround the bioceramic.

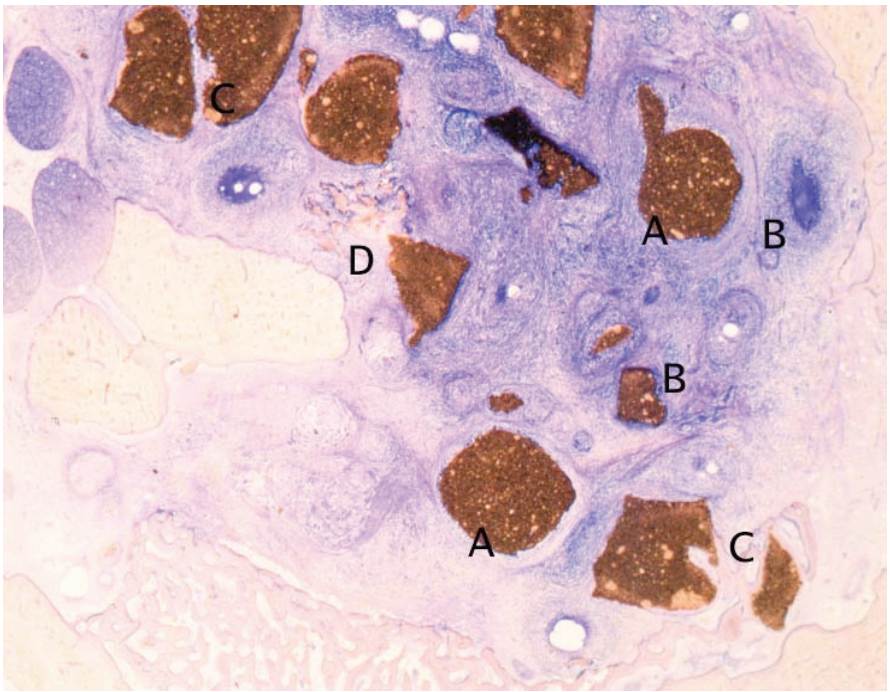


Figure 6.8: *Histological evaluation of the 5 weeks defect (Toluidine blue stain). The ceramic material (A) shows a mouth eaten appearance (C) and it is surrounded by bone (B)*

All our findings from the histological test can be confirmed by the results obtained with polarisation microscopy. In this examination, an embedded in glutaraldehid section from the edge of the defect (Fig. 6.9) is exposed to visible light under polarisation microscope, where according to light double breakdown, the different objects become coloured in dissimlar way. Figure 6.9 gives a typical view of the defect after five weeks, showing the ceramic piece surrounded by newly formed bone in the macropores, as well the very close contact between the bone and bioceramic. This is an evidence for direct bone apposition on the ceramic surface like nucleation (see the small blue dots over the ceramic material in Fig. 6.9).

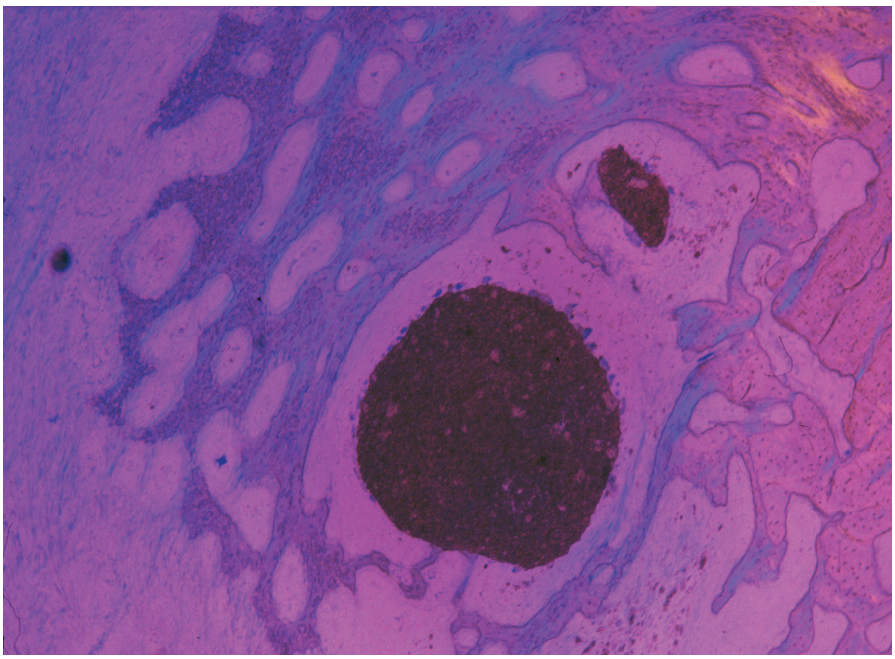


Figure 6.9: *Polarisation microscopy image of the bioceramic (in dark) showing the new bone formation directly on the ceramic surface (the small blue dots) and in the macropores*

Figures 6.10 and 6.11 are demineralized histological cuts stained with Hematoxylin Eosin staining. With the help of these images we can clearly see the process of material`s resorption. On Figure 6.10 the ceramic material (A) has a round shape, but in some parts became concave which is due to the osteoclasts activity (Oc). There is a very close contact between the ceramic and the bone matrix (BM), which after mineralization is turned into mature bone (B). The osteoblasts are also to be found (Fig.6.11, Ob), which cover the concave surface and are responsible for deposition of new bone.

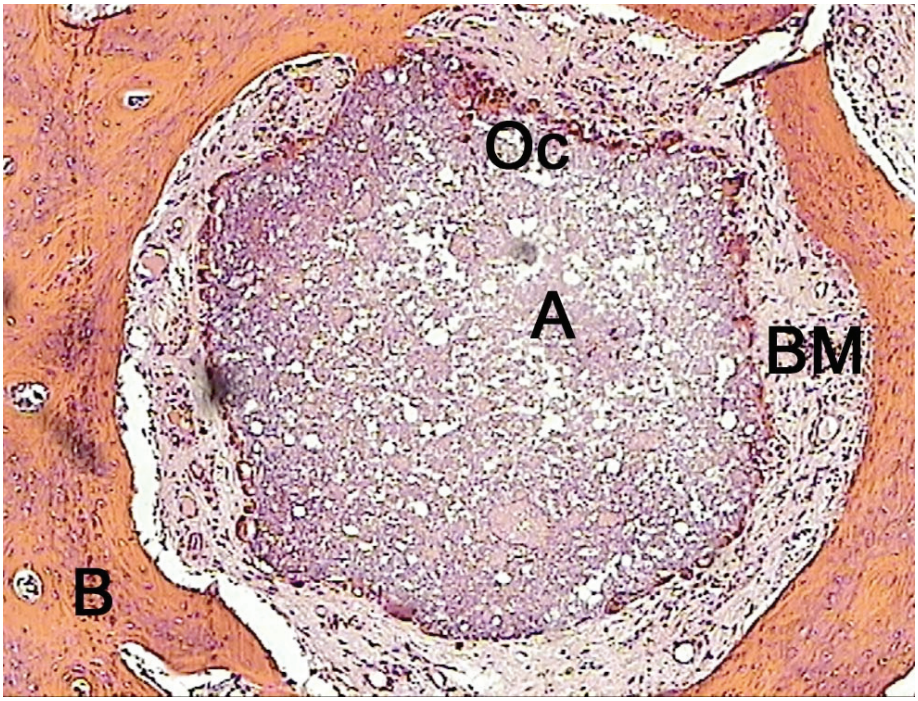


Figure 6.10: *Demineralized histolgical cut after 5 weeks, where A is the bioceramic, which is undergoing resorption by osteoclasts (Oc) with concequent formation of bone matrix (BM), which after mineralization is turning to matured bone (B), (Hematoxylin Eosin staining)*

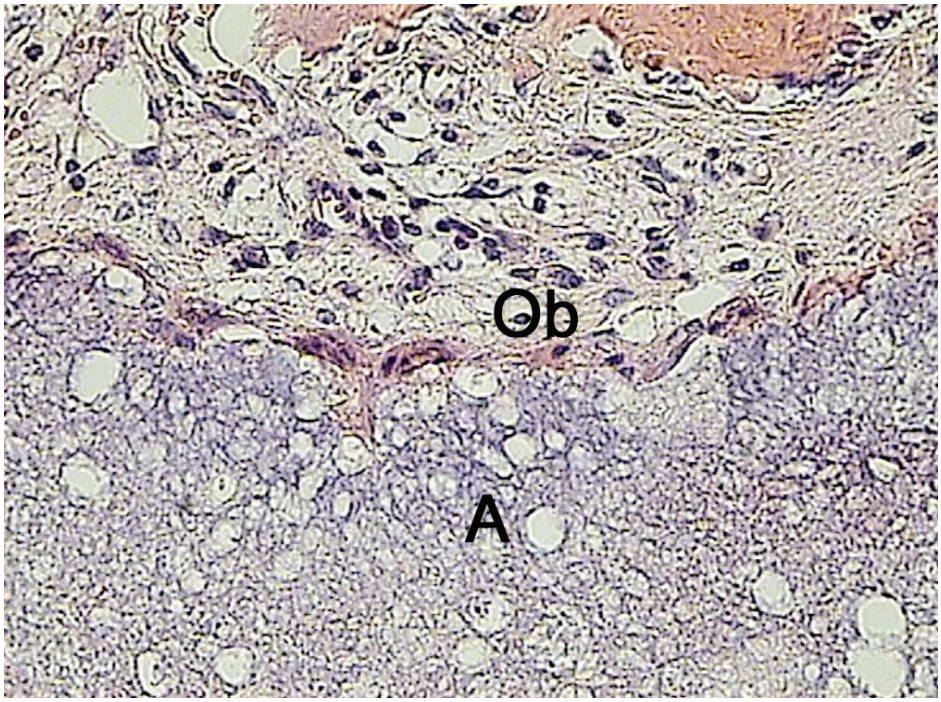


Figure 6.11: *Osteoblasts (Ob) lining the concave surface of bioceramic (A) (HE staining)*

The process of new bone formation in the macropores can be observed by staining the histological cuts with Goldner stain, which coloures the mineral component in dark green, and the collagen fibres in yellowish. Figure 6.12 is a cross-section of the surrounded by collagen fibres bioceramic (in green), and the trabecular structure of the remodelled bone. At this stage the orientation of collagen fibres is low, therefore they can be seen running in different directions, but in very close vicinity of the bioceramic.

Figure 6.12: *Lamellar bone structure around the bioceramic (in green) as seen with Goldner stain. The collagen fibres (coloured in yellow) run in different directions and around the bioceramic*

An enlarged fragment from Fig. 6.12 shows the mineralization of these collagen fibres (Fig. 6.13, the small green dots), which is connected with crystals formation (hydroxyapatite) and further maturation of these crystals to give rise to new bone. The collagen mineralization occurred as a front in direction from the edge of the defect to the middle. This is another proof that new bone tissue forms first at the edges of the defect and continue further throughout the whole defect.

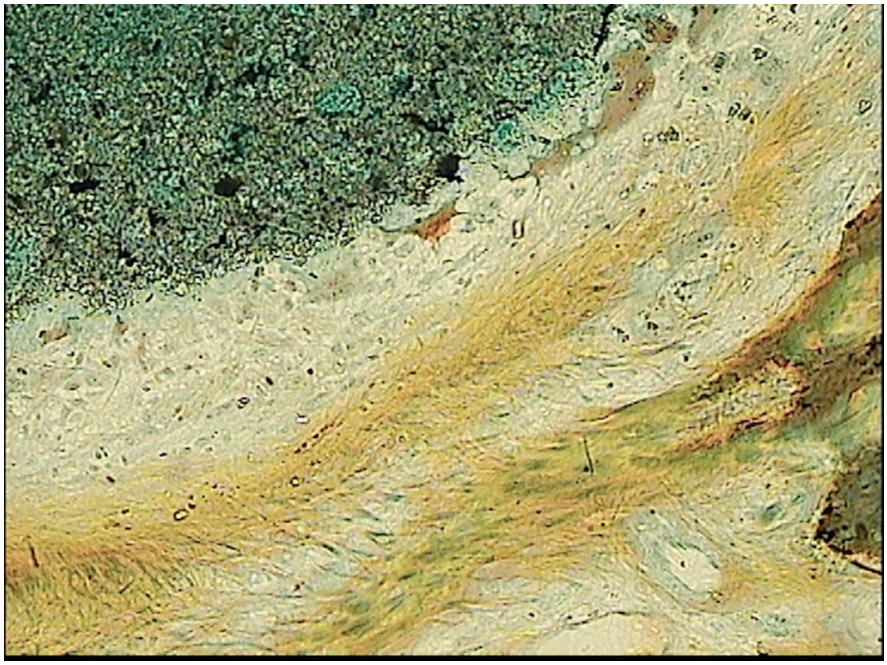


Figure 6.13: *Enlarged view from Fig. 6.1. showing the collagen mineralization (GS)*

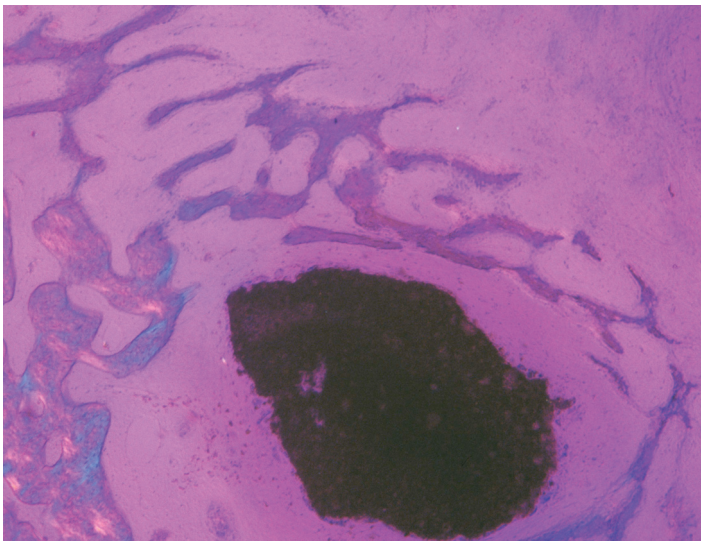


Figure 6.14: *Polarized light microscopy image indicating the process of material resorption and mouth eaten appearance left after osteoclast activity.*

The process of material`s resorption and degradation is accomplished by the activity of osteoclasts. These cells “digest” the ceramic material (and even the newly formed bone) by leaving a holes on material surface (Fig. 6.14). The multinucleated osteoclast cells invaded the material and as a consequense its round shape is destroyed (Fig. 6.15). At the same time, another object of the osteoclast activity is the new bone, which undergoes remodelling by the same manner as the bioceramic (Fig. 6.16). Basically, there is no difference in the osteoclast activity of the bioceramic and of the newly grown bone.

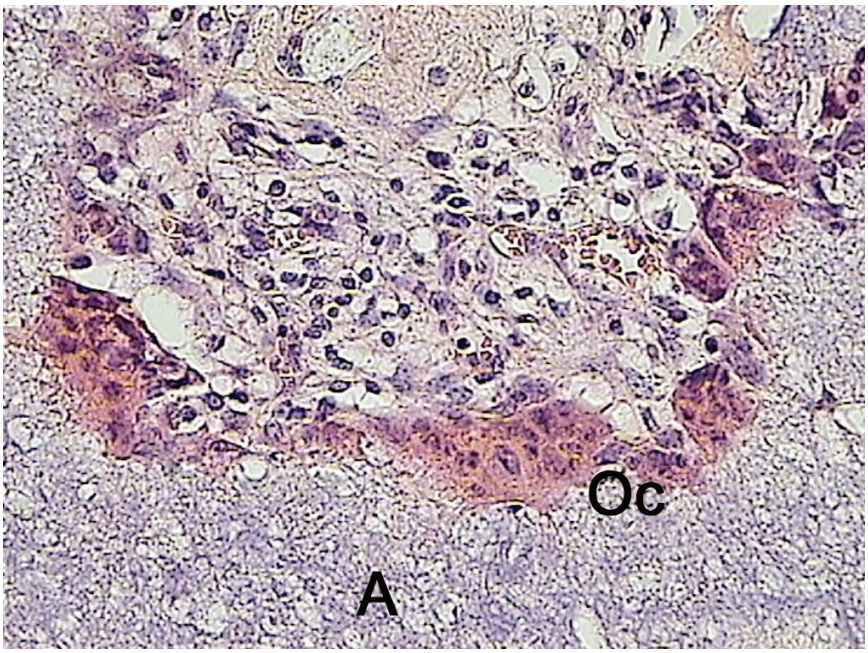


Figure 6.15: *Osteoclasts activivty (Oc) creates holes on materials`s surface (A), (HE staining)*

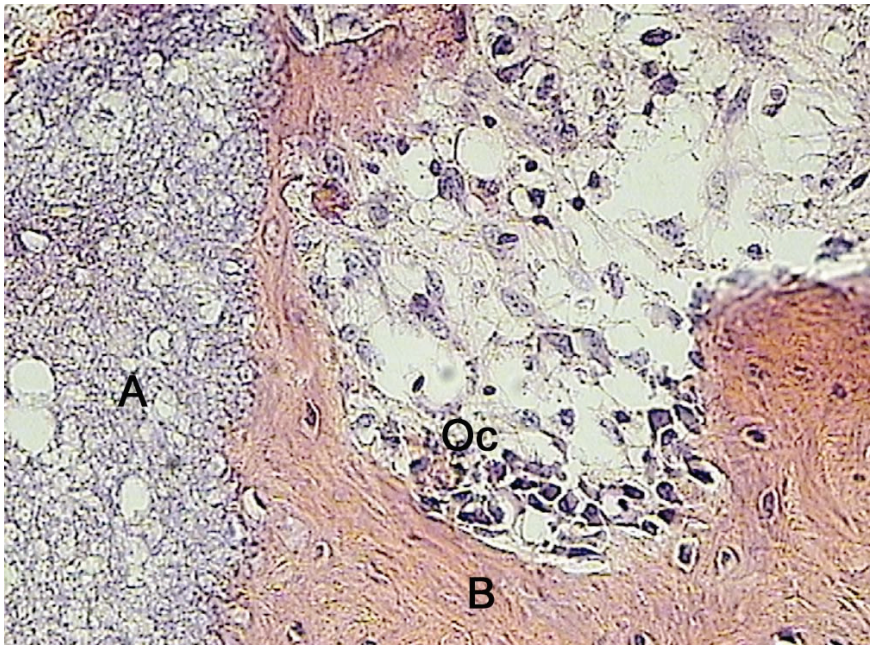


Figure 6.16: *Newly formed bone (B), which surrounds the ceramic (A) undergoes remodelling by osteoclasts (Oc), (HE staining)*

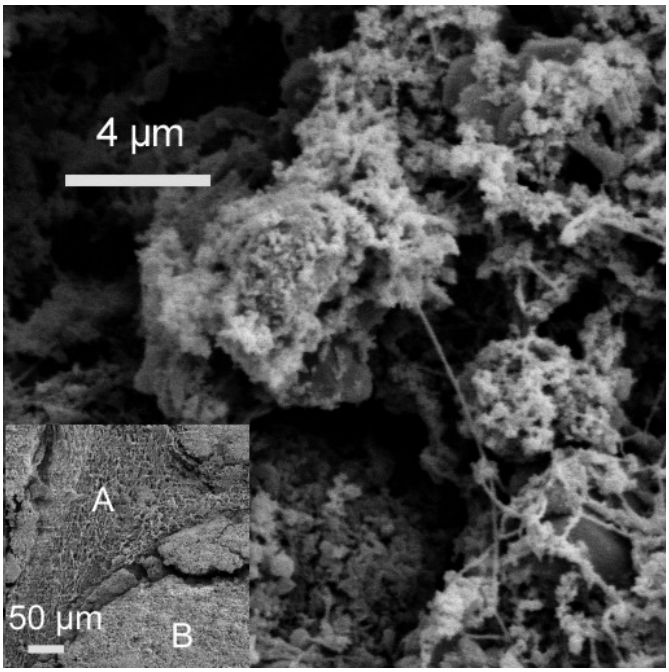


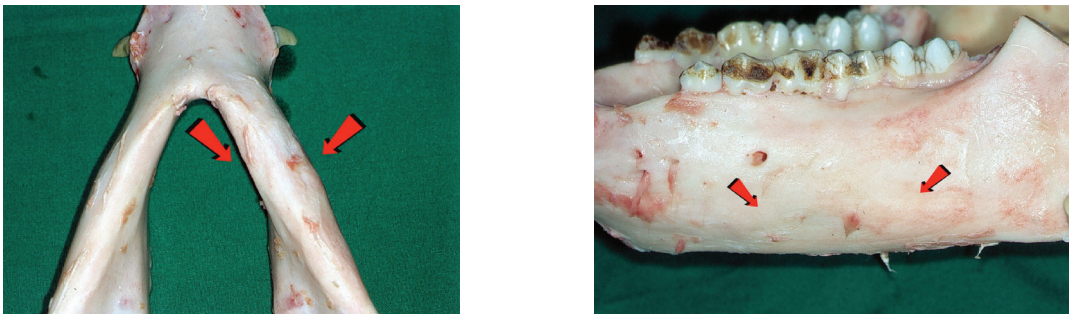
Figure 6.17: *Cross-section from the middle part of five weeks old defect, showing bioceramic (B) and tissue (A), SEM, scale bar 50 µm. At higher magnification collagen fibres undergoing mineralization filled up the micropores of the ceramic, scale bar 4 µm*

Figure 6.17 is a cross-section from the middle part of five weeks old defect, where the speed bone formation is low. These collagen fibres have permeated in the micropores of our bioceramic and are undergoing mineralization, which led to the formation of the biological hydroxyapatite crystals. Thus, the osteoclastic resorption of our material is very well expressed. The duration of these experiments is short, so that we cannot show whether an abundant new bone has been formed. It is well apparent that in five weeks the ceramic degradation has started, which proves to be with high surface activity. Therefore, short-term study is important for giving us information about:

1. Ceramic degradation and the way it occured
2. New bone formation as a sequence of collagen mineralization
3. Presence of two types of bone formation
4. Correlation between material degradation and bone remodelling

6.3 Results from long-term in vivo study (eight months)

In the previous paragraph it was mentioned that the degradation of our ceramic materials *in vivo* is a cell-mediated process, i.e. it is governed by the osteoclastic activity. The purpose of the long-term examination period is to show whether there is a healing of the artificially created bone defects (in the mandible and tibia), and whether the bioceramic is degrated and substituted by newly formed bone. Briefly, the surgical procedure was performed in the same way as the short- term period, with the only difference, that in this study in six of the animlas in their right prixmal tibia mono- and biphasic calcium phosphate silica-based ceramics were introduced additionally. For the eight months tests, a total of 12 mini pigs were used, of which 6 received BCP ceramic in the mandible, and 6 had received HA ceramic in the mandible. In eight of these pigs an additional defect in the right proximal tibia was created, where two of the operated pigs had received HA ceramic in the shin bone, and other six pigs had received BCP ceramic in the shin bone. The duration of this testing period was eight months, after which all the pigs were killed and thin sections from the implantation sites were cut in order to be studied with histological and microscopical methods. Figures 6.18 illustrate the mandible after 8 months, presenting very well clinically healed defect. The site which had received the bioceramic (marked by arrows) is thicker than the site without defect (Fig.6.18a), but there is no visible sign of the medical intervention (Fig. 6.18b).



a) *b)*

Figure 6.18: *Image of the mandible defect in 8 months: a) underneath view showing that the left site with defect is thicker, but clinically healed; b) direct view from defect place with no visible marks on the bone*

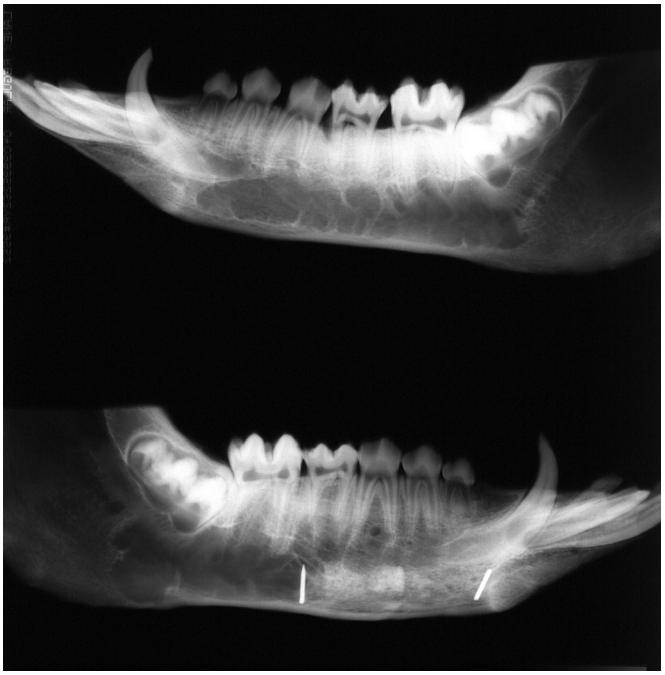


Figure 6.19: *Roentgenographical image of the mandible in 8 months: the upper part is the right part of bone where no defect was created, and the lower is the left bone part, with two titanium rods, locating the place of the defect.*

On Figure 6.19 the defect in the mandible is shown roentgenographically after the animal had been killed. The two white titanium rods on the lower jaw are locating the position of the defect. When compared with the roentgenographical image of the same animal taken postoperatively (Fig.6.20), one could think that in 8 months the material can be still found in the defect and that its degradation did not take place, which is not true. The histological cuts (Fig. 6.21, 6.23) show clearly, that the ceramic is fully resorpted.

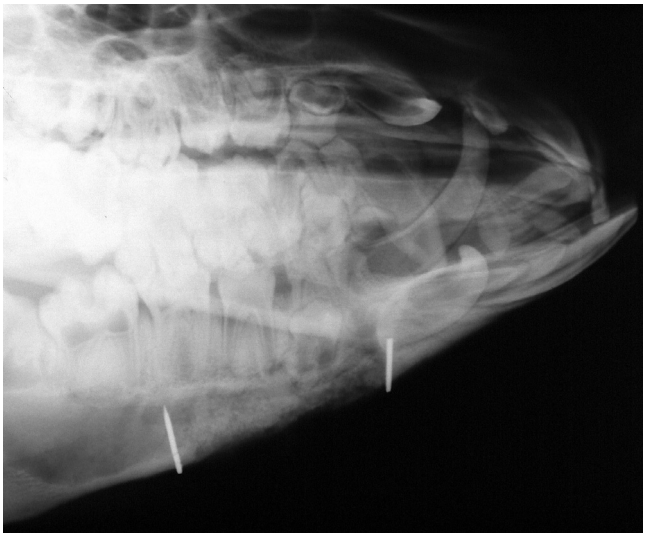


Figure 6.20: *Roentgenographical image of the mandible taken postoperatively. Two titanium rods indicate the place of defect, filled with BCP ceramic*

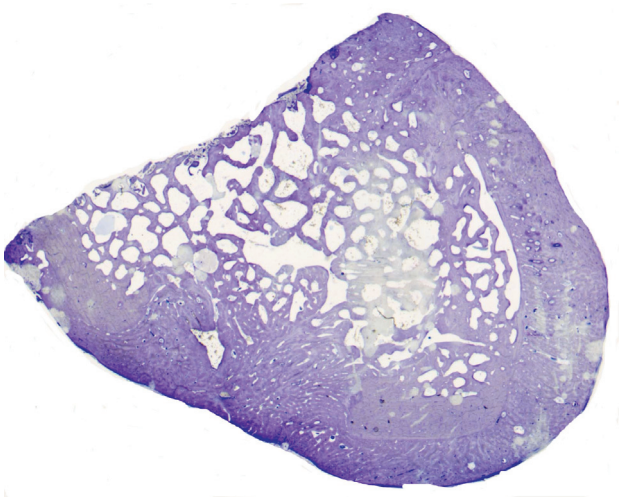


Figure 6.21: *Histological cut from the mandible 8 months after the operation, showing a completely resorpted bioceramic and clinically healed defect (Toluidine blue staining)*

The histological results confirmed that in eight months the bioceramic was fully resorpted and at the place of medical intervention a new trabecular bone was started to be formed. From Figure 6.21 one could see that the stucture of the newly formed bone is cancellous which is different from the old cortical bone. The defect is consisting mainly of this cancellous bone, while the old bone is involved in the remodelling process.

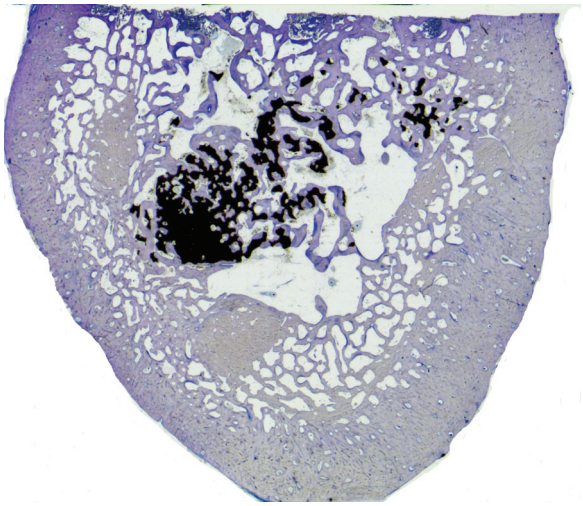


Figure 6.22: *Defect in the mandible in 8 months, presenting the worst case from all histological cuts of all tested animals, (Toluidine blue staining)*

The process of material degradation and resorption is not equally expressed in all of the operated animals. As an evidence we show another histological cut, which contain more ceramic material and its presenting the worst case of this study. (Fig. 6.22). This result only confirms that the acceptance of the material and its subsequent degradation is an individual process, which can vary between the testing animals. But it also could be due to manufacturing problems, where instead of “spaghetti”species we had gotten clusters, which degradation is slower for absence of macropores. In some animals, the histological cuts present less material (Fig. 6.23), which undergoes degradation and it is involved in the bone formation. Some statistic has been made to compare the results after 8 months, between HA and BCP ceramics implanted in the mandible. According to it, HA ceramic has been found to remain only 0.8 vol.% in the histological tests, while BCP ceramic is 3.4 vol.%.

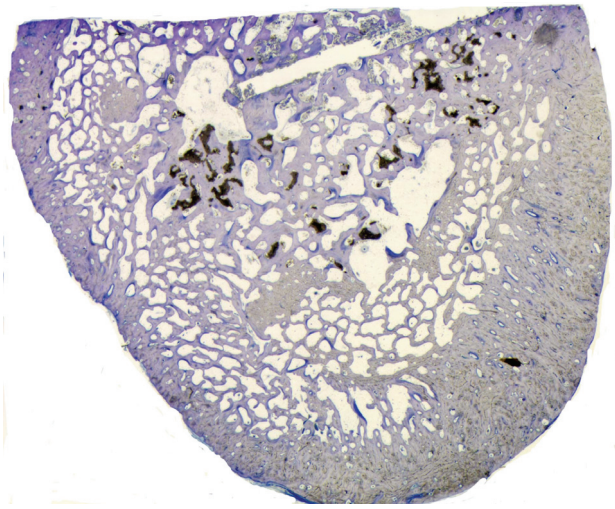
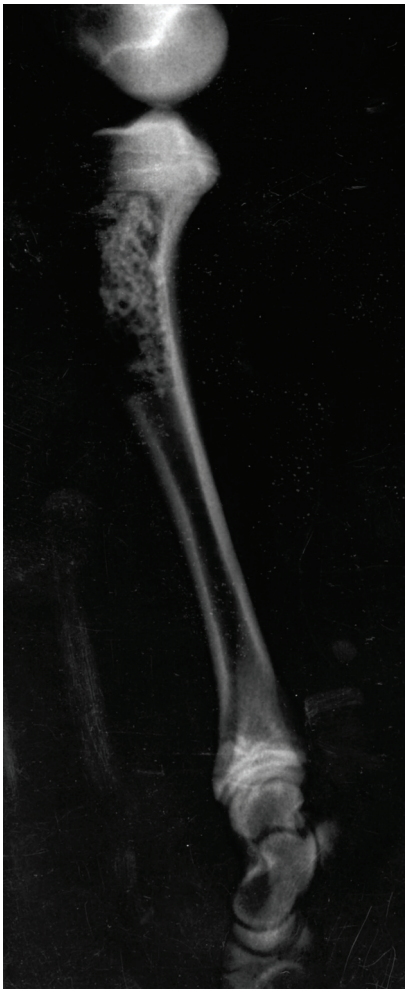
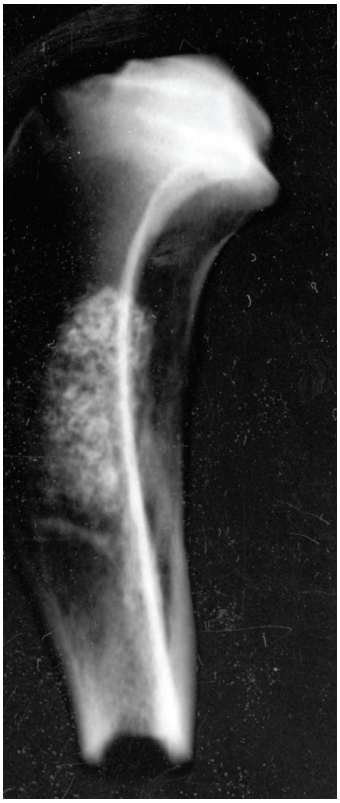


Figure 6.23: *An 8 months defect in the mandible with small leftovers from the bioceramic, which are involved in the remodelling process (Toluidine blue staining)*



a)



b)

Figure 6.24: R*oentgenographical image from the right tibia filled with the bioceramic (the top part of image a) taken after the operation; b) in 8 months the defect seems to be the same as after the operation. Note that the presence of some material is actually the trabecular bone, which is formed after the ceramic is being resorpted*

When the ceramic material was implanted in the tibia no requirements regarding the mechanical stability were completed. The surgeones even did not put an external fixations, which is an usual procedure in long bones surgery. It has been shown in Chapter 4 that the mechanical strength of our ceramic heated to 600°C remains almost the same, and has a values between 2,5 and 3,7 MPa, which is not sufficient for load-bearing applications. Although, the results obtained from the tibia confirmed the possibility of using our ceramic samples even in load-bearing sites with no preliminary fixations of the opeated place. Figure 6.24 present the roentgenograpical images of the tibia defect immediately after the operation (Fig. 6.24a) and in eight months (Fig. 6.24b). Here again we see a remained ceramic after 8 months, which leads to the misunderstanding that this ceramic has not been resorpted. In fact, the looking like-material appearance in Fig. 6.24b is the spongy bone, formed as a result of ceramic degradation.

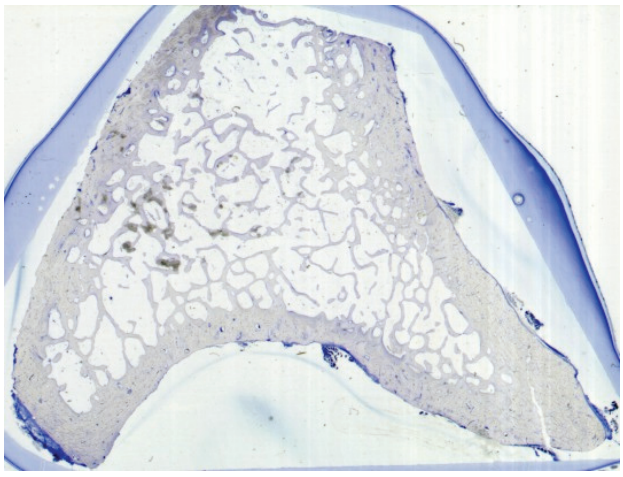


Figure 6.25: *Histological cut from the tibia defect in 8 months. Some small remainings from the ceramic are to be seen (Toluidine blue staining)*

Figure 6.25 shows a nice result from tibia defect, where the bioceramic is fully resorpted and involved in the bone formation. Note that the thickness of cortical bone in the tibia is less than the thickness of cortical bone in the jaw bone. This on one hand can be a reason for faster remodelling and formation, but on the other hand the posibility for complications in tibia is higher compared to the mandible. In Figure 6.25 the newly formed bone has almost the same thickness as the original host bone, which is an evidence for good material degradation and nondisturbed bone formation. In the practice, implantations in the long bones are followed by external fixations to prevent occuring of fractures. Such an external fixations had not been done in the tibia implantations, thus, some of tested animals had gotten fractures (Fig. 6.26). As a result deformation of tibia occurred, which was related to complete degradation of our ceramic material as shwon on Figure 6.26. The fracture has caused on other site mechanical

disorder, which forced the process of bone remodelling and repairing with the faster resorption of our ceramic.

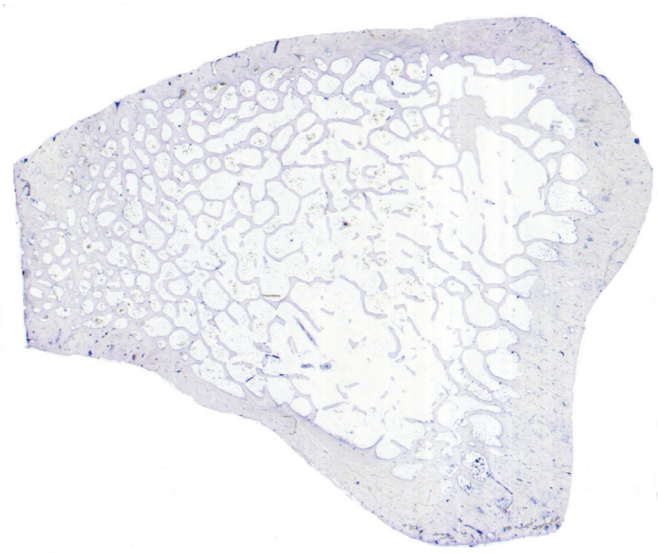


Figure 6.26: *Tibia defect in 8 months with fracture. Note the changed shape of the bone and the absence of bioceramic, which was resorpted faster in order to support the mechanical load (Toluidine blue stainig)*

In the tibia defect like in the mandible, in some cases there are evidences for higher presence of the ceramic graft bone material in the defect (Fig. 6.27). This should not be considered as a bad result, because in all these cases, a spongy bone is found around the bioceramic, showing the continuity of material resorption and bone formation.

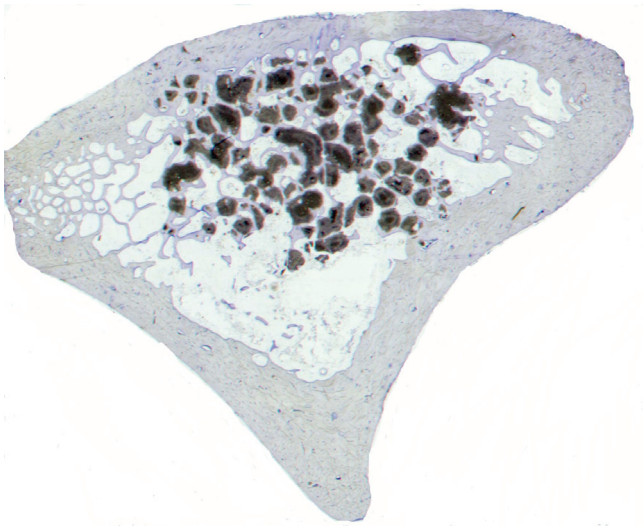
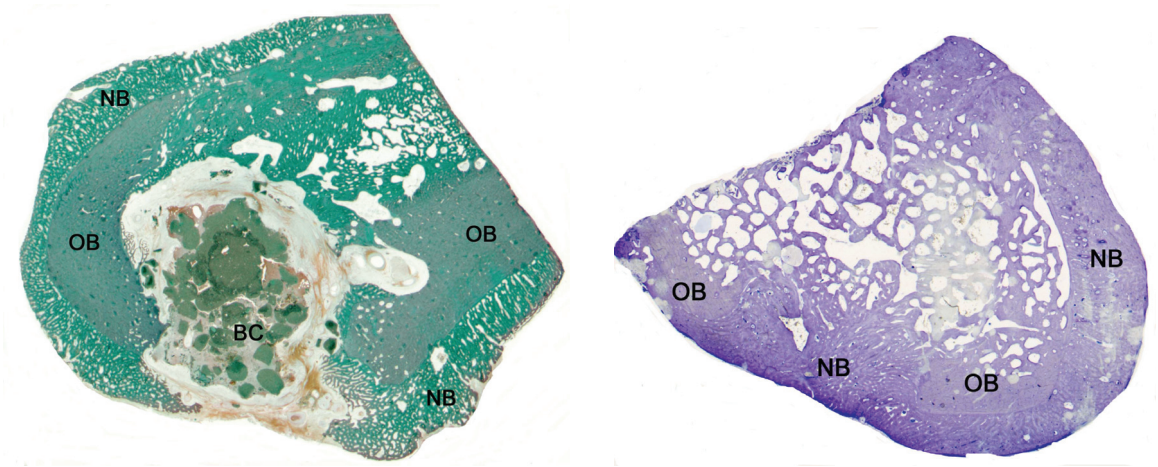


Figure 6.27: *Tibia defect after 8 months presenting the worst case of material degradation. The presence of spongy bone around the ceramic particles (in black) is a result for undergoing resorption and remodelling (Toluidine blue staining)*

If we compare how the process of bone healing and material degradation has been developed throughout the duration of long-term experiment we will find the following picture. In five weeks the bioceramic (Fig. 6.28a – BC) is present in the overcritical in size defect, coloured with yellowish green in the Goldner staining specimen. This is the organic part (collagen

fibres) of the newly formed bone. In the so created defect, due to its big size the activity of bone remodelling and bone formation processes is very high. The old host bone (OB), which is coloured in dark green is undergoing resorption and is slowly removed by the new bone (coloured in lighter green, NB). Thus, its role in supporting the mechanical stabiliy of the bone is overtaken by the new bone, which is forming at the periost in order to stabilize the mechanical function of mandible. The newly created bone at the periost, as well as in the whole defect, has a different structure from the host bone. As stated previously the new bone formation cannot be completed in five weeks, therefore in the beginning of our study we could find this structure difference. In parallel, the bioceramic macropores are permeated with collagen fibres, which differentiation guides the process of new bone formation. What is more important is the development of this bone formation within time, so that in eight months the defect in mandible has a disimilar appearance. Figure 6.28b proved, that after 8 months the ceramic graft material is resorpted and the precense of old bone is decreased, because of the undergoing remodelling. The defect is filled with trabecular bone, which is the result from the destroyed mechanical stability.



a) *b)*

Figure 6.28: *Evolution of the new bone formationi in 5 weeks on a), where there is a very clear difference between the new (NB) and old bone (OB), (Goldner staining); b) shows the completely healed defect, which is mainly consisting of the newly formed bone (NB), while the old bone (OB) is involved in remodelling, (Toluidine blue staining)*

6.4 Discussion of the results from *in vivo* study

The mineralization and deposition of the newly formed bone occures in the vicinity of the ceramic material, which is a very good proof that the ceramic is involved in the degradation-mineralization-bone formation processes. On Figure 6.15 one can notice the surrounded by the multinucleated osteoclasts (Oc) biomaterial (A), which are “digesting ” it by formation of bone matrix around it (Fig. 6.10, 6.12). As a result of the osteoclast activity, the ceramic became degraded into Ca and P ions, which are necessary for the deposition and mineralization of the bone matrix. The osteoblasts are lining on the concave material surface (Fig. 6.11), and due to the enzyme alkaline phosphatase the mineralization is attended. The formation of a seam (osteoid) around the bioceramic as shown in Fig. 6.10, is intimately related with the formation of the new bone, as it is consisting of collagen fibrils and fibres, which will mineralize to give the mineral component of the new bone matrix (Fig. 6.12). The relative speed of formation and mineralization of the osteoid determines its width. Its believed that if the supply with Ca and P ions is enough for the process of bone matrix (or osteoid) deposition, then its mineralization will be faster. In a naturally bone deposition and remodelling process, the suppy with Ca and P ions comes from the blood proteins, and in the case of foreign material which is inserted in the bone defect, Ca and P ions can be supplied as a result of materials dissolution. Thus, we believe, that according to the nice results which were shown from the histological study, in 5 weeks mineralization of new bone matrix occurs relatively fast, which is a proof for degradation of the implanted ceramic, for high activity of osteoclasts and osteoblasts, which “work” is not disturbed by the presense of calcium phosphate silica-based ceramic.

The resorption activity of osteoclasts is considered with the presence of island-like or track-like resorption lacunae on the surfaces of ceramic materials as reported by Yamada *et al.* (51). Depending on the ceramic surface properties and solubility, these resorption lacunae (or mouth eaten appearance on the ceramic surface) cannot be observed on each calcium phosphate ceramic surfaces. As it is very difficult to synthezise stoichiometric hydroxyapatite, CaO are usually found in commercial products. As the CaO impurities are very hydrosoluble, they may induce the formation of similar lacunae. In fact, those lacunae as shown by Soueidan *et al.* (54), are not due to cellular activity, but only to dissolution of CaO impurities.

Enlarged image (Fig. 6.15) gives better view from the activity of osteoclasts, which due to the secreted collagenase enzyme, are able to degrade the ceramic by forming a cave-look like appearance on its surface. Paralelly with the resorption of the bioceramic, there is also resorption of the host bone by osteoclasts (Fig. 6.16). We believe, that this result is very good, because it shows that the osteoclasts practically do not "make" any difference between the host bone and the ceramic material, and the resorption of both happen in the same way. In other words, the process of natural bone resorption and deposition is not affected by the presence of our implanted ceramic. However, from a material standpoint, we believe that characteristics, such as crystal structure, packing between the crystals, determine the resorptive ability of the osteoclasts to resorb HA and BCP silica-based ceramics.

According to our findings we can state, that the bone formation occurs in two ways: one is direct bone formation on the ceramic surface (Fig. 6.8, 6.9) and bone formation in the macropores of the biomaterial (Fig. 6.12, 6.13, 6.17). These results had proven once again that, our ceramic materials are able to promote both types of bone formation, which up to now has not been reported by anybody else. Tracy *et al.* (55) have reported that, the interface between hydroxyapatite cylindrical plugs implanted into rat femurs and bone showed a very intimate attachment of the bone to the hydroxyapatite implant, with no intervening tissue. After indicating the crystal type of bone close to ceramic interface, was posible to show that it was of the same size and shape as in the normal bone.

In Chapter 2 we have discussed the structure of bone, bone formation and bone remodelling processes. It was stated, that the bone as dynamic living tissue undergoes remodelling throughout life, which results in the continual formation of interfaces between new bone and older bone tissue. This bony interface is represented by a distinct histologic structure called the cement line and which is a mineralized, collagen-free matrix approximately 0.5 μm thick. While bone is known to interface with itself by the mechanism of the above mentioned cement line formation, it would follow that bone will interface with artificial materials by creation of different structures. The interface occurring between calcium phosphate-based materials and bone was also explained as an amorphous electron-dense appearance (56). Since all materials, used as bone graft substitutes are considered as biocompatible, one can assume that they have no major damaging effects on bone cell differentiation or, that cell differentiation and matrix elaboration will not be significantly influenced by the presence of the implant material. Each implanted material has a different surface reactivity, thus, some materials are considered to be with low surface reactivity, while others have high surface reactivity. Materials of low surface reactivity have shown to form the

cement line at bone-material interface. But what would happen with materials of high surface reactivity? Will they form a “better” bone-material interface? The differences therefore, will be in the degree of material surface reactivity. Some investigators (57) have rated calcium phosphate-based materials according to their bioactivity in the order: hydroxyapatite, apatite-wollastonite glass ceramic (AW-GC), Bioglass (BG). All these materials have formed a reactive apatite layer on their surfaces, which is variable and dependent on the supply of requisite ions. Thus, the thickness of the reactive zone would be expected to be greater with the more reactive material. Kitsugi *et al.* (58) have found that the reaction interfacial zone occurs in three steps: a) dissolution of the surface, making the ceramic irregular; b) Ca-P rich layer formation on the irregular ceramic surface; c) bone growth toward the smooth surface of Ca-P rich layer, completing bone-bonding. With our nice results we have shown already that, the surface of designed bioceramics is very active, therefore bone formation occurred directly on it, without the presence of such an interfacial zone (Fig. 6.9, 6.11)

The findings from our animal tests with the usage of HA and BCP silica-based ceramics, revealed that in 8 months in the jaw bone the bioceramic is fully degraded and resorpted, which led to the formation of a new bone (Fig. 6.19, 6.21). A photograph image of the jaw defect after 8 months, proved that it is clinically healed and the only difference between the right site of the jaw (no medical intervention) and the left one, which accepted our biomaterial, is in its thickness (Fig. 6.18). Basically, no other dissimilarity between the right and left site of the jaw can be found. As a misunderstanding can be interpreted the fact, which is seen on Figure 6.19 and 6.20. These roentgenographical images taken after 8 months (Fig. 6.19) and immediately after the operation (Fig. 6.20) are showing, that perhaps there is no difference between the state after 5 weeks and after 8 months. On both pictures one could see a certain amount of material, which is filling the cavity of the jaw bone. A logical quention is arising after the comparison between both images: Is there a resorption of the bioceramic, since it is possible to detect its presence in the defect after 8 months?

Mandible is the strongest bone of the face, which serves for the reception of the lower teeth. It consists of a curved, horizontal portion which is called the body, and two perpendicular portions, called rami. The body of the mandible is built up of cancellous bone, enclosed within a layer of compact bone. The middle part of the body has a cylindrical cavity, filled up with marrow which contain the bloodvessels (59). When the mandible is subjected to roentgenographical light, the middle part of the body looks like an empty channel. On Figure 6.20 the two titanium rods are put to determine the location of the defect, which is filled with our bioceramic. But still, on Figure 6.19 there is something in the defect, which looks like the

same ceramic. To the best of our knowledge, this is the trabecular bone formed after the bioceramic has been degraded within the duration of this study. The spongy (or cancellous) bone undergoes resorption around an implant, when a material is drilled into the compact bone. The amount of resorption is dependent upon the kind of the implant material and the surgical procedure. For hydroxyapatite and some other ceramics, the resorption of the cancellous bone is practically zero, due to the higher bioactivity of these materials. When the bioceramic is completely resorpted it is followed by deposition of a new bone, which is more compact than the surrounding spongy bone. Therefore, what we observed on Figure 6.19 is exactely the spongy bone remaining after the ceramic`s degradation and undergoing its own resorption to be remodelled and turned up to compact bone. Very similar case we reported 8 months after the bioceramic has been implanted in the shin bone (Fig. 6. 24b). Thus, with the above stated discussion we clarified the misunderstandings related to the roentgenographical pictures, which otherwise could lead to wrong interpretation of our results. Different case has been reported by Koshino *et al.* have used sintered HA blocks with 35-48% porosity for the treatment of patients with osteoarthritis. They have implanted the ceramic blocks below the upper end of the tibial plateau of patients aged between 66 and 71 years. After 36 months of implantation, the biopsy had shown that the density of HA was maintained, which in other words means that no absorption of HA had taken place, although they observed bone ingrowth into the pores of HA blocks progressively with time (60). For our study we can be satisfied with the obtained results, because the newly designed and advanced bioceramics had proven good biodegradibility and ability to support new bone formation.

Due to the equal number of pigs, which received HA and BCP ceramics in their lower jaws, it was possible to make statistical explanation of the results. According to it, hydroxyapatite silica-based ceramic had shown slightly better results and remained only 0.8 vol. % in the samples (e.g. nonresorpted), while for biphasic calcium phosphate silica-based ceramic this value is 3.4 vol.%. The small difference between the remainings of HA and BCP ceramics in the final samples can be neglected. Although, it can be explained with the composition of the both ceramics. In HA ceramic the silica percentage is 24 wt. % and the dry substance is 76 wt. %. In BCP ceramic the silica is 16 wt. %, and the dry substance 84 wt. % (from which 34 wt. % is TCP phase and 50 wt. % is HA phase). So, in HA ceramic there is higher silica content than in BCP. It has been shown by many researchers that silica plays an important role in bone formation and calcification (25, 61, 62, 63). Our aim thus, was to develop a material, which consists of certain amount of silica, and which at the same time is osteoconductive. Gibson *et al*. have proved that silica-substituted HA influences the process

of osteoconduction when compared with stoichiometric HA. At the implant interface on Si-substituted HA, the direct bone apposition exceeded in thickness the same on the stoichiometric HA implant (61). One way to explain the higher reactivity of Si-HA is through the decrease in grain size in the Si-HA compared to pure HA. These findings are particularly important, since grain boundary structure has been shown to have a predominant influence on the dissolution behaviour of biological apatites. Thus, the decrease in grain size and increase in surface area of the grain boundaries in Si-HA may be playing a significant role in increasing the solubility of HA and the subsequent rate at which bone apposes HA ceramic (62). Similarly, our results have shown less presence of degrated HA silica-based ceramic after 8 months in the biopsies, compared to BCP silica-based ceramic.

Many authours have reported bone formation around different materals, which were tested *in vivo.* What is interesting to be remarked is that the speed and quantity of bone formation of Bioglass (BG) were 8 times greater than AW-GC and 17 times greater than hydroxyapatite in the first week after implantation (63). They explained the obtained results as dependent on the amount of silica in the composiion of the material. Bioglass contains more silica than apatite-wollastonite glass-ceramic, and even more than hydroxyapatite. We assumed, that the fast mineralization and bone formation after 5 weeks of implantation in the mandible of mini pigs, are most probably due to the high level of silica in our biphasic calcium phosphate silica-based ceramic.

Another comparative study between TCP, porous HA and natural coral implants in surgical defects in mandibular bone of pigs led to the conclusion that, in all cases, bone formation started from the bony walls and progressed towards the implanted particles. The structure and the composition of these biomaterials had varying but important roles in bone conduction. Their influence depends on the experimental model, the site of implantation and the delay after implantation (64). Regarding our *in vivo* study we could state similar with the literature findings, which prove the above mentioned. Only in five weeks time we observed almost 50 % decrease of the TCP peaks intensity, which is well shown on Fig. 6.7. By comparing an X-ray diffraction pattern taken from the edge of the defect with diffraction pattern of the bioceramic, it is clear that the intensity of the correspondng TCP peaks is different. This leads to the conclusion that, the resorption of our bioceramic (via dissolution of the TCP portion) started soon after its implantation, and is almost the same at the edge of the defect and in the middle of defect (Fig. 6.7-lower part). TCP dissolution has shown to happen independently on the new tissue growth.

A common characteristic of calcium phosphate biomaterials is their osteoconductivity, i.e. a guided bone formation on a biomaterials surface and formation of a chemical bonding between the newly formed bone and biomaterials. Recently, it has been found by many researchers that calcium phosphate biomaterials can also induce bone formation, i.e. they are osteoinductive. The osteinductivity is reported for coral-derived hydroxyapatite ceramic, synthetic hydroxyapatite ceramic, biphasic calcium phosphate ceramic, tricalcium phosphate ceramic, calcium pyrophosphate ceramic and calcium phosphate cement. As bone conduction by calcium phosphate biomaterials is both material- and animal dependent, in the same manner bone induction is material- and animal–dependent. Yuan et al. have reported good results for the bone-induction of hydroxyapatite ceramic in soft tissues, but not for tricalcium phosphate ceramic. They explained their findings to be related to the higher solubility of TCP in comparison with HA ceramics, which in the case of bone-induction in soft tissues can be harmful. Thus, these authors concluded that resorbable calcium phosphate biomaterial is not recommended for bone induction (65). Another study by the authors (64) revealed information that, when the TCP, porous HA and natural coral implants have been implanted in extraskeletal sites, non of them was able to induce bone formation, i.e. HA (in dense and porous form), TCP and natural coral are not osteoinductive. The difference between these results is to be found in the nature of the materials used, in the implantation site and animal model.

SUMMARY AND CONCLUSIONS

Bone is the second most frequently transplanted tissue in humans. In the United States alone, it is estimated that over 250 000 operations per year are performed that requires bone grafts. The golden standard for graft material is autogenous bone graft, due to its excellent biocompatibility, nontoxicity and high osteogenic potential. However, its limited amounts and the complications related to the surgical procedure upon removing, are very big disadvantages for surgeons and patients all over the world. Therefore, bioceramics have been created during the 60`s and 70`s of twentieth century as a result of new demand for materials to improve the quality of life. This new demand concerns the innovative use of specially designed ceramics for the repair and reconstruction of diseased or damaged parts of the body. Our work represents a small attempt to contribute to the field of bioceramics: how to improve their tissue bonding quality and to understand tissue bonding mechanisms.

We have designed two different calcium phosphate ceramics, which contain a certain amount of silica. One is called hydroxyapatite silica-based ceramic and contains 24 wt % silica, and the second one is biphasic calcium phosphate silica-based ceramic with 16 wt % silica. The biphasic calcium phosphate (BCP) silica-based ceramic consists of both hydroxyapatite (HAP) and tricalcium phosphate (TCP). Hydroxyapatite is found in the mineral part of hard tissues, therefore it became a material of choice in the field of biomaterials. Tricalcium phosphate on the other side has shown to be highly biodegradable. Many researchers have proven that silica has a positive role in the mineralization process, which makes it a suitable additive for medical purposes. Hence, we have produced in our laboratories a novel bioceramic which had overcome the latest inquiries for highly soluble, porous and osteoconductive biomaterial, and may succesfully be used as bone grafting substitute material.

Our HAP and BCP silica-based bioceramics were manufactured by mixing calcium phosphate powders with sol-gel-derived silica in an ultrasonic bath. After ripening the mixture for a certain time, its viscosity was checked in order to find the optimal time for shaping the ceramic. Via extrusion, the ceramic samples were shaped in spaghetti-like form. This form optimally allows the loose packing between the crystallites, therefore the bioceramics posseses a high level of porosity in the range 60 % to 80 %. From the material point of view,

high-porosity can be considered a disadvantage when aiming at load-bearing applications. However, one of the factors presumed to govern biodegradation is the degree of porosity. Our bioceramic materials have a well expressed porosity in nanometer range, due to the silica gel acting as a matrix; in micrometer range, after removing the organic part with heating; and in milimeter range due to the unique spaghetti-like shape.

Another risk factor which influences the porosity level, but strengthens the mechanical properties is sintering. We did not sinter our ceramics, for preventing thermal decomposition which could occur. The results obtained with X-ray powder diffraction of specimens exposed at different temperatures, had shown that temperatures higher than 1000°C are always related with decomposition of mono- and biphasic calcium phosphate ceramics to other phases, most often to beta-tricalcium phosphate. Therefore, to preserve the phase purity and the starting composition of the material we do not recommend heating at temperatures higher than 1000°C.

Calcium phosphate ceramics are considered fragile, i.e. they cannot be used for load-bearing applications. After testing the compressive strength of our specimens heated at different temperatures, we have found out that until 600°C the strength remained the same: in the range 2.5-3.7 MPa. When heating at temperatures up to 1200°C, the strength changes drastically to 50 MPa, which is much more than the compressive strength of cancellous bone (1.86 MPa). As stated already, high mechanical strength is always reached after sintering, however this leads to thermal decomposition of the specimens. Since the mechanical stability was not an aim to achieve, we believe that mechanical strength of 2.5-3.7 MPa is sufficient for the purposes of our application. The material characteristics of both ceramics (HA and BCP) like porosity, shrinkage, thermal decomposition and compressive strength have shown similar results, although the ceramics have slightly different chemical composition.

The biological performance of our ceramic samples was investigated at the Department for Oral and Maxillofacial Surgery and the Department for Orthopaedics at University of Rostock. Sixteen, adult Goettinger mini pigs were used for the animal tests. Four pigs were used as a control group into which empty defects without material were made. In the other twelve animals, defects in the left lower jaws and in the right proximal tibia were created. These defects had an overcritical size of 5 cm^3. The *in vivo* test was divided into two periods: a short-term five weeks period and a long-term eight months period. The short-term test period was performed in the mandible of six pigs using both ceramic types. The purpose of the short-term *in vivo* study was to observe material degradation and subsequent bone formation. Since calcium phosphate ceramics are bioactive, their degradation is done by the

activity of osteoclasts, which was observed for our ceramics. In five weeks we found out that 50 % of the TCP corresponding peak from the biphasic ceramic decreased, a process independent on the tissue in-growth. The result proved once again the good choice of combining the more soluble tricalcium phosphate with hydroxyapatite, more favourable to bone formation. New bone formation usually occurs either around the biomaterial, or directly on the material surface. Our study found evidence for both types of bone formation when mono- and biphasic calcium phosphate ceramics were implanted in the mandible or tibia of mini pigs.

The long-term *in vivo* study had shown complete resorption of the bioceramic implant and abundant bone formation at the place of medical intervention. In the tibia defect, for example, where no mechanical requirements were accomplished, the process of bone formation and remodelling had been going on throughout the entire test period. Here it is worth mentioning, that in agreement with several other research groups, our results support the idea that mechanical strength of calcium phosphate bioceramics improve rapidly *in vivo*, due to bone ingrowth.

Some statistical procedures have also been carried out. According to these, there is a very slight difference between the remaining mono- and biphasic ceramics in the mandible after eight months. Leftovers from hydroxyapatite have been found to be 0.8 vol.%, while 3.4 vol.% biphasic calcium phosphate ceramic remained nondegraded. Similar statistics concerning the tibia were not performed, due to the unequal number of tested animals treated with hydroxyapatite and biphasic calcium phosphate silica-based ceramics. Nevertheless, on both sites of medical intervention (mandible and tibia) a complete new bone formation and remodelling with the involvement of our ceramics has been demonstrated. Thus, we believe that both hydroxyapatite and biphasic calcium phosphate silica-based ceramics have a very promising future as high-performance bone graft substitute materials.

The good results mentioned in this work have given a background for clinical applications, which have started already. In parallel, we will continue working on improving the mechanical stability of these bioceramics and on optimizing their manufacturing process in order to launch the bioceramics on the market. We are proud to be a part of such an interdisciplinary project, aiming to increase the quality of human beings life by designing new state-of-the-art bioceramic materials.

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Part A - List of Figures

Part B – List of Tables

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Erklärung

Hiermit versicherte ich, dass ich die vorliegende Arbeit selbständig angefertigt und ohne fremde Hilfe verfasst habe, keine außer den von mir angegebenen Hilfsmittel und Quellen dazu verwendet habe und die den benutzten Werken inhaltlich und wörtlich entnommen Stellen als solche kenntlich gemacht habe.

Rostock, den 22.02.2003