Chapter I

Introduction

1.1 Bone

Bone is a natural composite material, which by weight contains about 60% mineral, 30% matrix and 10% water (Athanasiou *et al*. 2000). Bone is also a living tissue, with about 15% of its weight being due to the cellular content (Lodish 1995). The matrix of bone is comprised primarily of Type I collagen that is highly aligned, yielding a very anisotropic structure. This organic component of bone is predominantly responsible for its tensile strength. The mineral component of bone is a form of calcium phosphate known as hydroxyapatite (HA). Stoichiometric HA has the formula $Ca_{10}(PO_4)_6(OH)_2$**,** and adopts a hexagonal geometry with the unit cell crystal dimension 9.42 Å in the a and b directions, and 6.88 Å alongthe c-axis (Ong and Chan 1999). However, bone mineral is rarely stoichiometric, containing many substitutions such as magnesium, sodium, potassium, fluorine, chlorine, and carbonate ions (Guyton 1991). The apatitic mineral in bone is closely associated with the collagen fibers and is made up of long, flat, plate-like nanocrystals that are approximately 40 nm long, 10 nm wide and 1-3 nm thick (Guyton 1991). This mineral component gives rise to the compressive strength of bone. In the body, bone serves a number of functions, such as providing the cells, found in the marrow, that differentiate into blood cells, and also acting as a calcium reservoir (Guyton 1991; Yaszemski *et al*. 1996). Nevertheless, its primary purpose is to provide mechanical support for soft tissues and serve as

an anchor for the muscles that generate motion. There are two types of bone, compact (cortical) and cancellous (trabecular) also known as spongy bone (Figure 1.1). Compact bone is very dense, consisting of parallel cylindrical units (osteons), and is found in the shafts of the long bones as well as on the outer surface of the smaller bones in the body. Trabecular bone is less dense and is made up of an array of rods and struts that form an open-cell foam, the pores of which are filled in by marrow. This type of bone is found at the ends of the long bones and inside the smaller bones (ribs and spine) (Athanasiou *et al*. 2000; Yaszemski *et al*. 1996). The anisotropic structure of bone leads to mechanical properties that exhibit directionality. This directionality results from the fact that bone has evolved to be both tough and stiff, two competing properties which are optimized in bone but with an inherent loss in isotropy (Currey 1998). Nevertheless, bone exhibits extraordinary mechanical properties, displaying both viscoelastic and semi-brittle behavior (Zioupos 1998; Athanasiou *et al*. 2000).

1.1.1 Bone Structure and Function

Bone structural integrity is essential to skeletal function. The human skeleton (Figure 1.2) is made up of 206 named bones that account for about 20% of body mass (Weiner and Wagner 1998).

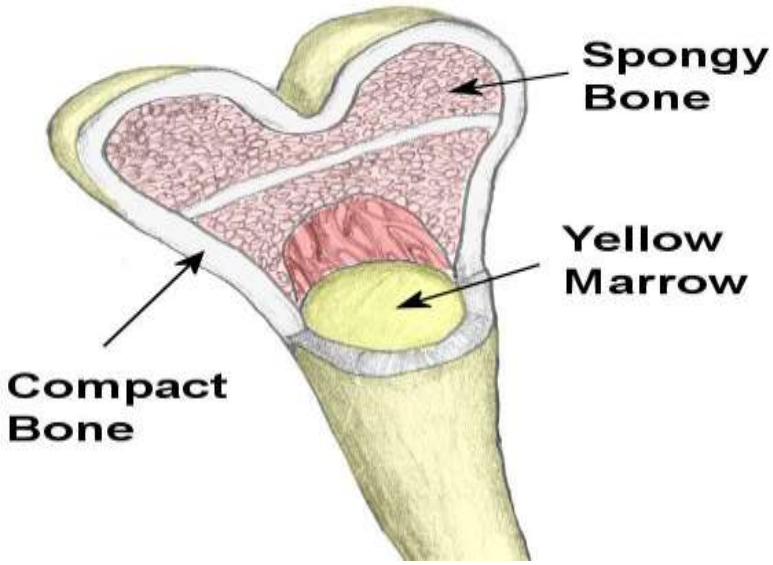

Figure 1.1: The structure of bone. The spongy bone (trabecular bone) surrounds the yellow marrow, and then the compact bone (cortical bone) surrounds the trabecular bone. http://www.teachpe.com/

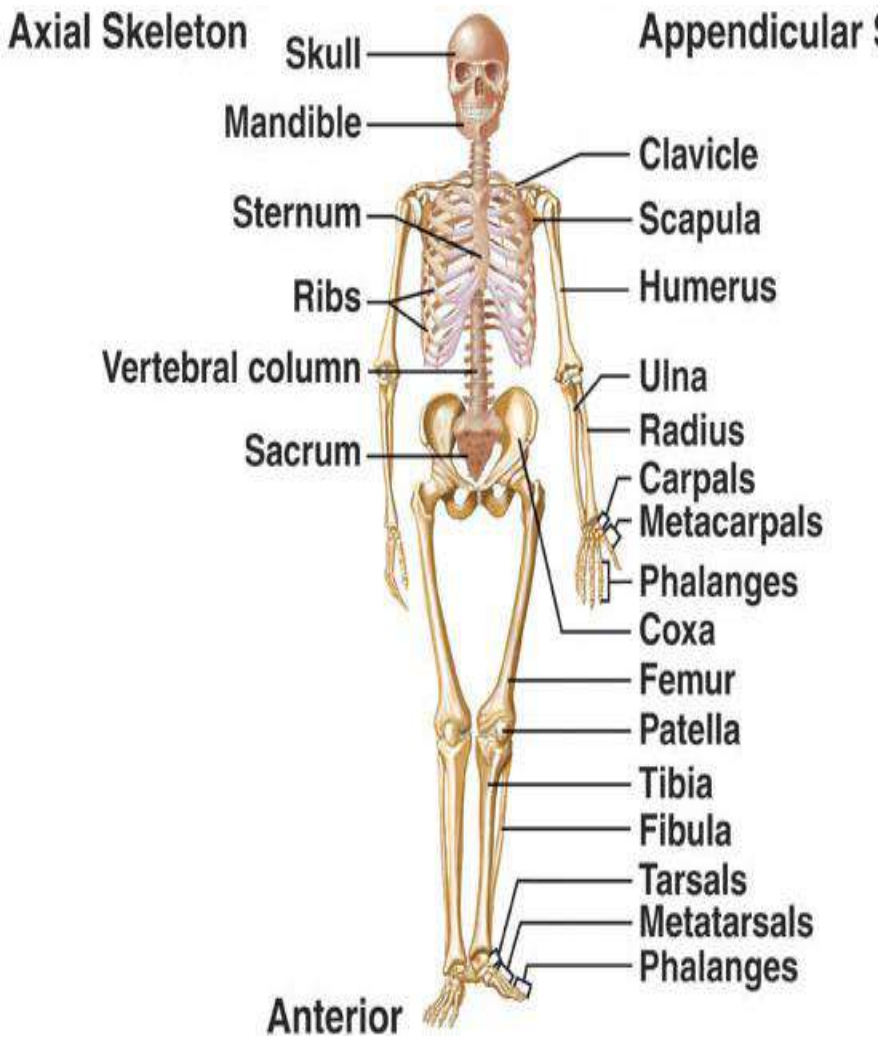

Figure 1.2: The human skeleton. Replicated from Adam Healthcare. http://www.ijces.com/

A bone is composed of two main parts, the cortical bone and the trabecular bone. Each presents different mechanical properties and different structures. The cortical bone also is called compact bone whereas the trabecular is sometimes called

cancellous or spongy bone (Weiner and Traub 1992) (Figuer 1.2) shows that the cortical bone surrounds the trabecular bone, and that the yellow marrow is at the center of the bone.

The cortical bone represents 80% of the bone mass and allows the protection of the internal part of the bones (spongy tissues). This part is much denser, with porosity ranging between 5% and 10%. The trabecular bone represents 20% of the bone mass and it consists of the bones raw materials. There are three types of cells inside the bone tissue: the osteoblasts, the osteoclasts and the osteocytes. The spongy bone is much more porous than the cortical bone, with porosity ranging between 50% and 90% (Polly 2008). The volume fraction of the trabecular bone is smaller than the volume fraction of the cortical bone since the compact bone is denser (Table 1.1) in comparison the values of the surface/volume and the total bone volume, trabecular bone has a greater surface than cortical bone. The structure of the bone is divided into different levels, as shown in (Figure 1.3) Level 1 represents the basic building blocks of the bone which form an organic matrix (Sharir, Barak, and Shadar 2008; Polly 2008). At a higher length scale, the bone is composed of mineralized collagen fibrils arranged in regular staggered array (levels 2 and 3). Level 4 represents the structural types; it is the sub-microstructure scale (lamellae). These lamellae are arranged in several ways to form cylinders which contain the blood vessels and the nerves (level 5). Then, the next levels are at the microstructure scale, the compact and trabecular bone forms the whole bone.

Table 1.1: Structural features of bones. University of Michigan BME/ME 456 Biomechanics (Jee 1983).

	Cortical	Trabecular
Volume fraction (mm^3/mm^3)	0.90 (0.85 – 0.95)	0.2 (0.05 – 0.6)
Surface / volume of bones (mm^2/mm^3)	2.5	20
Total bone volume (mm^3)	$1.4x10^6$	$0.35x10^6$
Total internal surface	$3.5x10^6$	$7.0x10^6$

Figure 1.3: The structure levels of bones (Sharir, Barak, and Shadar 2008).

1.2 Classification of Bone

1.2.1 Classification by Structure

Primary bone tissue (non-lamellar bone) is also known as ‘coarse fibred’ or ‘woven’ bone or immature bone. It is characterized by the presence of randomly oriented coarse collagen fibres clearly visible by polarisation microscopy. Non-lamellar (woven) bone is seen in the bones of foetuses and young children. It is the osseous tissue first deposited on the calcified cartilage matrix in endochondral ossification. It is also the first tissue to appear in the repair of bone (fracture healing) (Nather, Ong, and Aziz 2005).

Secondary bone tissue (lamellar bone) is also known as mature bone. It is characterized by the presence of collagen fibres arranged in parallel layers or sheets (lamellae) readily apparent when viewed by polarisation microscopy. Lamellar bone is present in both structured types of adult bone, cortical (compact) bone and cancellous (spongy or trabecular) bone (Nather, Ong, and Aziz 2005).

Cortical or compact bone is made up of a structure of Haversian systems or Osteons (Figure 1.4). Each Haversian system or osteon is a cylinder running parallel to the long axis of the diaphysis. In the centre of each osteon is the Haversian canal which is lined by endosteum containing blood vessels, nerves and loose connective tissue. Surrounding each canal are 4–20 concentric lamellae of collagen fibres. The Haversian canals are round or oval in cross-section. They generally run in a longitudinal direction. Each osteon communicates with the marrow cavity, the periosteum and with each other through transverse or oblique canals the Volkmann's canals (Figure 1.5). The osteocytes are arranged circumferentially around the central

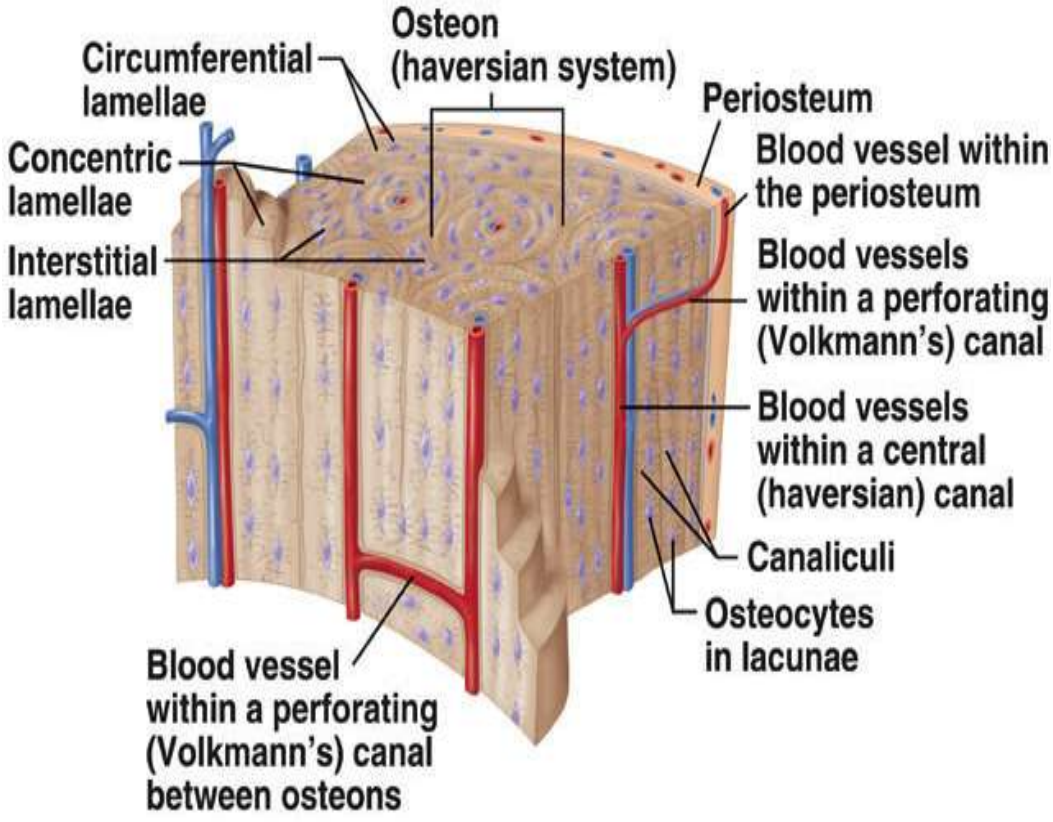

Figure 1.4: The structure of a long bone.

(http://nihroadmap.nih.gov/initiatives.asp).

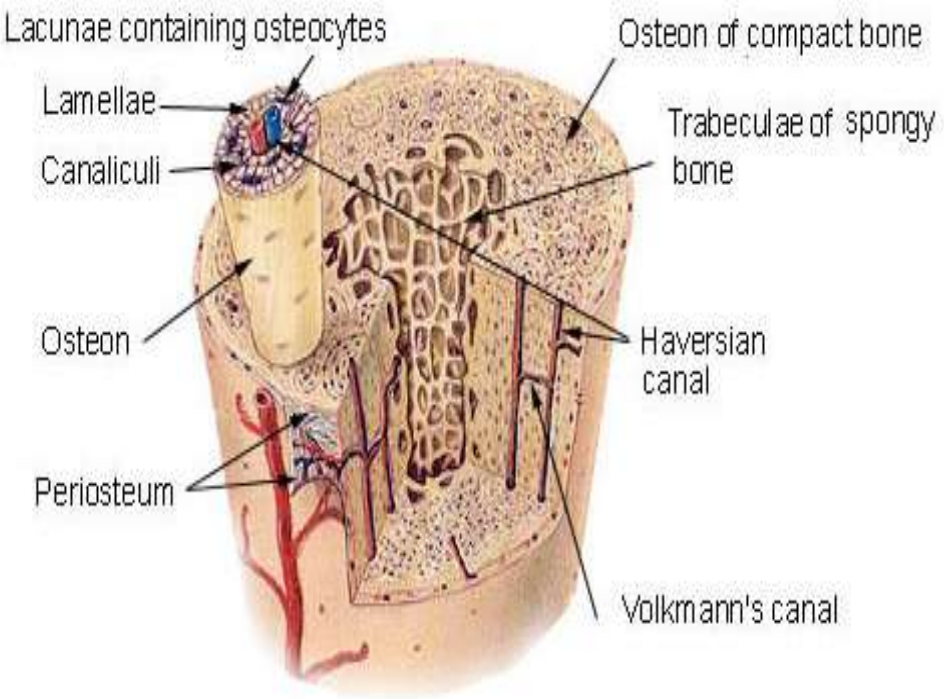

Figure 1.5: Compact bone consisting of cylindrical units (Nather, Ong, and Aziz 2005).

canal in parallel with the lamellae and are interconnected by fine processes of osteocyte Haversian canal in its center and Surrounded by a cement line. In between the Osteons are the interstitial lamellae (Nather, Ong, and Aziz 2005).

Bone matrix is organic matter consisting of type I collagen fibres embedded in the ground substance containing proteoglycans and glycoproteins. The collagen fibres are made up of bundles of fibrils to resist pulling forces and inorganic matter which is made up of stiffening substances to resist bending and compression.

The bone mineral is an analogue of crystals of calcium phosphate hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ which is a substance that can only be seen under electron microscopy. It is the association of hydroxyapatite with collagen fibres which is responsible for the hardness of bone (Chipchase, Mccaul, and Hearn 2000). Cancellous or spongy bone consists of a series of interconnecting plates of bone. Each bone trabecular contains collagen fibres arranged in parallel lamellae. The surface of the trabecular is covered by an attenuated layer of flattened cells, the resting osteoblasts. Such a structure, in addition to providing a large surface area for metabolic activities of bone, gives mechanical strength without the disadvantages of undue weight. The thickest and strongest trabecular is arranged in the direction subjected to the greatest stress (Wolff's Law) (Nather, Ong, and Aziz 2005).

1.2.2 Classification by Shape

Bones can be classified by their shape as long, short, flat or irregular, as shown in (Figure 1.6). The long bones such as the femur (Figure 1.7) are tubular in structure, and consist of the following regions:

Diaphysis: The central shaft of the long-bone is called the diaphysis. It is located between both metaphysis, consists of compact bone walls, and has a hollow medullary cavity that is filled with yellow bone marrow in adults, and red bone marrow in children. The external surface of the diaphysis is covered by the periosteum.

Epiphysis: The rounded end or head of a long-bone that consist of mostly cancellous bone covered by a relatively thin layer of cortical compact bone.

Metaphysis: The body of cartilage that separates the epiphysis and the diaphysis. Epiphyseal plates (growth plates or physes) are located in the metaphysis and are responsible for growth and lengthening of the bone during childhood.

At roughly 18 to 25 years of age, the metaphysis stops growing altogether and completely ossifies into solid bone.

Diametaphysis: The region between the metaphysis and the diaphysis, where the bone shaft begins to widen and curve. Short bones such as the finger bones, wrist and ankle bones, and the patella have a similar structure to long-bones, except that they have no medullary cavity. The Flat bones in the skull and ribs consist of two layers of compact bone with a zone of cancellous bone sandwiched between them. Finally the irregular bones are bones such as the vertebrae and pelvis, which do not fit into any of the previous categories (Chipchase, Mccaul, and Hearn 2000)**.**

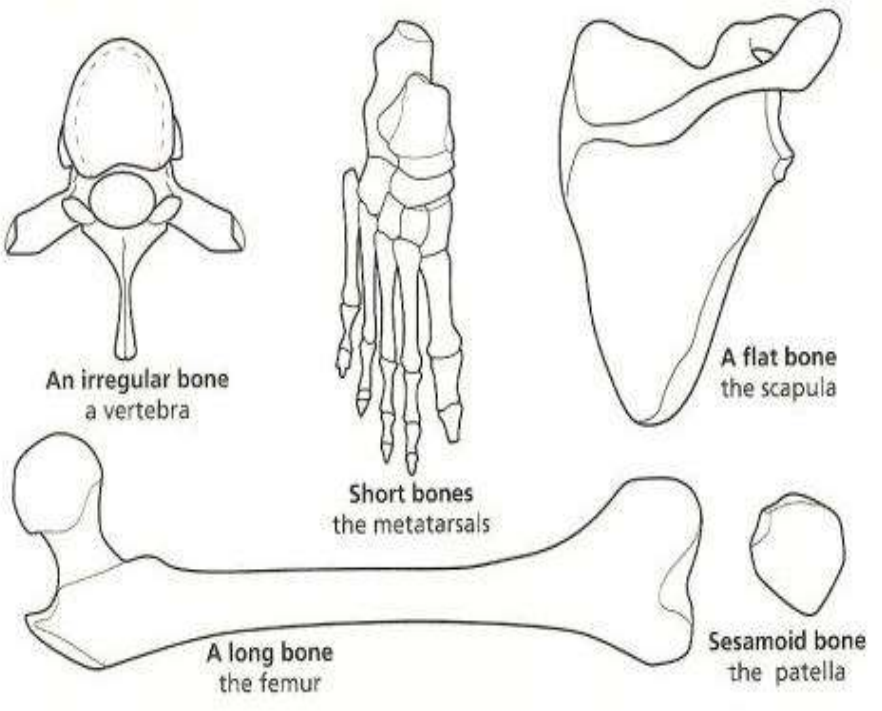

Figure 1.6: Classification of bones based on shape, (ADAM Health care Center, 2006).

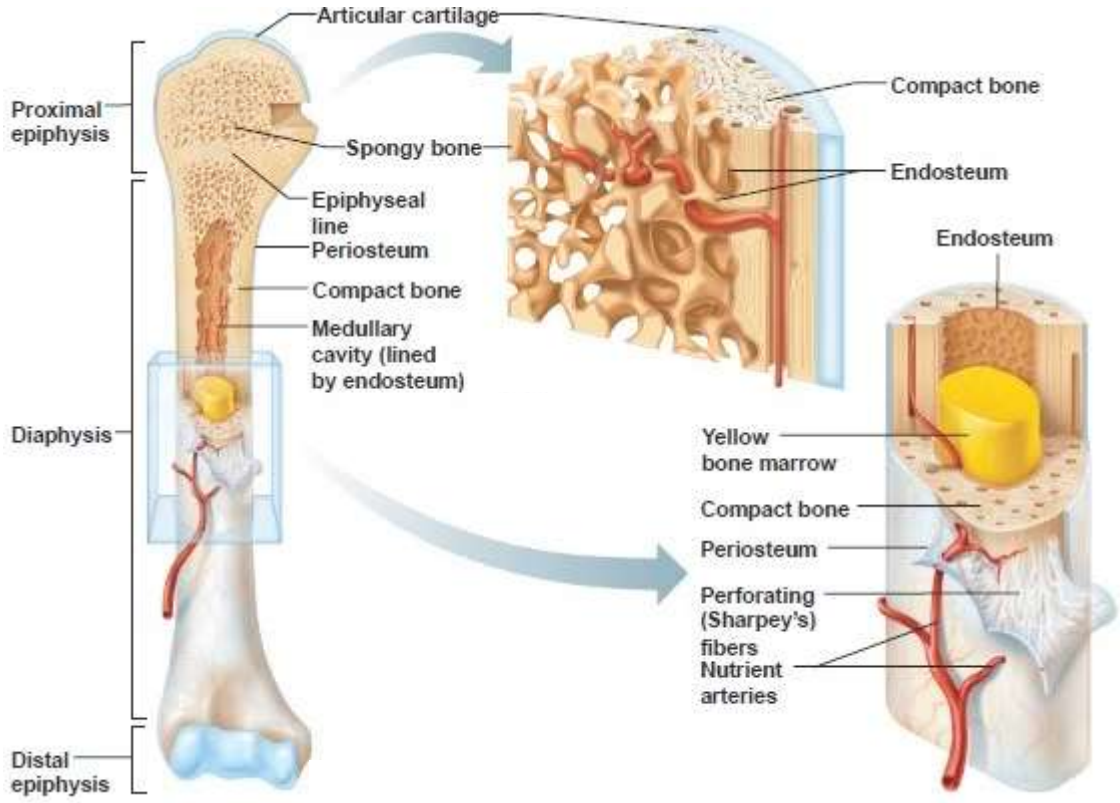
Articular cartilage
Proximal epiphysis
Spongy bone
Epiphyseal line
Periosteum
Compact bone
Medullary cavity (lined by endosteum)
Diaphysis
Distal epiphysis
Compact bone
Endosteum
Endosteum
Yellow bone marrow
Compact bone
Periosteum
Perforating (Sharpey's) fibers
Nutrient arteries

Figure 1.7: The structure of a long-bone.

(http://nihroadmap.nih.gov/initiatives.asp).

1.3 Mechanics of Bones

Mechanical properties of bones show how the bones are used and how their properties can be quantified.

Bone is a complex, highly organized and specialized connective tissue. It is characterized physically by the fact that it is a tissue that is hard, rigid and strong, and microscopically by the presence of relatively few cells and much intracellular substance formed of collagen fibers and stiffening substances. It is important to understand this complex structure in detail in order to comprehend how the complex process of bone healing occurs when fractures heal. Furthermore, it is only by understands the biomechanical and biological properties of bone and what type of bone grafts or bone substitutes could be best used to reconstruct large defects of normal bone. The best grafts and bone substitutes are naturally those with biomechanical and biological properties closely resembling those of normal bone. When damaged, bone demonstrates a remarkable capacity for regeneration. Though the detailed process of fracture healing is complex, it can be simplified into three stages: inflammation, repair, and remodeling (Hulth 1989; Yaszemski *et al*. 1996). In the first stage, the normal wound healing response occurs, resulting

in a fibrin clot, when ruptured blood vessels flood the region with growth factors and signaling molecules, attracting cells such as macrophages to digest the damaged tissue. During the repair stage, a callus is formed by bone-making cells recruited to the site, osteoblasts, that produce the cartilage-like bone matrix, which eventually mineralizes through deposition of a non-stoichiometric (calcium deficient) HA. In the final and longest stage, the bone remodels through a process of resorption and deposition, this enhances the bone to resist the applied environmental stresses. This remodeling process is ongoing throughout an individual's lifetime. A tunnel is first created by cells called osteoclasts that digest the old bone by releasing acids and enzymes. This space is then invaded by osteoblasts and a blood vessel to supply nutrients and remove waste. The osteoblasts line the walls with new bone matrix that eventually entraps them. These entrapped cells, called osteocytes, are interconnected by microscopic processes called canaliculi, and are nourished by the blood vessel that exists in a cylindrical space called the Haversian canal. This leads to the osteonal structure of bone made up of concentric cylindrical layers (Figure 1.8). As a result of this inherent regenerative capability, bone is a prime candidate for tissue engineering and reconstruction strategies in dealing with trauma to bone tissues.

Bone is a relatively hard and lightweight composite material containing both organic and inorganic components consisting of living cells embedded in a mineralized organic matrix. The organic components include the cells (osteoblasts, osteocytes and osteoclasts) and the osteoid. One third of the matrix is made up of osteoid, which include the proteoglycans, glycoproteins and the collagen fibers, all of which are created by osteoblasts. The organic components, in particular collagen, are responsible for the flexibility and tensile strength that allow the bone to resist twisting and stretching. Without them, bones would be hard, but extremely brittle. The inorganic components of bone consist mostly (65% by mass) of calcium phosphate mineral salts

chemically arranged as calcium hydroxyapatite. The calcium salts are present as tiny crystals that lie in and around the collagen fibers in the extracellular matrix. These components produce the exceptional hardness of bone, and allow it to resist compression. The proper combination of the organic and inorganic components makes bones exceptionally durable and strong, without being brittle.

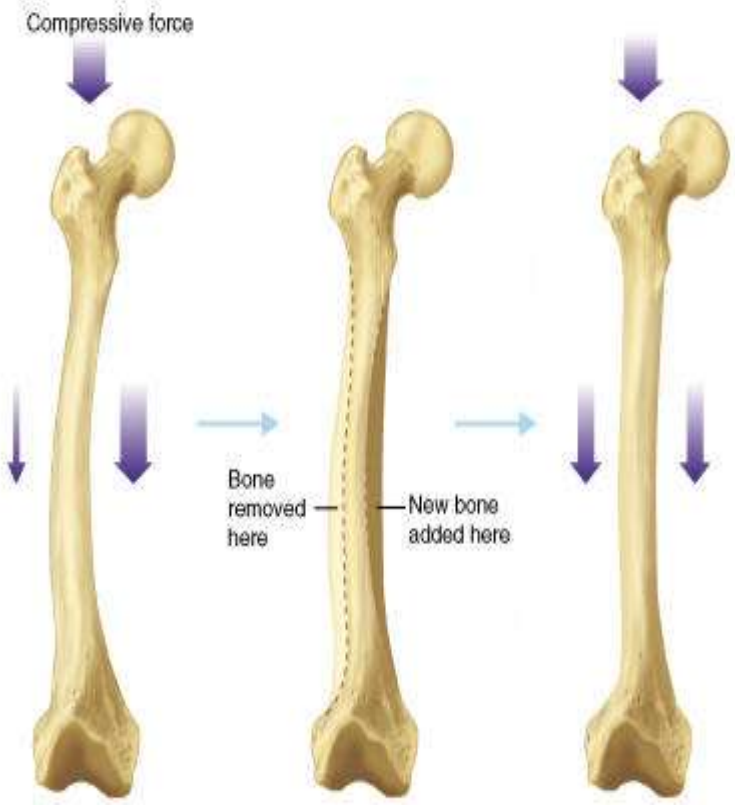

Figure 1.8: Mechanics of Bone Tissue (Colletti 1989)

1.4 Bone Graft

The principals involved in successful bone grafts include osteoconduction (guiding the reparative growth of the natural bone), osteoinduction (encouraging undifferentiated cells to become active osteoblasts), and osteogenesis (living bone cells in the graft material contribute to bone remodeling). Osteogenesis occurs only with autografts. Bone grafts and bone graft substitutes include autografts (a tissue or organ that is transplanted from one part to another of the same body), allografts (transplantation of tissue between genetically nonidentical individuals of the same species) and alloplastic or synthetic bone grafts (Laurencin *et al*. 2006). Although autografts are still considered as the gold standard in bone transplantation and allografts are attractive alternatives to autografts, their inherent problems, such as limited availability, donor site morbidity and risk of disease transmission from donor to recipient, have limited clinical application (De Long *et al*. 2007).

Therefore, synthetic bone grafts composed of polymers, ceramics or composites, with or without cells and growth factors, have been used for bone regeneration. The ideal bone graft substitute should be biocompatible, bioresorbable, osteogenic, able to provide structural support,

easy to use clinically and cost-effective (Parikh 2002; De Long *et al*. 2007). Among all synthetic bone graft methodologies, tissue engineering, which combines osteogenic cells, osteoconductive scaffolds and osteoinductive biological signals together in an orchestrated way to facilitate bone regeneration, is a promising approach (Griffith and Naughton 2002; Hou *et al*. 2007).

1.4.1 Biology of Bone Grafting

In 1668, the first recorded bone graft procedure was performed by Job van Meekeren, a Dutch surgeon (Meekeren 1668). The transplantation of bone or biosynthetic materials to repair skeletal defects is now an accepted surgical technique. Materials reported to have been used include cancellous, and corticocancellous bone, intraarticular grafts have been reported using cancellous bone cortical, and osteochondral grafts. Bone grafts provide osteogenic potential (primary osteogenesis or osteoinduction) and scaffolding for ingrowth of new elements (osteoconduction) (Kold and Hickman 1983; Stevenson 1985; Goldburg 1989; Johnson 1991). Primary osteogenesis is the formation of new bone stemming from transferred living cells (Alexander 1987) which may survive up to 1 mm from the implant surface.

Osteoinduction is the phenotypic conversion of host mesenchymal cells to osteogenic cells in response to biochemical factors from the graft such as bone morphogenetic proteins (BMPs) (Delacure 1994). **Osteoconduction** is the process by which the bone graft provides passive support for host neovascularization and osteogenic elements orienting the structure of the newly

forming bone (Alexander 1987). **Osseointegration** is the formation of a direct, intimate and lasting connection between the host bone and graft (Delacure 1994). An additional purpose of a graft may be structural support (Stevenson 1985; Stevenson 1990) which is mainly derived from cortical, corticocancellous or osteochondral bone grafts. Bone has the unique ability to heal completely and regain its original structure and mechanical properties; repair tissue consists of new bone rather than scar tissue (Delacure 1994). The net biologic activity of the graft is the sum of its inherent biologic activity, its capacity to activate surrounding host tissues and its ability to support the ingrowth of host osteogenic tissue (Stevenson, Emery, and Goldberg 1996). During incorporation, the graft site goes through several concurrent phases. Within minutes platelet aggregation and degranulation initiates the release of cytokines and growth factors causing inflammation (Stevenson 1990). Neutrophils, macrophages and fibroblasts are recruited via chemical messengers such as kinins, complement, histamine, serotonin, prostaglandins and leukotrienes. Macrophages and giant cells debride the wound of devitalized protein while osteoclasts begin removing dead bone. Ischemic death of lacunar osteocytes and subsequent release of lysosomal enzymes results in osteoid destruction (Wornom and Buchman 1992). The inflammatory phase lasts up to one week in cancellous autografts (Stevenson 1985). Centripetal vascularization begins as early as the second day. In cancellous bone, vascularity advances at a rate of 0.2-1.0 mm/day and may be completed within one to three weeks. Cortical grafts become revascularized much more slowly requiring four to eight weeks for fresh autogenous grafts and greater than 4 months for frozen grafts or allografts with a histocompatibility antigen difference (Stevenson, Emery, and Goldberg 1996). Mesenchymal cells begin to proliferate by day 3, differentiate into chondroblasts by day 5, and osteoblasts by day 10. The osteoinduction of inducible pluripotent stem cells by BMP and transforming growth factor-b is complete within the

first one to two weeks. Vascular ingrowth also brings osteoclastic activity initiating graft resorption. Osteoclasts resorb the dead bone, while osteoblasts deposit an osteoid seam along the remnants of the dead trabeculae. The osteoid is then mineralized into new host bone. During this phase the installed graft trabeculae are gradually replaced by new host bone either by 'creeping substitution' in cortical bone or by surface resorption in cancellous bone. **Osteoconduction** lasts several months in cancellous grafts and may take years in cortical bone (Stevenson 1985). Bone graft success depends on the host recipient site, local growth factors of the host bed, bone graft viability, the volume of bone grafted and the structural function of the bone graft. The host recipient site influences the graft physiologically and mechanically. The number of host osteoprogenitor cells and the quality of the perivascular connective tissue determine the hosts ability to respond to graft (BMP) and other growth factors. Factors adversely affecting graft incorporation include trauma, infection, insufficient vascular supply and graft or fracture instability. Motion at the graft/host bone or soft tissue interface will impede or prevent revascularization (Stevenson, Emery, and Goldberg 1996).

Growth factors are polypeptides that bind to specific cell membrane receptors and stimulate or inhibit certain cell functions. Five important growth factors that have been identified include platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-b), insulin-like growth factor, basic fibroblastic growth factor and epidermal growth factor (Hollinger and Seyfer 1994). Within the TGF-b super family, the BMP which are secreted by osteoblasts have emerged as the trigger needed to stimulate bone formation (Hollinger and Seyfer 1994). BMP-2 appears to be the most potent member of the family inducing heterotopic bone formation as early as 5 days. BMP-2 induces bone formation at heterotopic as well as orthotopic sites) and has been evaluated in segmental bone defects in rats, (Yasko, Lane, and Fellinger 1992; Linde and Hedner

1995) dogs, (Toriumi, Kotler, and Luxen 1991) rabbits, (Bostrom, Lane, and Tomin 1996; Lu, Zhang, and Wang 1996) and monkeys, (Aspenberg and Turek 1996; Boyne 1996) with similar results.

BMP induced bone formation is dose dependent and exposure to an immediate source of osteoprogenitor cells is crucial (Yasko, Lane, and Fellinger 1992); BMP plus bone marrow yields the highest bone production and may be 3 times as effective as autogenous cancellous grafts. BMP increases the recruitment of bone forming cells but does not increase individual cell activity (Welch 1996). BMP activity of the bone graft and the host bed induce proliferation of perivascular connective tissue and facilitate osteogenesis. Because graft viability improves success, the graft should be transferred directly or wrapped in a blood-soaked sponge. Air, saline or antibiotics damage the grafted cells (Bassett 1972). Even with ideal handling, up to 90% of living cells die after bone graft transfer, (Auer 1992). But those up to 1.0 mm from the bone surface usually survive. The open structure of a cancellous graft allows diffusion of nutrients and limited capillary microanastomosis, whereas denser cortical bone is a greater barrier. Autogenous cancellous and vascularized corticocancellous bone grafts have a greater chance of survival than do allografts and xenografts which lack early vascularization and elicit an immune response (Auer 1992). Larger grafts require longer for complete incorporation which increases the likelihood of complications and failure. Conversely, transferring more cells means that more osteogenic cells survive (Auer 1992).

1.5 Synthetic Bone Graft

During the past 30 years a variety of synthetic bone graft substitutes has been developed with the aim to minimize these complications. The benefits of synthetic grafts include availability, sterility and reduced morbidity. There are four characteristics that an ideal bone graft material should include:

(i) osteointegration, the ability to chemically bond to the surface of bone without an intervening layer of fibrous tissue.

(ii) osteoconduction, the ability to support the growth of bone over its surface (Constantino and Freidman 1994).

(iii) osteoinduction, the ability to induce differentiation of pluripotential stem cells from surrounding tissue to an osteoblastic phenotype (Cypher and Grossman 1996). (iv) osteogenesis, the formation of new bone by osteoblastic cells present within the graft material (Constantino and Freidman 1994).

Only autogenous bone graft satisfies all of these requirements. Allograft is osteointegrative and osteoconductive and may exhibit osteoinductive potential, but it is not osteogenic because it contains no live cellular component. Synthetic bone graft substitutes currently possess only osteointegrative and osteoconductive properties.

There are several substances such as ceramics, calcium phosphate and other synthetic materials that have similar biomechanical properties and structure similar to that of cadaver bone

and may be used as a bone graft substitute. They allow for bone growth on their surface and then they are resorbed by the body, with the patient's own bone remaining in place. However, these products do not have all the properties necessary to stimulate a spinal fusion when used alone. They are usually used in combination with the patient's own bone to augment the amount of bone graft available. When the patient's bone marrow cells (bone marrow aspirate) are added to ceramics, clinical studies have demonstrated that these products are effective. Unlike allograft (cadaver bone), ceramic-based products do not present a risk for disease transfer. However, they may occasionally cause inflammation (Hak 2007).

1.5.1 Calcium Sulfate

Calcium sulfate is actually plaster of Paris. It was first documented as being used for fracture treatment by the Arabs in the 10th century, who would surround the affected limb in a tub of plaster. In 1852, a Dutch army surgeon by the name of Mathysen incorporated plaster into a bandage. In 1892, a German by the name of Dreesman successfully used plaster of Paris medicated with a 5% phenol solution to treat tuberculous osteomyelitis of long bones, the majority achieving successful healing (Peltier and Bickel 1957).

Calcium sulfate is thought to act as an osteoconductive matrix for the ingrowth of blood vessels and associated fibrogenic and osteogenic cells. For this to occur it is critically important that the implanted calcium sulfate is adjacent to viable periosteum or endosteum (Coetzee 1980). Over a period of five to seven weeks the calcium sulfate is reabsorbed by a process of dissolution (Bell 1964). Its rapid reabsorption may be used to advantage in the context of osteomyelitis where an antibiotic-impregnated form could be used in place of gentamicin beads, thus

alleviating the need for a second operation. A medical grade of calcium sulfate impregnated with tobramycin is commercially available (Osteoset; Wright Medical Technology, Arlington, TN, USA 2001). Calcium sulfate, however, requires a dry environment to set and if it is re-exposed to moisture it tends to soften and fragment. For this reason it has no reliable mechanical properties *in vivo* and its application should be limited to a contained area. Hence the primary use of calcium sulfates should be as a bone void filler (Coetzee 1980). Calcium sulfate has been used since 1892 as bone defect filler and is known to be osteoconductive (Greenwald *et al*. 2001; Kelly *et al.* 2001). Demineralized bone matrix is known to be osteoinductive (Greenwald *et al*. 2001) in a canine model. The combination of calcium sulfate pellets and demineralized bone matrix was more effective as a bone-graft substitute than is either calcium sulfate or demineralized bone matrix alone (Turner *et al.* 2001). The extensive work of Peltier (1961) on calcium sulfate led to the development of a medical grade alpha-calcium sulfate hemihydrate cylindrical pellet (OsteoSets; Wright Medical Arlington, Tennessee, USA) for the treatment of bony defects. Experimental and clinical studies have shown its positive effect in the filling of dead bony spaces (Alexander, Manson, and Mitchell 2001; Mirzayan *et al*. 2001). A disadvantage of calcium sulfate is its transient cytotoxic effect leading to inflammatory reactions, which was shown in several studies (Coetzee 1980; Robinson *et al*. 1999). Calcium sulfate has also been used as an antibiotic carrier material. Petrova (1928) loaded plaster of Paris with the antimicrobial agent Rivanol and treated bone infections in dogs. In 1928, Nystrom achieved successful healing in children with the same composite material 3 years later. In more recent literature, good results have been reported for antibiotic-loaded calcium sulfate in experimental (Dacquet *et al*. 1992) and clinical settings (Turner *et al*. 1998; Mckee *et al*. 2002).

However, in all studies antibiotic loading of the pellets was done before hardening of the calcium sulfate jeopardizing antibiotic activity after the hardening and sterilization processes (Dacquet *et al*. 1992). To date, there is only one kit commercially available allowing antibiotic loading of already sterilized calcium sulfate powder (Osteoset sBVF-Kit; Wright Medical, Arlington, Tennessee, USA) (Gitelis and Grebach 2002).

In summary, there are two major disadvantages in the use of calcium sulfate pellets with a fixed antibiotic preloading. Firstly, there is at least a short-term cytotoxic effect of calcium sulfate and secondly, addition of the appropriate antibiotic before the hardening and sterilization procedures may reduce its activity during the sterilization procedure. The advantages of the use of calcium sulfate, are that an unusually biocompatible material and is completely resorbed following implantation. It does not evoke a significant host response and creates a calcium-rich milieu in the area of implantation. These calcium ions may provide some stimulation to osteoblasts, which may account for some of the positive results reported with the material. Calcium sulfate can be used as a delivery vehicle for growth factors and antibiotics, although this application has not been thoroughly exploited in the clinical setting (Thomas, Puleo, and Al-Sabbagh 2005).

1.5.2 Calcium Phosphate

The calcium phosphate family of synthetic bone grafts has both osteointegrative and osteoconductive properties. Osteointegration results from the formation of a layer of HA shortly after implantation. The Ca^{+2} and PO_4^{-2} ions required to establish this layer are derived from the

implant and surrounding bone. The pathways of both Ca^{+2} and PO_4^{-2} ions have been traced in serum and urine without any significant elevation in serum levels from which it can be concluded they are handled as part of the normal body ion pool. They have an excellent record of biocompatibility with no reports of systemic toxicity or foreign body reactions (Hollinger and Battistone 1986).

The main driving force behind the use of CPs as bone substitute materials is their similarity to the mineral component of bone. As a result, in addition to being non-toxic, they are biocompatible, not recognized as foreign in the body, and most importantly, exhibit bioactive behavior, being integrated into the tissue by the same processes active in polymer-calcium phosphate composites for use as an injectable bone substitute remodeling healthy bone. This leads to an intimate physicochemical bond between CP implants and bone, termed Osseo integration (Ong and Chan 1999). CPs are also known to support osteoblast adhesion and proliferation (de Groot 1993). This unique bioactivity is highly desirable and hence CP compounds are widely studied and used as bone replacements or as coatings on implants (de Groot 1993; Ong and Chan 1999; Bohner 2000).

Even so, the major limitations to CP use as load-bearing biomaterials are its mechanical properties. Typical of ceramics, CP biomaterials are brittle with poor fatigue resistance. It is for this reason that these materials are used primarily as fillers and coatings. The poor mechanical behavior is even more evident for highly porous CPs, yet porosity greater than 100 mm is considered a requirement for proper vascularization and bone cell colonization of a CP scaffold. Porosity also improves fluid penetration of the material and in turn enhances its dissolution, which is an important characteristic of a CP since the solubility of CPs largely determines their properties *in vivo* (Ducheyne and Qiu 1999; Bohner 2000).

1.5.3 Zinc Ions

Zinc is an essential trace element with stimulatory effects on bone formation *in vitro* and *in vivo*. Zinc contents range from 0.0120 to 0.0250 weight (wt %) in human bone, which is relatively high compared with the average zinc content of whole fat-free adult tissues (0.0030 Zn wt %) and that of plasma (0.78–1.0 Zn mg/ L) (Sadler 1977; Bettger and Dell 1993). At a concentration of 6.5 mg/L, zinc resulted in an increase in bone protein, calcium content, and alkaline phosphatase activity in rat calvaria *in vitro* (Yamaguchi 1988; 1987). A low dose of zinc administered to rats resulted in an increase in alkaline phosphatase activity and DNA content in the bone tissue (Yamaguchi and Yamaguchi 1986). On the other hand, zinc is a highly potent and selective inhibitor of osteoclastic bone resorption *in vitro* (Kishi and Yamaguchi 1994; Moonga and Dempster 1995).

1.6 Medicinal Plants

Throughout the ages, humans have relied on nature for their basic needs for the production of food-stuffs, shelter, and clothing, mean of transportation, fertilizers, flavours, fragrances, and medicines. Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years and that continue to provide mankind with new remedies. Although some of the therapeutic properties attributed to plants have proven to be erroneous, medicinal plant therapy is based on the empirical findings of hundreds and thousands

of years. The use of traditional medicine is widespread and plants represent a large source of natural antioxidants that might serve as leads for the development of novel drugs (Conforti *et al.* 2009).

1.6.1 Lepidium Sativum

Lepidium sativum (LS), otherwise known as garden cress, pepper weed or garden pepper weed, is an annual herb that belongs to the Cruciferae (Brassica) family (Gokavi, Malleshi, and Guo 2004). The seeds are consumed wildly in salads and as a spice (Maghrani *et al.* 2005) and are reddish in colour, oblong, somewhat angular and curved slightly on one side with a rugous surface (Figure 1.9) (Shukla, Singh, and Bigoniya 2011). LS seeds contain volatile essential aromatic oils, fatty oils, carbohydrates, proteins, fatty acids, vitamins (β-carotene, riboflavin, niacin, and ascorbic acid), flavonoids and glycosides (Nadkarni 1995). In addition, glucosinolates, a class of naturally occurring thioglycosides, have been identified as principle constituents of *Lepidium sativum* (Fahey, Zalcmann, and Talalay 2001).

Figure 1.9: ***Lepidium sativum*** **seeds.**

(http://botanyjohn.org/gallery/v/ubcbgseed/2006_680_0101.jpg).

imidazole alkaloids and essential oil composition of the plant have been investigated (Maier, Gundlach, and Zenk 1998; Mirza and Navaei 2006).

Lepidium sativum plant and seeds are considered one of the popular medicinal herbs used in the community of Saudi Arabia, Sudan and some other Arabic countries as a good mediator for bone fracture healing in the human skeleton. A number of recent studies pointed out the traditional uses of *Lepidium sativum* seeds extract in controlling many clinical problems. They were used as anti-asthmatic antiscorbutic, aperient, diuretic, galactogogue, poultice and stimulant. The leaves are antiscorbutic, diuretic and stimulant (Eddouks *et al*. 2002). It was found that oral administration of the aqueous *Lepidium sativum* extract exhibited a significant decrease in blood pressure (Maghrani *et al*. 2005). *Lepidium sativum* seeds increase weight gain as they are found to contain 18-24% of fat. Thirty four percent of the total fatty acids are alpha linolenic acid; and the oil has alpha linoleic acid which could give it nutritional advantages (Diwakara *et al*. 2008). The primary fatty acids in *Lepidium sativum* oil were oleic (30.6 wt %) and linolenic acids (29.3 wt %) and was found to contain high concentrations of tocopherols. It contains good amount of lignans and antioxidants, which can stabilize the n-3 polyunsaturated fatty acids in its seed oil. The primary phytosterols in *Lepidium sativum* were sitosterol and campesterol, with avenasterol (Bryan *et al*. 2009).

Lepidium sativum seeds are publicly used in Saudi Arabia as a traditional medicine, mostly for the treatment of recent traumatic fracture (Czimber and Szabo 1988; Ahsan *et al*. 1989). Good results of healing of fractures were observed over decades by the hands of traditional folk medicine practitioners. Traditional uses of LS include treating some inflammatory conditions, like asthma, skin disease, diabetes (Eddouks *et al.* 2005; Archana and Anita 2006), hypertension,

and renal disease (Jouad *et al*. 2001; Maghrani *et al*. 2005; Tahraoui *et al.* 2007). Previous studies have demonstrated the protective action of LS against carcinogenic compounds (Kassie *et al.* 2003).

1.7 Vibrational Spectroscopy

Vibrational spectroscopy is a very powerful analytical technique for both quantitative and qualitative analysis of components. FTIR spectroscopy allows the examination of the molecular structure and conformation of biological macromolecules because it measures the absorption energy, which produces an increase in the vibrational or rotational energy of atoms or groups of atoms within the molecule. The infrared spectrum is formed as a consequence of the absorption of electromagnetic radiation at frequencies that correlate to the vibration of specific sets of chemical bonds from within a molecule. The fundamental requirement for infrared activity, leading to absorption of infrared radiation, is that there must be a net change in dipole moment during the vibration for the molecule or the functional group under study. There have been several previously published FTIR investigations on bone (Paschalis *et al*. 2003). Fourier transform infrared absorption spectroscopy has been extensively employed in these fields in order to characterize the structural and chemical/physical properties at the molecular level (Crupi *et al.* 2001).

Another important form of vibrational spectroscopy is Raman spectroscopy, which is complementary to infrared spectroscopy. The selection rules for Raman spectroscopy are different to those for infrared spectroscopy, and in this case a net change in bond polarizability must be observed for a transition to be Raman active. There is a reasonably large literature on

vibrational spectroscopy of bone (Carden and Morris 2000; Carden *et al*. 2003). The major Raman band assignments for bone include vibrational modes for phosphate, carbonate and mono-hydrogen phosphate. The important matrix bands are mostly Absorbance/Wavenumber (cm^{-1}) markers for the collagen backbone. The band positions, intensities and shapes report on the state of maturation as well as on abnormal composition resulting from genetic defects or disease. Vibrational spectroscopy therefore allows convenient measurement of mineral/matrix ratios, as well as alterations in mineral content associated with aging and/or mechanical deformation (Sahar 2005).

1.8 Gas Chromatography-Mass spectrometry

GC-MS enable the investigation of thermally stable – volatile materials. The derivatization of OH and NH functional group is known to be completed with many reagents. The most reactive and stable reagent is N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA). Exploring of non-volatile materials contain –OH or NH, were first derivatized and analyzed by using GC/MS. Mao *et al*. (2009) detected *Lepidium sativum* using GC/MS and Elguera, *et al*. (2013) GC/FID.

1.9 Aims of the Study

1- To improve the synthetic calcium sulfate dihydrate based composite as a biodegradable bone substitute material *in vitro*.

2- To apply the uses of vibrational spectroscopy for qualitative and quantitative analysis of the new biopolymer.