Synthesis and Characterization of Whitlockite Containing Polymeric Injectable Conductive Hydrogels for Biomedical Applications

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Synthesis and Characterization of Whitlockite Containing Polymeric Injectable Conductive Hydrogels for Biomedical Applications

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DEDICATION

I dedicate this thesis to my beloved Family for their unconditional support and love

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All admiration to Allah Almighty. He is the One, who bestows and gives the power to us to think, utilize our expertise in knowledge in achieving remarkable solutions for mankind in every field. Therefore, I express my greatest thanks to Almighty Allah the universal and the architect of the world. Allah Almighty says in Quran:
"Read! In the name of your lord" (Alaq; 1st revealed ayah)

This Quranic verse sums up the entire importance of education in the lives of humans. I like to express my gratefulness to my very supportive and respected supervisor **Dr. Zakir Hussain** for his clear and patient guidance that directed me to fulfill my project and this thesis. His cool and calm behavior motivated me to do my best. His valuable suggestions, constructive advice and feedback contributed to this thesis. Also, I am very grateful to my Co-Supervisor **Dr. Usman Liaqat** and GEC members including **Dr. Aftab Akram** and **Dr. Bilal Khan Niazi** who helped me and motivated me to do my best. I would also like to thank my parents, family members, and friends for their help, prayers, and their valuable suggestions

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- Aleema Marrium

ABSTRACT

Human Skeleton contains 206 bones which provide support, strength and mobility to human body. These bones may fracture as a result of trauma, sports injury or disease. Treatment of the fractured and defected bones is always a matter of concern for mankind since early history. Conventional Methods of treatment like autograft, allograft used may cause potential problems for host. So researchers developed the bone tissue engineering (BTE) techniques for bone regeneration which combines use of biomaterials with principles of Engineering. Usually BTE scaffolds are static in nature and implanted in host body by surgical Invasiveness. It is needed to develop the material which can be used at irregular traumatic sites of bone with minimum invasiveness during its implant. For this purpose, we developed an injectable conductive polymeric hydrogel system that contains bone whitlockite (WH) which is a bioceramic material. WH is used to enhance the biological activity as well as conductivity of hydrogels. Prepared composite hydrogels were than characterized using FTIR which confirmed the blending of materials. Injectability of prepared hydrogels was found to be less than 30 secs with controlled gelation rate which ranges between 10-30 minutes. Viscometric analysis supported the results of Injectability and gelation rate of injectable hydrogels, as viscosity of hydrogel decreases with increase in amount of WH. Ionic conductivity of injectable hydrogels was also found to increase with increase in whitlockite concentration. Cytotoxicity of hydrogel was measured using MTT assay which confirmed that our prepared biomaterial is nontoxic and crosslinking in synthesized injectable hydrogel was confirmed by SEM imaging.

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List of Abbreviations

Bone Tissue Engineering…….……..…...BTE

Bone Tissue Regeneration……...BTR

Nanoparticles……...NP’s

Food and Drug Administration…….... FDA

Whitlockite……...WH

Polypyrrole…… PPY

Alginate…….....ALG

Hydroxyapatite……. HA

Calcium Phosphates……. Ca

Phosphate Buffered Saline……...… PBS

Extra Cellular Matrix…….……. ECM

Chapter 1

Introduction

1.1. Background

Bone tissues are naturally capable to heal in case of small injury with the aid of bone remodeling process however bone defects caused by trauma or pathological reasons cannot be healed naturally and need surgical intervention. [1-3]. For the repairing of the affected bone site several bone repair methods are in practice i.e. auto grafts involve use of subject's own bone tissue from donor site and graft it on receiver site while in allografting bone tissues are transplanted from one individual to other of similar species. Although tissues and cells used in these methods have advantages like same structure as human bone, can induce osteoconduction and some of them can induce osteoinduction these methods earned negative reputation due to risks associated with them i.e. potential infection, donor site morbidity , transmission of bacteria and viruses etc.[4-7].

To cope with the complications associated with traditional methods it is vital to develop new methods and material for bone tissue regeneration and fracture healing. One of the promising and efficient approaches is Bone Tissue Engineering(BTE) which "combine the principles of engineering with life sciences to develop biomaterial for repairing and improving the tissue functions". Major components of BTE are osteogenic potential containing cells and Bioactive molecules for osteogenic differentiation of cells and biocompatible scaffolds possessing similar extracellular matrix as bone tissue. BTE scaffold must contain certain characteristics like biocompatibility, biodegradability, mechanical strength, non-toxicity and, stability [8, 9].

Among various material developed as BTE scaffolds, **hydrogel** possess most unique three dimensional matrix which is structurally like to extra cellular matrix of bone tissues. Hydrogels are 3-dimensional hydrophilic/hydrophobic network of polymeric chains which are capable to hold large amount of liquid alongside maintaining their structural integrity. Originally hydrogels were implanted to the fracture site using

invasive surgical methods. These methods are not cost effective and can lead to severe complications. To overcome this issue, researcher have now developed hydrogels that can be injected directly on the traumatic site from its resource (syringe). These injectable hydrogel shows minimal invasiveness to the defect site and can be used at sites with irregular bone defects.[10, 11]

As only one material cannot possess all the characteristics required for BTE scaffolds so we incorporated and manipulated different biomaterials with unique properties to fabricate composite hydrogel scaffold with desired features[12].

Injectable hydrogels are usually formulated by using FDA approved or cytocompatible polymers which can be natural polymers like gelatin or collagen or synthetic conductive polymers like polypyrrole, polylactic acid, polyaniline etc. after realizing the importance of endogenous electrical signals in micro environment of bone tissues, attention of researchers is averted towards new class of organic polymers which are electrically conductive in nature and are widely used in biomedical applications due to their compatibility, stability in ambient environment and cost effectiveness[13].

Conductive polypyrrole attracted the interest of researchers because it is stable in its oxidized form and water soluble with low processing cost. It is an electrically active material which can meet physio-electric requirements of bone tissues. Conductive polypyrrole is fragile and crystalline in nature. it is susceptible for irreversible oxidation. As only one material cannot possess all the required characteristics of BTE scaffolds so different biomaterials were incorporated and manipulated to gain the desired properties in our research project.[14-17]

Sodium alginate is a biocompatible material and is favored by researchers as it is biodegradable and biocompatible material with non-antigenicity. It is highly hydrophilic in nature and helps the microenvironment to remain moist. It helps in controlled gelation rate of hydrogels and provide stability for *in vivo* function of hydrogels. sodium alginate based Ionic gels can be synthesized when polyvalent cations are used as cross-linker due to formation of polyelectrolyte complexes as a result of bond between carboxyl group of sodium alginate with positively charged cations when polyvalent cations are used as cross linker [18, 19]. Along with its unique features alginate but it is limited by poor heat and mechanical stability as well as to

achieve desired porosity and pore size it is blended with other materials like gelatin and bioceramic for bone tissue engineering as well as biomedical applications[20].

Use of calcium phosphate based bioceramic for treatment of bone defects has long history**. Bone Whitlockite** is a bioceramic material which is naturally present in human bones. It is magnesium containing calcium phosphate mineral that plays an important role in bone regeneration and integration. For a long time, it was confused with β-TCP because of their structural similarity. It is a bioresorbable, bioactive and osteoconductive material with good mechanical properties compared with other calcium phosphates which also helps to provide mechanical properties to the scaffold. Alginate is also limited in providing sufficient sites for adhesion and cell proliferation. so other biomaterials are added in it to promote attachment of cells. [21-23].

Gelatin is also biocompatible and biodegradable polymeric material which is often used in fabrication of scaffolds for bone regeneration it is water soluble material. In BTE gelatin finds its applications by promoting adhesion, proliferation and differentiation of cells on the bone surface [23, 24].

In our studies above mentioned biomaterials were used to fabricate a novel composite hydrogel material for bone regeneration.

Chapter 2

Literature Review

2.1. Bone:

Bone is a unique type of bio mineralized connective tissue which provides strength and support to the animal body. it also helps to maintain the Body's structural framework.[25-28]. Bones play crucial role in maintaining body's homeostasis, regulating the pH of blood, protecting the critical organs and act as store house for essential minerals for proper functioning of body. Naturally the entire tissue content of bone is composed of two major phases, inorganic phase (70%), usually comprises of calcium phosphate salts attributed to strengthen bones and organic phase (20%) , contains collagen fibers, providing the flexible properties to bones and rest of 10% contains water and overall cells.[29, 30]

Table 1 Composition of Bone [31]

Composition of Bone	
Components	**Wt.%**
Calcium phosphate	**60-70**
Collagen	**10-20**
Non-collagenous Proteins	**3-5**
water	**9-20**
Carbonates	**4-6**
Sodium	**0.7**
Magnesium	**0.5**
Other organic material (cytokines, polysaccharides, lipids)	**Trace**
Inorganic ions (Fe^{+}, Cl^{-},K^{+}, Cu^{+2},F^{-}, Pb^{+2}, zn^{+2}	**Trace**

2.1.1. Macrostructure of Bone:

On macroscopic level, depending upon the porosity of bones, adult human bones can be divided into two types, **cortical bones** and **cancellous bones**. Around 80% of the

mass of human skeleton is made up of cortical bones. Cortical Bones are dense in nature and show mechanical resistance to shock. This type of bones forms the outer layer of compact bones in human body with the porosity of 3-5% in the form of blood vessels, canaliculi and osteocytes. The rigid properties of compact bones are attributed to collagen fibrils which form lamellae. These lamellae organized themselves perpendicularly to enhance the mechanical support provided by the bones to our body. Cancellous bones are also known as spongy bones as they have highly porous interior like honey comb with the porosity of 50-90% and form 25-30% mass of human skeleton Human bone marrow is surrounded by these bones. these types of bones shows several metabolic functions [32-36]. But in comparison of cortical bones, the spongy bones do not have much mechanical properties.

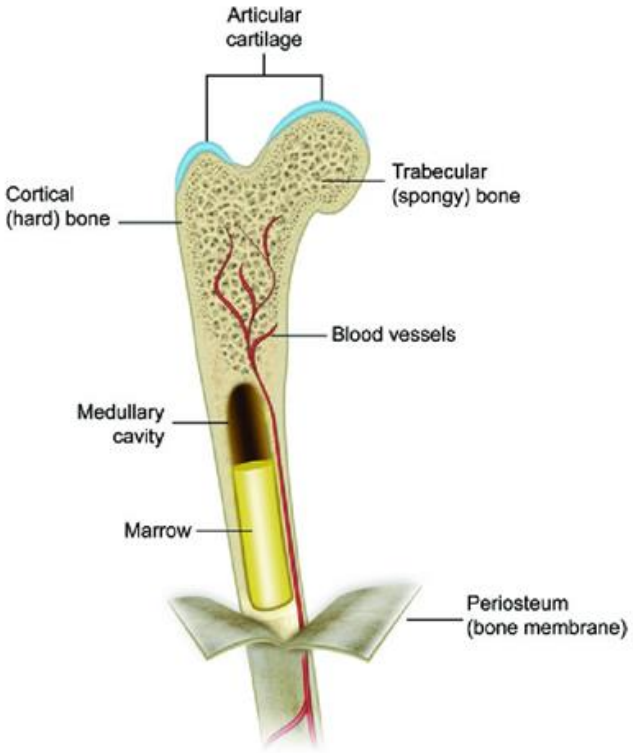

Figure 2.1 Bone Tissues [37]

2.2. Bone Fracture and Regeneration:

2.2.1. Fracture:

Failure of bones to bear the external pressure exerted on it leads to bone fracture which breach the structural continuity of cortex of bone. It may happen because of sport

injury, any accident or due to medical reasons like bone cancers or osteoporosis. Depending upon the injury, bones fracture can be divided into two types i.e. open fractures and closed fractures. In open fracture soft tissues as well as wounds are present on skin which may lead to infections due to high contamination. In closed fracture the skin at the fracture site remains intact and thus less potential threats for contamination

In general, complete healing of fractured bones takes 6-8 weeks. One of the unique properties of bone healing is that after complete healing bone tissue restore their original function completely without any scars[36, 38, 39]

Based on forces which cause the bone fracture, fractures can be further divided into three types

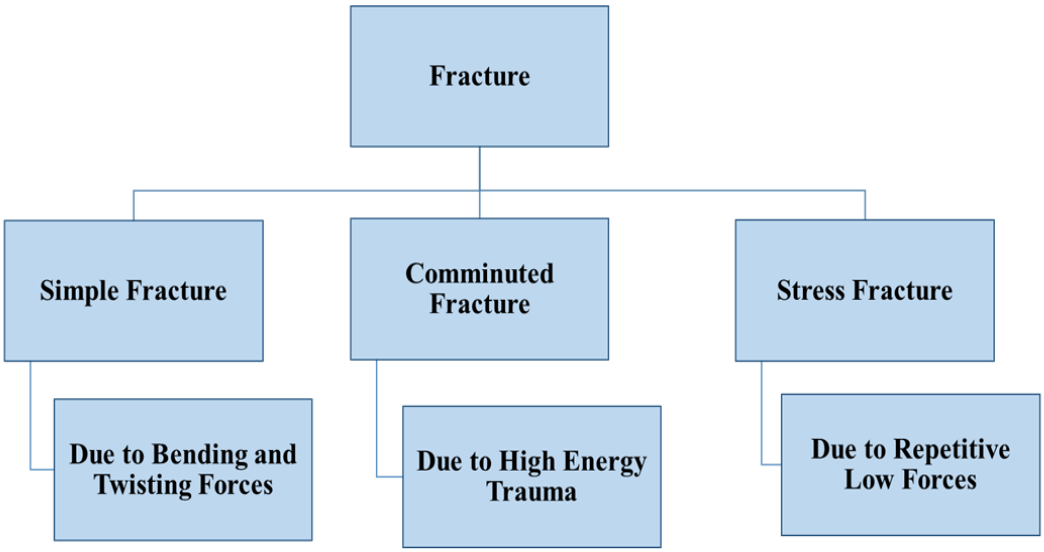

Figure 2. 2 Types of Fractures

2.2.2. Bone Regeneration:

Fracture healing and Bone regeneration is a series of complicated process which includes removal of bone tissue debris, production of extracellular matrices of bone and restoring the vascular supply in the bone. For bone regeneration several local factors should also take into account such as degree of trauma faced by bone, tissue

surrounding the trauma site, type of affected bone, bioelectric factors, amount of bone lost because of fracture. Trauma induced as result of bone injury led to upset the vascular structure of bone as well as nutrient supply chain of bone is also affected which in return causes lack of oxygen at the trauma site and architectural disruption of bone marrow. For the regeneration and remodeling of traumatic bones, several essential factors like mesenchymal cell, growth factors, extracellular matrix and bleeding plays their role. Internal bleeding due to injury causes the coagulation at the site which turned out as hematoma development at the traumatic site. After that mesenchymal stem cells and fibroblast takes action and the expansion of pre-existing blood vessels takes place for the generation of new blood vessels. Cytokines, adhesion cells and growth factors are released after this process for regeneration of bone tissues. [36, 40, 41]

Following are the several stages of bone regeneration.

- Hematoma development (1-5 days)
- Formation of fibro cartilaginous callus5-11 days
- Development of bony callus from day 11-28
- Bone Remodeling from day 28 till complete healing

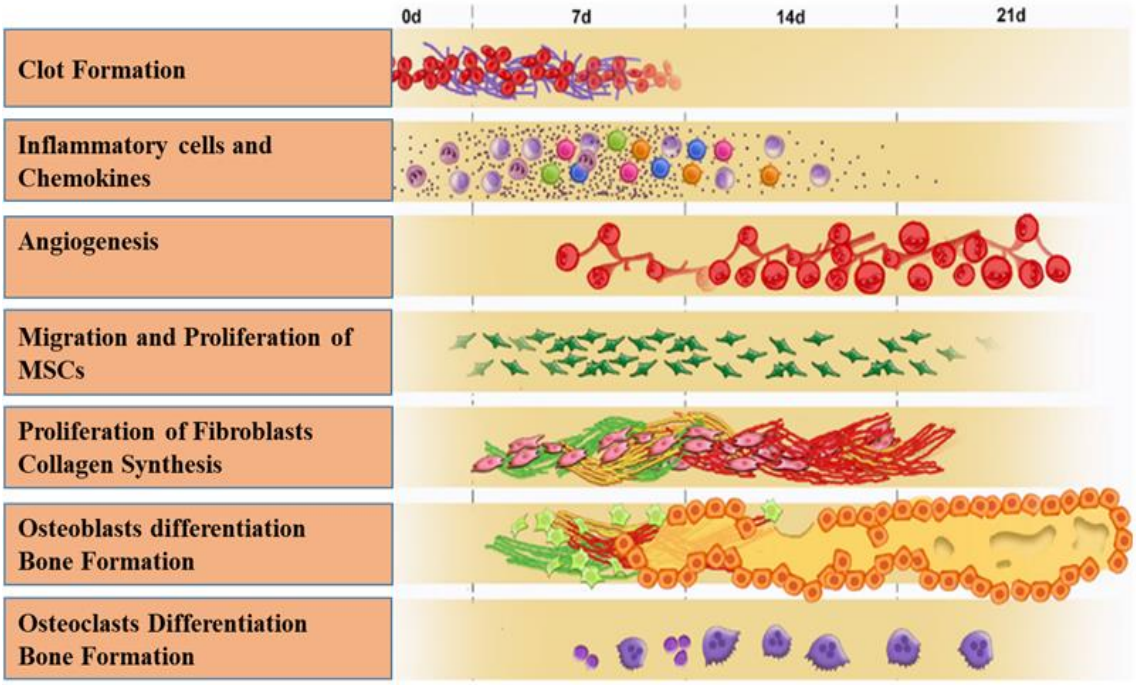

Figure 2. 3 Stages of Bone Regeneration[42]

2.3. Bone Remodeling:

Bone remodeling occurs for entire life and its major purpose is to renew the bone tissue. Annually 5-10 % volume of bone in human skeleton is renewed. Remodeling of bones involves replacement of older or traumatic bones into the new bones. During this process Osteoclasts and osteoblasts continue to migrate that results in coupled remodeling. The older or fractured bones are removed by osteoclasts by the process of bone resorption, meanwhile osteoblasts do the necessary action for the generation of osteoid. In bone remodeling process, the edges of callus were transformed into lamellar bones and central soft part is turned into the hard compact bones. Phases involved in bone remodeling are mentioned below[32, 39, 43, 44].

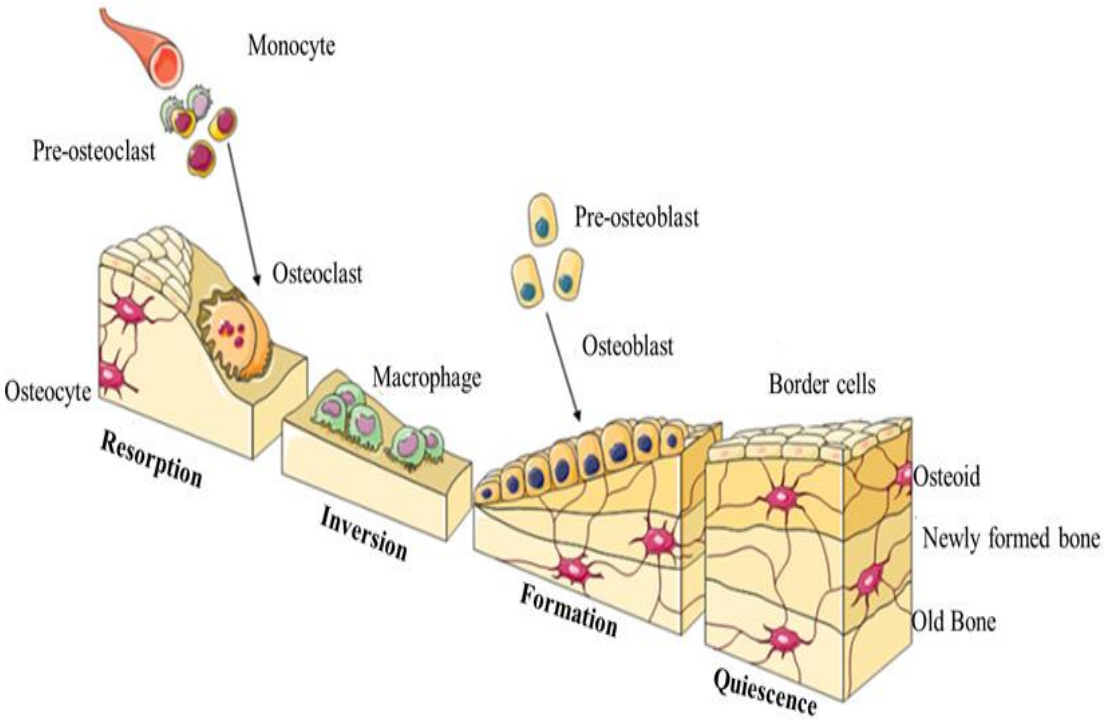

Figure 2. 4 Phases of Bone Remodeling [45]

2.4. Bone Regeneration Techniques:

Bone is one of the most frequently transplanted body tissues despite being used for a long time. Clinically bone grafts exhibit some disadvantages that limit their application. Different techniques for bone regeneration are

Allografts: Allografts is the process of bone regeneration by taking some part of bone or tissue from a living process. But it is a very difficult process; it is hard to find donors and can generate abnormal immune responses in the acceptor[46].

Auto-grafts: Auto grafting is the process of taking cells or tissues from a patient's own body. It can be highly efficient as no donor complications are involved. But auto-grafts generate another potential site for infection in the body[47].

Xenografts: Xenografts involve taking cells from animals i.e. cow or pig, it can be very harmful as grafting from animals increases potential risk of harmful viruses and pathogens entering in the human race.

2.5. Bone Tissue Engineering:

Bone Tissue engineering is a multidisciplinary field which aids in maintenance, restoration and fixation of bone tissues that combines the biological material and principle of engineering[48]. BTE involves the understanding of bone architecture, dynamic environment, mechanics to synthesize the biologically active and degradable material for the fixation of damaged bones. Main focus of BTE is to find the alternate and improvise solutions for fixation of damaged bones to avoid the potential damage caused by traditional bone grafting[48].

Generally, for the fabrication of engineered tissue below mentioned stages are followed.

1. Fabrication of scaffolds
2. Cells isolation or removal
3. Cells proliferation to increase the number of cultured cells.
4. Use of proper scaffold with appropriate growth factor for seeding
5. *In vitro* culturing of tissues
6. Implantation of fabricated scaffold to the traumatic body.
7. Assembly of implant [31, 49]

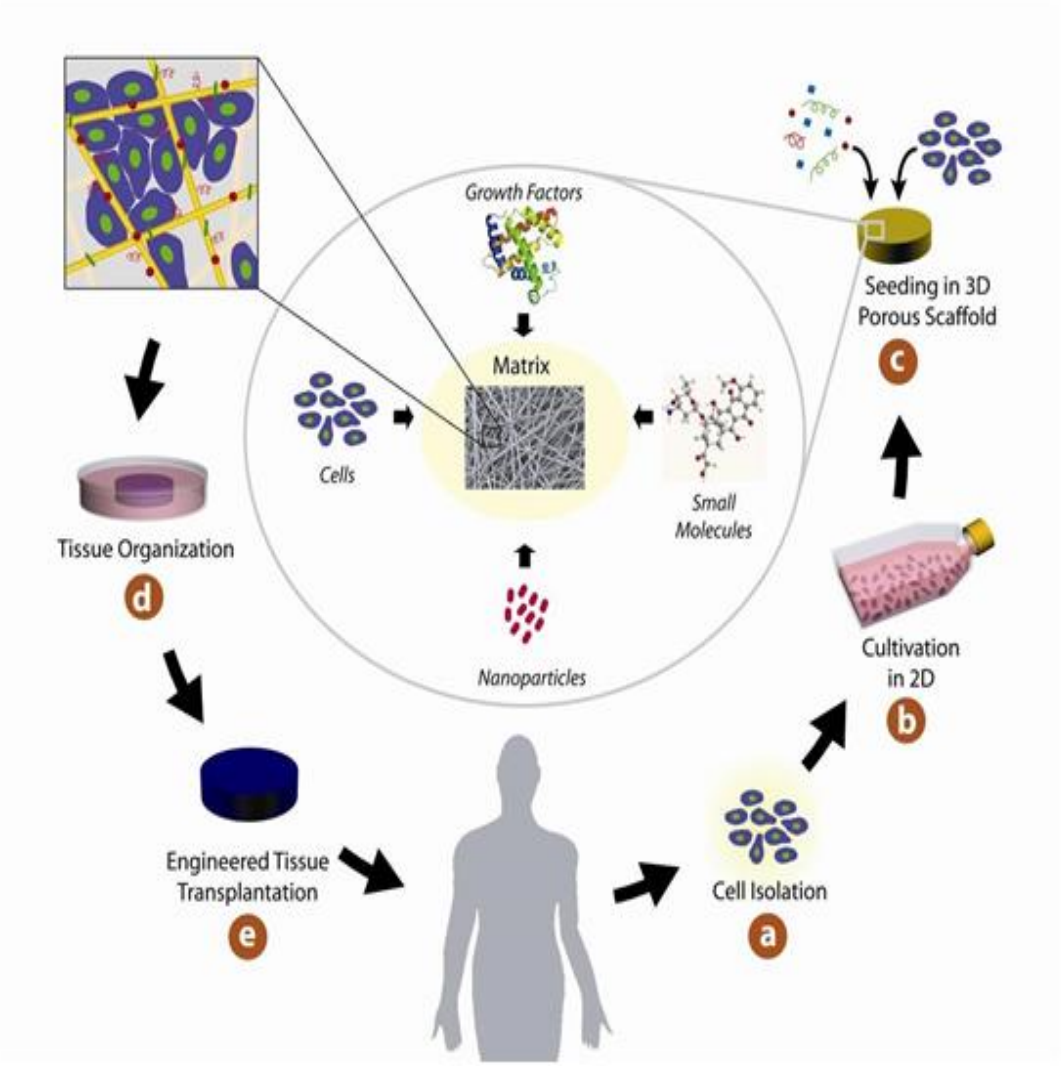

Figure 2. 5 Pathway of Tissue Engineering [26]

Bone tissue engineering is all about interaction of three major components. i.e.

1. **Bioactive Molecules** and material are essential substances for differentiation and growth factor or adhesion molecules are as they help the cells and provide them guidance to generated the required tissue
2. **Cells** which can be either implanted or cultured for the generation of new tissues
3. A **scaffold** which shows close resemblance with extracellular matrix of bone and have ability to hold the cells altogether[50, 51].

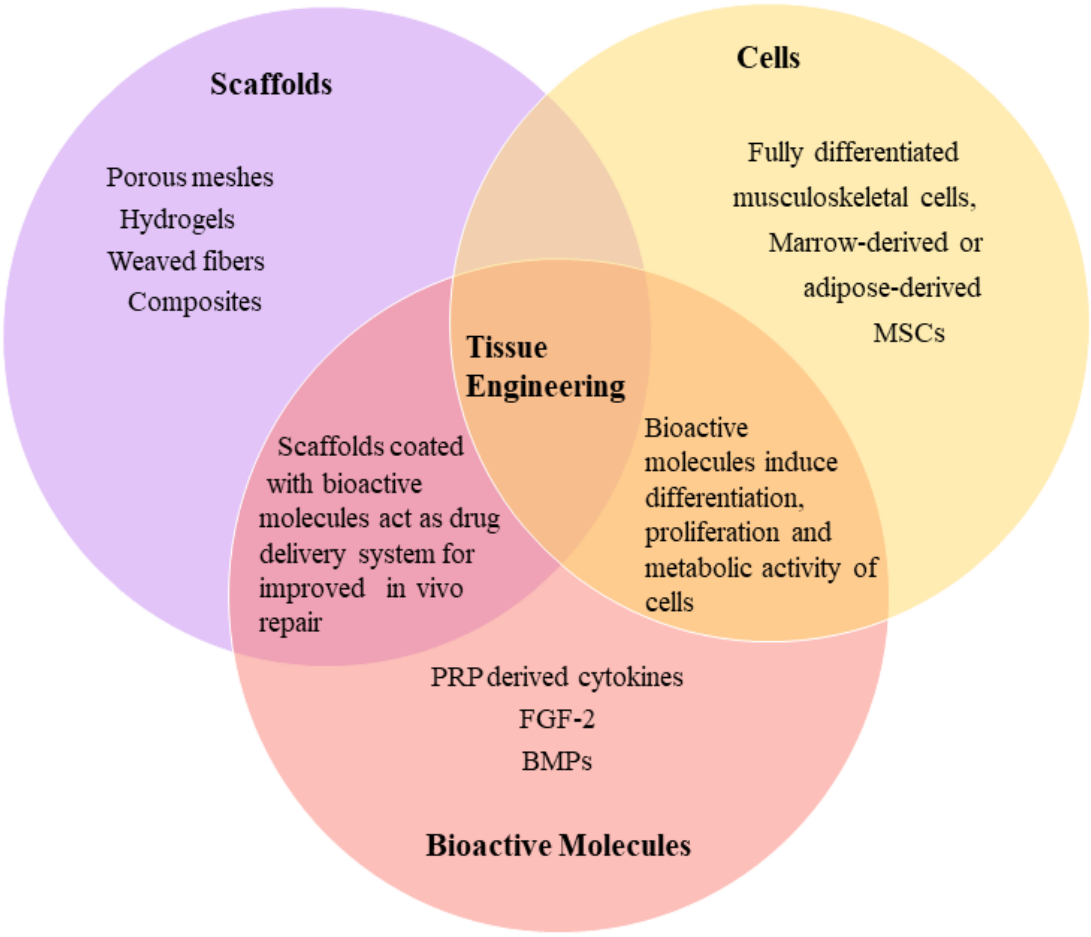

Figure 2. 6 Tissue Engineering Triad [52]

Usually the cells are used to develop new tissues with the help of scaffolds that fulfilled the environmental requirements for tissue generation meanwhile the growth factor make sure that cells follow appropriate differentiation pathways [52]

2.6. Growth Factors:

These refer to a clan of proteins which takes part and aids in the process of healing of tissues by controlling several cellular processess. Some of them are listed below

- Growth of cells
- Differentiation of cells
- Migration of cells
- Metabolic Activity
- Apoptosis
- Signaling between the cells and molecules

- Information transferring i.e. between cells and their microenvironment which lead to speed up the process of restoring the traumatic tissues

Types of Growth Factors used in BTE:

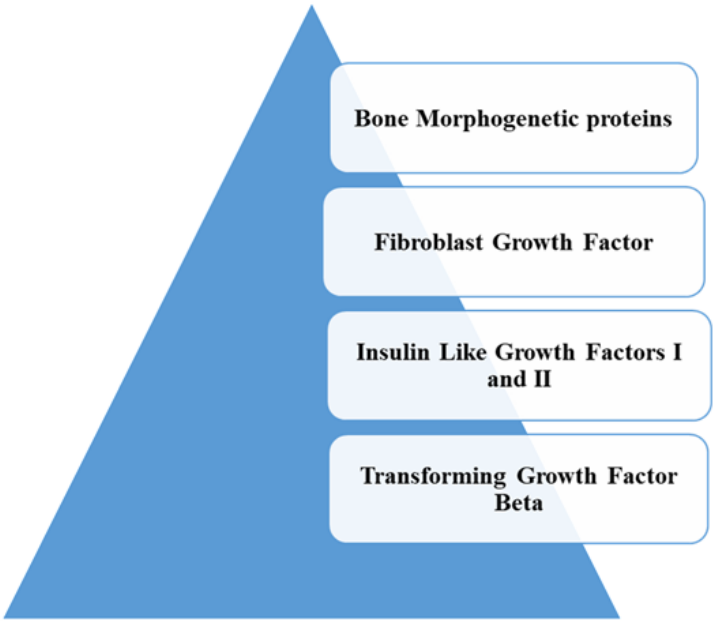

Figure 2. 7 Types of BTE Growth Factors

Growth factors are when incorporated with BTE scaffolds they enhance the healing process of injured tissues. the growth factors have short life span and capable to diffuse into other tissues due to which they have to be used in large quantities. However, the use of growth factors in scaffolds is limited as they are not able to bear harsh processing condition of the scaffolds as well as are highly expensive. Special protocols must be also followed for their preservation that increase the overall cost of the scaffold in return [53, 54].

Side effects of BMP-2 growth Factors:

Bone morphogenetic proteins are vigorously studied as they lead osteoprogenitors and mesenchymal cells to undergo mitogenesis which will then differentiated to osteoblasts out of 15 BMPs which are found in human body, BMP-2 is the most suitable one for the bone formation. But BMP-2 also shows some drawbacks which are listed below

- Formation of ectopic bone

- Swelling of site where surgery is done
- Neurological side effects[55].

2.7. Cells for BTE Scaffolds:

For repairing of damaged bone, designing a delivery system of progenitor/stem cells from cell source to repairing site is one of the vital need for bone tissue engineering. As the cell sourceand their type affects the quality BTE scaffolds. [56]. Use of active and alive cells in BTE results in providing the desired extracellular matrix required by tissue for repairing and regeneration

Properties of cell source:

Cells source for BTE must possess the following properties

- Numbers of cells should be high.
- Should not initiate immune reactions in the body
- Should not form tumors
- Must possess osteogenic potential and must be able to differentiate in to bone tissues under given conditions[57]

(a) Autologous cells

one of the safest method is to use autologous cells which are obtained from patient by isolating the osteoblasts cell of biopsies and then *in vitro* limited expansion is done. these cells are used as they are non-immunogenic [58].

Disadvantages of autologous cells

- Time consuming
- Low expansion rate
- Provide small amount of cells after completing the tissue dissociation
- Cell seeding on scaffold is effected by poor expansion rate.[58]

(b) Xenogeneic Cells

These are the cells which are donated by non-human sources. These sources can provide large amount of cell but there are several disadvantages associated with it

Disadvantages of xenogeneic cells

- Immunogenic reactions
- High chances of transmission of infectious agent
- Association of social and ethical problems[57]

(c) Stem Cells

Stem cells are capable to undergo self-renewal as well as has ability to differentiate into several types of cells. A lot of work is done on using stem cells is scaffolds of BTE. Studies are made on differentiation of stem cells to follow the osteogenic pathway so that it can result in intramembranous ossification Some of properties of stem cells are mentioned below[58, 59].

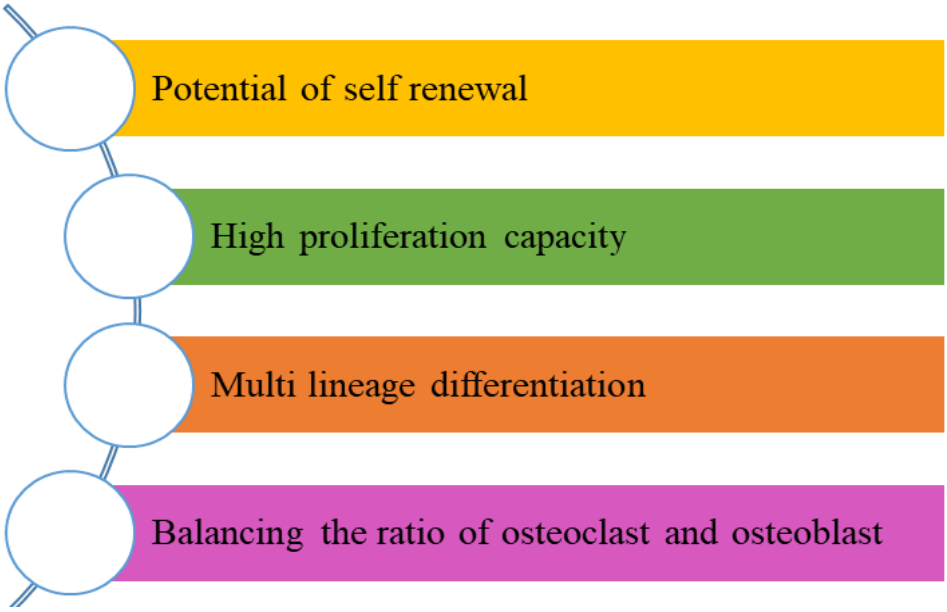

Figure 2. 8 Properties of stem cells

In natural system, bone remodeling is also one of responsibilities of stem cells as they balance the ratio of osteoclast an osteoblast throughout life.

Cell sources usually used for BTE are given below listed [60].

Cell Source	Advantages	Disadvantages
Bone marrow derived Mesenchymal stem cells	High osteogenic potential Studied extensively	Low abundance Requires extensive in vitro expansion
Embryonic stem cells	Pluripotency Capable of differentiating into all cell types in bones	Ethical and regulatory constraints Can produce teratomas when transplanted in vivo
Adipose derived stem cells	Similar osteogenic characteristics as BM-MCSs Highly abundant; easy to harvest surgically	More studies needed to test their use in bone repair
Umbilical cord blood mesenchymal stem cells	High availability Broad differentiation and proliferation potential Higher in vivo safety than embryonic stem cells	More difficult to isolate than MSCs from marrow More studies are needed to test their use in bone repair
Induced pluripotent stem cells	Pluripotent Capable of differentiating into all bone cells types	Reprogramming efficiency is low Require extensive expansion Safety concerns; limited clinical applications
Adipose derived stromal vascular fraction	Abundant; easily harvested via liposuction Able to form vascularized bone	Cell population varies among donors 2-3 hours multistep isolation process

Figure 2. 9 BTE Cell Sources

Recruiting stem cells to the trauma site is referred by the term "**Stem cell Homing".**

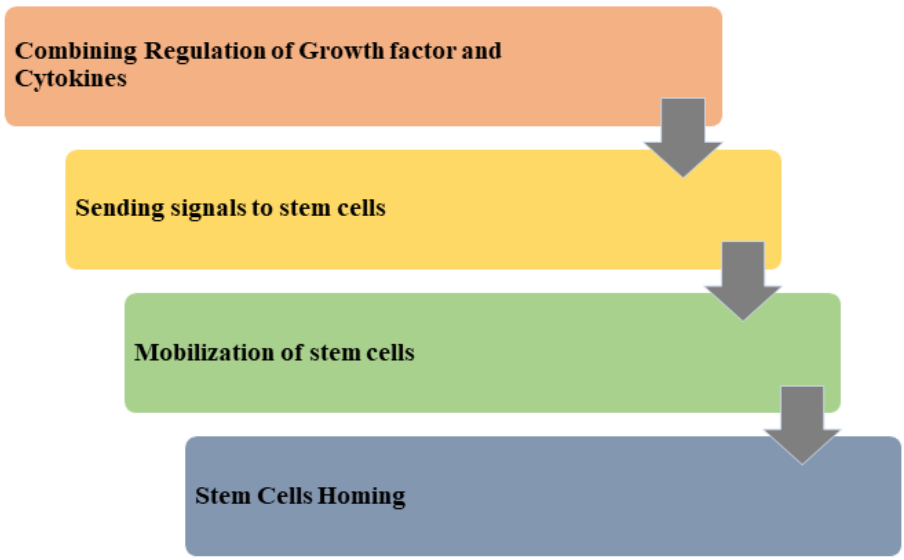

Figure 2. 10 Pathway of Bone Healing

To enhance the cell homing at the affected site two types of approaches are usually followed

Cell Based Approach: stems cell are engineered in a way to show markers which will guide them to regenerative site.

Scaffold Based Approach: engineered scaffolds are implanted at trauma site which are responsible for releasing growth factor and cytokines results in mesenchymal stem cells homing[61, 62].

2.8. BTE Scaffolds

Scaffolds required for bone tissue engineering must possess certain structural properties at micro and macro level to make sure the apt tissue growth. some of these properties are as follow

- **Osteogenic properties**: Scaffold should be osteo-integrated and have osteogenic, osteoinductive and osteoconductive properties. which allows adhesion, cell proliferation and extracellular matrix formation at the surface
- **Biocompatible**: BTE scaffold should favors cellular activity and must be well integrated with biological system of bone without showing any toxicity.

- The **microstructure** of BTE scaffold must support the ingrowth and vascular activity of bone
- **Porosity:** appropriate porosity will lead to the vascularization of new osteoid and results into stability in oxygen and nutrient supply that is essential for cell proliferation. The scaffolds with a porosity of more than 80% and pore size between 50-100 micrometer shows best activity for cell proliferation and tissue ingrowth.
- **Biodegradation**: a controlled *in vivo* degradation rate of BTE scaffold is required to provide new bone tissues sufficient space for growth. The by-products of degradation must also not induce any toxic effect in the body.
- **Mechanical Properties:** these properties of BTE scaffold should be in contrast to the mechanical properties of bone under fixation to ensure the load transfer to the bone
- **Drug Delivery**: Bone damage provide good site for microbial growth. To avoid infections and to speed up the process of bone healing the scaffold must be able to actively deliver the drugs or biologically active molecule at the required site[52, 63-65].

2.9. Hydrogels as BTE Material:

Latest research in BTE emphasis in use of Hydrogel based scaffold as they are biodegradable and their structural irregularity making them perfect for the of individual personalized medication. Hydrogels are hydrophilic three dimensional polymeric network possessing several covalent/noncovalent interactions. Hydrogels has ability to be swelled up to hundreds to thousands of times of its original weight without disintegrating into water. the structure of hydrogels shows close resemblance with the extracellular matrix of bones so these are widely used in bone tissue regeneration[66, 67]. Fabrication of hydrogels usually takes place by polymerization of hydrophilic polymers or by crosslinking of existing polymers. To make the hydrogels biologically compatible to the system, the use of toxic monomers and cross linkers must be avoided. During the appropriate process of crosslinking, encapsulation of different drugs, growth factors or nucleic acid can also be done in hydrogel[25, 68, 69].

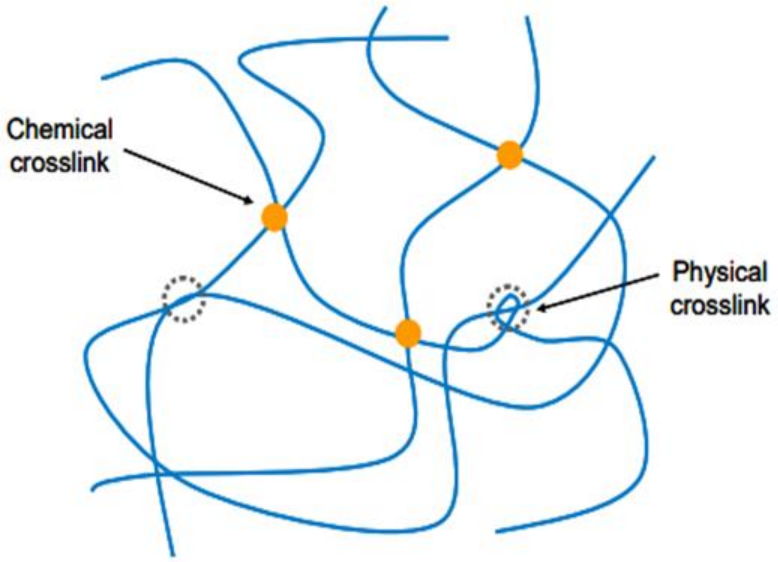

Figure 2. 11 Hydrogels

2.9.1. Requirement for Hydrogels:

- To enhance the bone regeneration, hydrogels must be osteogenic, osteoconductive osteoinductive and osteocompatible.
- For best adhesion of cells, their propagation and differentiation, hydrogels should impersonate natural extracellular matrix of tissues at implant site.
- Should be non immunogenic so that inflammatory response can be avoided.
- Porosity and pore size of hydrogels must be controlled so that it can control the release amount of bioactive factor.
- Hydrogel should be stable must maintain their structural integrity.
- Should be biodegradable. i.e. by hydrolysis or endogenous enzymes result in providing enough space for the formation of new bone
- Should have simple administration process[70, 71].

2.9.2. Hydrogels Configurations used for Bone Regeneration

A lot of hydrogel configurations are currently used for bone remodeling and regeneration which can be fabricated by using different techniques. Some of hydrogel structure used in bone regeneration are as below.

Hydrogel Microbeads: Polymeric hydrogels can be used in the form of micro beads and can be synthesized by emulsification, coaxial air jetting and polymerization (in situ)[56].

Hydrogel Fibers: these possess fibrous morphology with the diameter between nanometer to micron. Fabrication of these hydrogel involves spinning followed by crosslinking. These types of hydrogels can be implanted to the trauma site by injecting [72, 73].

Nano gels: these are also known as hydrogel nanoparticles as they are spherical particles formed by polymeric crosslinking either by physical or chemical means. These have high applications for bone regeneration as they are biocompatible and have good mechanical properties. As for the other properties like surface , size etc. they show same characteristics like nanoparticles[60, 74] .

2.9.3. Injectable Hydrogel

The hydrogels which can be directly introduced on the fracture site shows many potential advantages like avoiding the nonunion of long bone, potential damage to surrounding environment, and morbidity of donor site. In biomedical uses, especially for bone tissue engineering, hydrogels are cross linked in in situ environment by means of covalent bonding where gelation of hydrogel takes place under the influence of physiological conditions[75, 76]. To facilitate bone regeneration and cell proliferation hydrogel required some necessary properties which are as follow

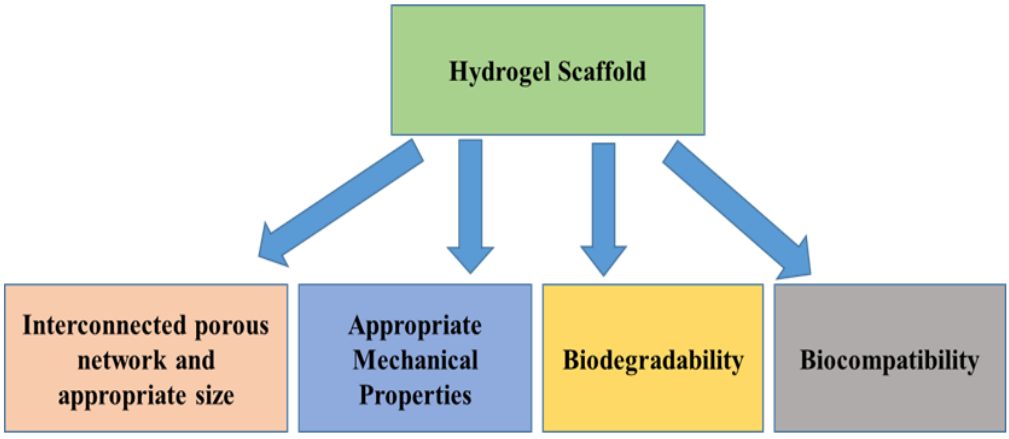

Figure 2. 12 Properties of Hydrogel Scaffolds

2.10. Biomaterials for BTE :

Biomaterials are defined as "Materials, natural or synthetic, that can be incorporated within the living organisms to replace or treat organs, tissues or functioning of body are considered as biomaterials". The artificial materials, e.g., artificial limbs and hearing aids, are not considered to be as biomaterials because they are in contact with the skin only and are not incorporated into the body[32, 43].

biomaterials interact with body and perform certain functions like repairing, replacing, treating, and regenerating the given site of body by the aid of their constituents. Each biomaterial has its own characteristic properties which is used to perform specific functions in body. Going all the way back to the dawn of civilization, gold was utilized in dental applications by the Romans, Aztecs, and Chinese. The Mayans were discovered to have made dental implants out of seashells. Biomaterials are utilized to supplement, repair, or replaces any bodily tissues, organs, or functions that has been lost due to trauma, illness, or damage.

2.10.1. Polymers:

Natural as well as synthetic both types of polymers have been extensively studied for Bone tissue regeneration (BTR) scaffolds. Natural polymers are attributed with the characteristics of biocompatibility, biodegradation, can enable adhesion and are capable to migrate cells in their unique morphological structure. Whereas advantages related to synthetic polymers are that their properties like elasticity, porosity, stiffness, binding groups, and architecture can be controlled according to the requirements. Various types of biocompatible polymers like alginate, gelatin, chitosan, polyvinyl alcohol, poly lactic co glycolic acid, collagen, polycaprolactone, hyaluronic acid, Polyethylene glycol etc., are utilized in bone tissue engineering applications. Some of naturally degradable polymers are listed below [76].

Table 2 Naturally Degradable Polymer

NATURAL: DEGRADABLE POLYMERS	
Polymer	**Applications**
Collagen	Drug delivery, cell guidance to tissue engineering in dental Application, gene delivery, orthopedic applications, blood vessels reconstruction scaffolds, homeostatic agent, closure of wounds, repair of spinal Dural
Gelatin	Coatings encapsulation to aid oral drug delivery
Fibrin and Fibrinogen	Cell delivery and tissue sealant
Hyaluronic Acid	Drug delivery, synthetic grafting of bones, tissue Engineering, wound dressing, tissue Engineering
Elastin	Coatings for vascular grafting, Drug delivery
Polysaccharides	Wounds dressing, drug delivery, cell Encapsulation, healing of wounds, cells Encapsulation
SYNTHETIC: DEGRADABLE POLYMERS	
PGA and Copolymers	Hormone Delivery, encapsulated coatings for dental care, orthopedic applications, drug delivery, urological stents, injectable fillers, sutures, membrane barriers
Poly(propylene fumarate)	Orthopedic applications
Poly (ortho ester)	Stents, Drug delivery, fracture fixator of non-weight bearing bones
polydioxanone	Wound clips, sutures,
PLA	Drug delivery, orthopedic applications

Electrically Conducting Polymers:

The electrical signal produced endogenously in human body has an important place in micro environment of bone. It's been in practice for the treatment of many osteoporotic

diseases and fracture, electrical stimulation is provided externally as external stimulation is capable to enhance

- osteoblast differentiation.
- Cell proliferation
- Adhesion
- Spreading of cells

In last 15 years' researcher's attention are diverted towards developing a new family of organic polymers which have capacity of conducting electrical current. The reason behind their extraordinary conducting properties is the presence of conjugated backbone where overlapping of pi orbitals take place at high degree. With the help of doping, neutral polymers can also be charged positive or negative. The oxidize backbone of polymers along with polarons and bipolarons are responsible for generating and propagation of charged polymers. Polypyrrole is one of the most renowned polymers materials used as electrically conductive polymer

Polypyrrole;

Polypyrrole is an aromatic polymer known as poly(heterocyclic). Synthesis of polypyrrole of polypyrrole can be done by both i.e. electrochemical method and chemical method.[77] Diaz along with his coworkers was the first one to synthesize polypyrrole while its neutrality is maintained by the help of dopant ions that are incorporated in the backbone of polymer. While Donald Weiss and his colleagues were the one who reported the conductivity of polypyrrole in 1960's[78]

Conductive polypyrrole is excellent electroactive biomaterial which can be used in coatings directly and useful to maintain the cell behavior along with metabolism. It is biologically inert but biocompatible and can transmit electrical signals effectively in the body.[15, 16]

Mechanism of polypyrrole conduction:

Polypyrrole conduction occurs by removing the electrons, generating free radicals which lead to oxidation of polymer chain . Delocalization of these ions over the polymer backbone portion creates a change or defect in structure termed as polaron, this polaron have both i.e. spin as well as charge.

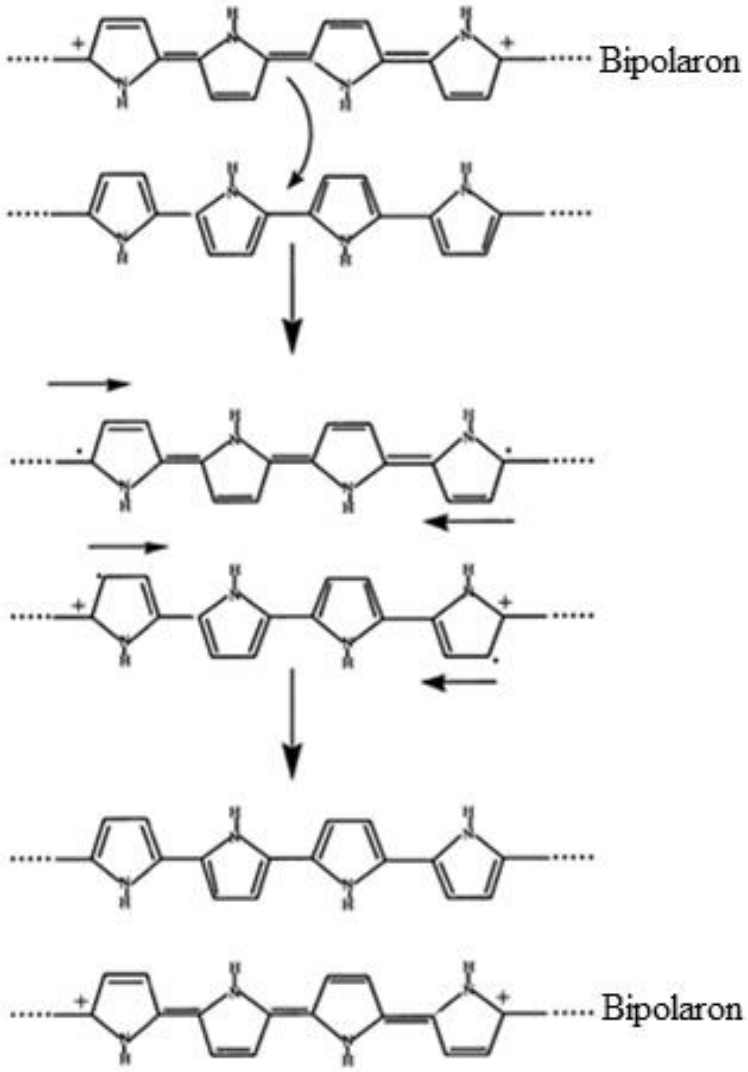

Figure 2. 13 Mechanism of Polypyrrole Conduction[16]

Diffusion of two polarons take place and bond is formed by combining the spin of both polarons. Electrical conduction of polymer is usually done with the help of charge carriers. Here positive ions act as charge carrier

Polypyrrole shows thermal as well as chemical stability. It is biologically active material which is comparatively easy to synthesized. By changing the polypyrrole oxidation state or wettability using appropriate dopants . it is possible to control the interactions of cell with surface and function of PPy thin films[16]

Applications of polypyrrole

- Amperometric Glucose sensors[79]
- Enzyme based immobilized analyte [80]
- Creatinine biosensors[81]
- Biological compatible for human cells
- Bone regeneration applications[82]

BTE focuses on improving the osteogenic cells proliferation on a growth factor containing BTE scaffold which should be porous and shows osteoconductivity. As the osteocytes shows response towards the electrical charges, research studies are made to fabricate scaffolds which contains conductive biomaterials to meet the electrophysiological necessities of bone tissues[59, 64]

Langer R Group reported a studies of using 2 dimensional polypyrrole films for osteogenic cells stromal cells differentiation obtained from bone marrow[61]

Zanjanizadeh et al. utilized Polypyrrole to enhance the mechanical properties of a scaffold mesoporous silica containing polypyrrole scaffold was fabricated which improved the mechanical strength of scaffold from 7MPa which is comparatively equivalent to mechanical strength of cancellous bone[62].

Pelto et al. reported a research on enhanced cells proliferation as a result of PPy based scaffold[83].

2.10.2. Sodium Alginate:

Alginate is the polymer which can be extracted from brown algae. It is a linear copolymer whose backbone consists of successive blocks linked at (1-4)a-L guluronate which form G-Block and b-D mannuronate generating M-Block which are then followed by alternate MG Block[84, 85]

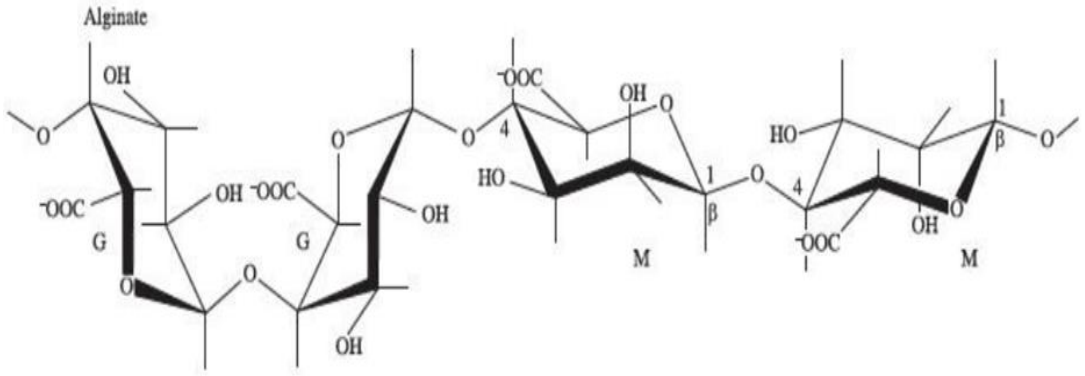

Figure 2. 14 Alginate Polymer[76]

Mechanism of alginate hydrogel formation:

Mechanism of Alginate transition from solution into gel form is ionic in nature. Under existence of divalent cations, G-Block present in the chain of alginate polymer G-Block changes its structure forming egg like morphology. The change in morphology is the result of intermolecular bond formation of divalent cations with the two OH(hydroxyl groups and two deprotonated COO^- (Carboxyl groups) of G-Block[84, 86-88].

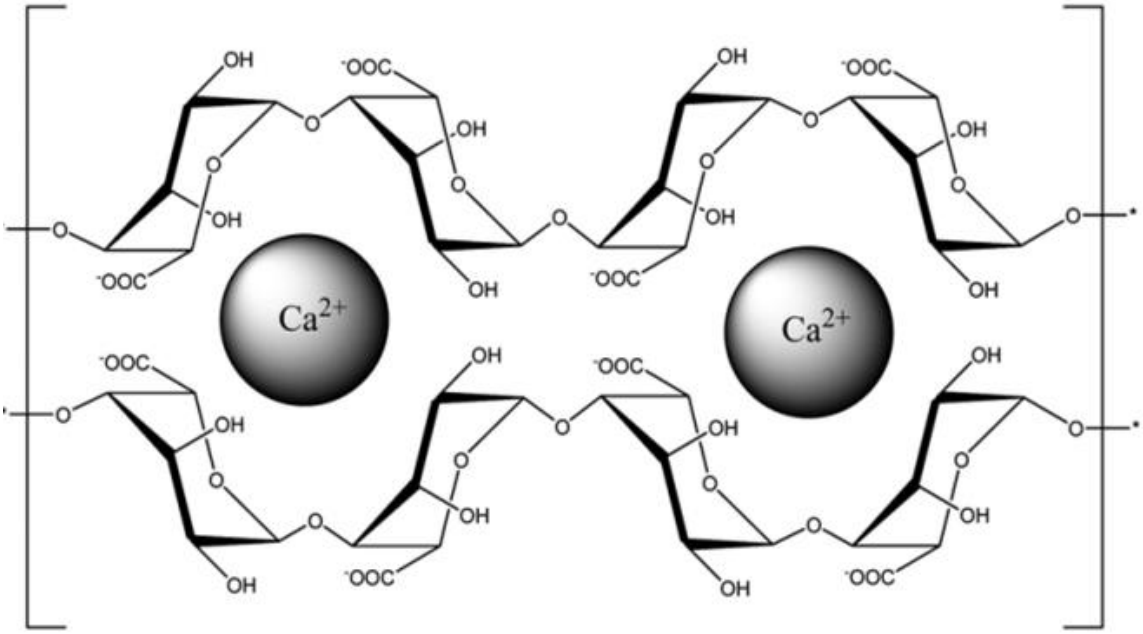

Figure 2. 15 Mechanism of Alginate Hydrogels Fabrication[77]

Selection of Alginate for injectable Hydrogels:

Alginate hydrogels are widely used for fabrication of injectable hydrogel in tissue Engineering. One of its advantages is that gelation rate of hydrogel can be controlled and mechanical stability can be achieved within desire time. As due to trauma , bones often fractures in irregular manners but Irregular defect morphology is not a problem for injectable scaffold as compare to other conventional static scaffolds used for tissue engineering.[89, 90]

Mamujudar was the first one who reported fabrication of alginate based 3-D matrix for culturing of Bone cells in 1991. In this studies cell lines of osteoblasts and calcium chloride cross-linked alginate gel was used [91].

In 2001 **kuo & Ma** made an insitu studies on gelation of alginate where gelation kinetics was controlled. In this studies MC3T3 cells were encapsulated by alginate[18].

In 2018 **segredo et al.** prepared injectable scaffolds for bone regeneration of damaged sites caused by osteoporosis. these scaffolds were thermos responsive in nature. These hydrogels were cross-linked ionically[92]

2.10.3. Gelatin:

Gelatin is the natural polymer which is attained as a result of partial hydrolysis along with denaturation of collagen. The random helix morphology of collagen is disrupted and results in random coils of gelatin which can be renatured under the influence of cooling. Although morphology of collagen is transformed, the chemical composition of gelatin is almost similar to collagen

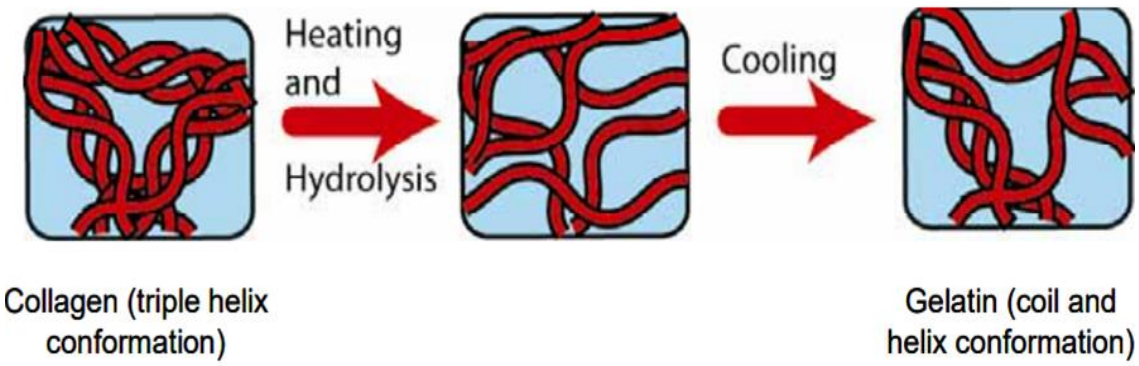

Figure 2. 16 Gelatin Morphology

Due to lesser aromatic groups present in gelatin structure it is almost non immunogenic in nature. It is regarded as safe product by FDA and biodegradable in nature. The byproducts which are formed as a result of enzymatic degradation of gelatin are also biocompatible in nature[93-96].

In water, Gelatin thermos-reversible network. The transition temperature of gelatin (conversion of sol into gel) is around 30^0 C. the rigidity of gelatin hydrogels is not only temperature dependent but also concentration, any additive, pH also plays its part to provide a rigid structure.

Several studies are made to use gelatin scaffolds for biomedical applications like wound healing, cardiac tissue regeneration, and skin regeneration. Different methods including fiber bonding, freeze drying, foam templating, electrospinning were used for fabrication of gelatin based scaffolds.[93, 96]

2.10.4. Bioceramics:

Ceramics used as biomaterials are referred to as bioceramics [24]. Use of bioceramics for treatment of bone defects have long history. back in 975AD, calcium sulphates were used for fabrication of cements used in bone setting. existence of calcium phosphates in bones were reported in 1769. And then in 1800, researchers put a lot of efforts to analyze the calcified tissues present in bones and then classified these calcium phosphate derivatives. First scientists started to utilize ceramic material as substitutes and supporting material. In 1970's after successfully synthesizing bioglass, scientist started to work on using bioceramics for bone regeneration instead of substitution

As 80-90% of bone inorganic phase consists of calcium phosphate based minerals such as hydroxyapatite. CaP's are biocompatible Ca^{+2} & PO_4^{+3} ions released from calcium phosphates aids in binding with bone and lead them to the bone tissue formation. CaP's allow bone the proteins absorption as well as differentiation, migration and attachment of cells. The ideal pore size for osteogenic differentiation lies between 300-500 micrometer which allows the good exchange of nutrients from pores but one of the drawback is it cause brittleness.

For bone regeneration, in comparison with other bioceramic, hydroxyapatite with chemical formula is $Ca_{10}(PO_4)_6(OH)_2$ is the one which extensive studies are made as it is present in bones abundantly as well as have easy synthesis methodology and bioresorbable. However, HA shows bio inertness and uncontrolled degradation. some draw backs such as poor mechanical stability, Other mineral phases are di-calcium phosphate, di-basic calcium phosphate, tri-calcium phosphate, and a number of amorphous calcium phosphates [25].

In general, ceramics containing calcium phosphate are biocompatible and their chemical composition shows strong resemblance with native bone chemistry. These features make them ideal candidates for knee joints, orthopedic implants dental implants and stents [26].

Table 3 Commonly used CAPs in Biomedical Applications [97, 98]

Commonly used calcium phosphate in biomedical application		
Name	**Formula**	**Ca/P Ratio**
Monobasic calcium phosphate monohydrate	$Ca(H_2PO_4)_2\ H_2O$	0.5
Monetite	$CaHPO_4$	1.0
Dibasic Calcium phosphate dehydrate	$CaHPO_4.2H_20$	1.0
α-TCP	$Ca_3(PO_4)_2$	1.5
β-TCP	$CA_3(PO_4)_2$	1.5
Whitlockite	$(Ca_{18}Mg_2\ (HPO_4)_2(PO_4)_2)$	1.43
Hydroxyapatite	$Ca_{10}(PO_4)_6(OH)_2$	1.67

2.10.5. Whitlockite:

For regeneration and integration of bones, magnesium plays a vital role. So to enhance the biological as well physical properties of materials used for bone repair, many derivatives of Hydroxyapatite and β-TCP synthesized in which magnesium was incorporated but during their synthesis original morphology of HA was compromised. In the meantime, it was learnt that bone contains another inorganic mineral which contains magnesium and shows crystal structure similar to class of mineral whitlockite. Bone whitlockite is differentiated from mineral whitlockite as it contains both magnesium and hydrogen and have chemical formula ($Ca_{18}Mg_2(HPO_4)_2(PO_4)_2$) and calcium to phosphorus ratio of 1.43. β-TCP was utilized by scientist for long period of time as it shows same crystalline structural similarity with WH but its chemical composition is different from bone whitlockite.

Whitlockite surface has negative charge and it shows stability in acidic environment. It is responsible for delayed osteoporotic bones and confirmed its solubility in physiological solutions.[21, 22]

Characteristics of whitlockite:

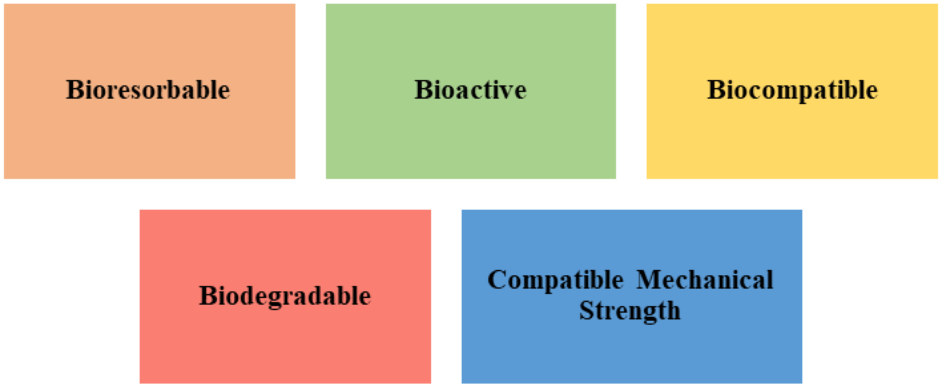

Figure 2. 17 Characteristics of Bone Whitlockite

Whitlockite is the material which is difficlt to synthesize as it exists in several phases and all of these phases are stable unfder ambinet environment. However in recent studies optimum condition for pure phase of bone whitlockite was reported along with its application as adsorbent for heavy metals, Bone tissue engineerinf and homeostatic agent .

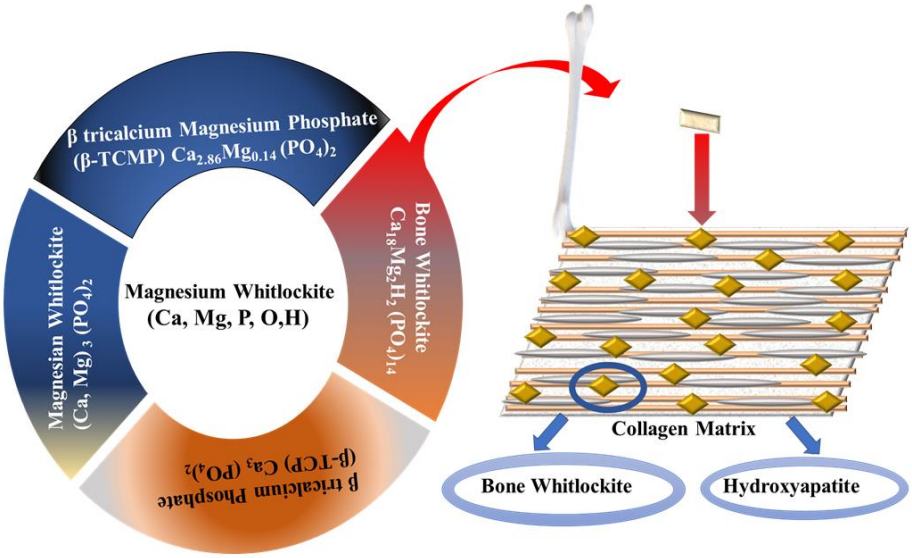

Figure 2. 18 Schematics of Mg(WH) and Bone WH [21, 22]

$Ca_{18}Mg_2H_2(PO_4)_{14}$

Advantages of Whitlockite Scaffolds

- Whitlockite Crystals has hexagonal geometry so it shows excellent mechanical stability as hexagonal geometry helps in superior stress distribution.
- Negatively charged surface of whitlockite aids in good cell adhesion.
- Whitlockite is a bioactive material so helps in osteoconduction[21, 22].

2.10.6. Composite Biomaterials:

The creation of smart biomaterials for tissue regeneration arouse the interest of many researchers. The composite method of integrating biomaterials in the form of biopolymers and/or bio ceramics, either synthetic or natural, opens more possibility. The creation or identification of materials capable of encouraging desired cellular and tissue behavior is a key problem in tissue engineering. Given that few biomaterials have all the essential qualities to operate optimally, engineers and physicians alike have sought the creation of hybrid or composite biomaterials to synergize the positive attributes of various biomaterials elements into an improved matrix. The mixing of natural and synthetic polymers with a variety of different materials has been shown to improve cellular contact, stimulate integration into host tissue, and give adjustable material characteristics and degradation kinetics. The ideal tissue engineered scaffold is porous interconnected structure that supports cell growth, cell proliferation and cell

differentiation. To achieve this, superposition of two or more materials is required. Ceramic-polymer composites are gaining popularity as possible fillers for bone deformities. Tricalcium phosphate, hydroxyapatite and whitlockite are three of the most widely used calcium phosphate ceramics, have proven acceptable biocompatibility as well as osteoconduction and osteointegration. [21, 99]

Bioactive glass and Bioceramic, have also been shown to stimulate bone bonding and vascularization. However, these materials are shows some limitations as they are too stiff and brittle to be utilized on their own. So one of the smart approach is mixing of a ceramic to a polymeric materials to impart desired characteristics as bioceramic – polymer composite can provide i.e., combining the inorganic phase's osteoconductivity and bone-bonding potential with the porosity and interconnectivity of the polymer [99-101]

Nanocomposites enable for larger quantities of ceramics to be used, resulting in improved mechanical characteristics such as increased tensile strength, bending strength, impact energy, and moduli closer to the order of natural bone while preserving an interconnected architecture [102]

2.11. Bone piezoelectric Effect

Piezoelectric effect refers to ability of electricity production by certain materials when they undergo mechanical stress. Bones are capable to induce piezoelectric effect in them by producing electrical energy from mechanical stress. In early 1950's, phenomena of bone piezoelectric effect was reported by the scientist Several *in vitro* as well as *in vivo* studies has been made to search about piezoelectric characteristic of bones by using different piezoelectric biomaterials along with trying different pathways of deformation in order to speed up cells migration and mineral deposition on regenerative site. Several studies on difference of piezoelectric potential of dry and wet bones awere also made[103-108].

As bones consists of contains 30% organic and 70 % inorganic minerals contents. When mechanical stress is applied , the collagen fibers (organic phase) of bone induce potential gradient causing the particles of surrounding area to be charged. These electrically charged particles started to travel on bone surface or scaffold and provide stimuli to osteoblasts for formation of new bone [109-111]. Mechanism of

piezoelectric conductive bone scaffold effect on mechano-responsive features of bone is shown in the figure given below [109]

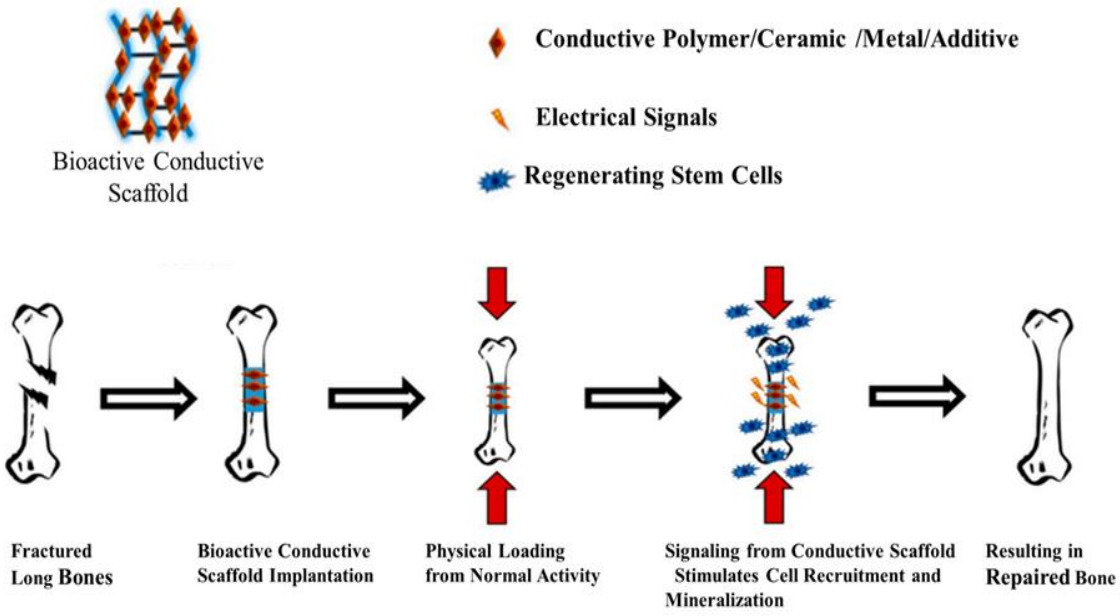

Figure 2. 19 Mechanism of Piezoelectric Conductive Scaffolds [109]

2.12. Research Gap

Many researches are made previously to use calcium phosphate based bioceramic material especially hydroxyapatite (a bioceramic that is present in our bones naturally) for regeneration of bones. Another bioceramic material known as bone whitlockite which constitutes 26-28% of bone and its synthesis in pure form without phase mixing was a challenging process for researchers. Successful Synthesis of whitlockite which is osteoconductive, biodegradable and bioresorbable magnesium containing bioceramic material open a new path to use this material and its effect on bone regeneration. Unlike other bioceramic materials which are usually insulator in nature whitlockite shows piezoelectric properties. As endogenous currents that play vital role in bone regeneration and fracture healing and previous studies proves that conductive biomaterials are more helpful in process of bone regeneration. Along this, in past research studies most of the BTE materials and scaffolds are static in nature so cannot be used at the sites with irregular bone defects as well as they are implanted at defective sites by invasive surgical methods which can cause potential harms. So it is need of time to prepare an injectable scaffold which can overcome this problem. A

single material cannot possess all these properties, so in our studies we developed a hybrid material to obtain our desired properties.

2.13. Problem Statement

Synthesis of BTE scaffolds for healing of irregular bone defects and bone regeneration is always challenging for researchers. Although many biomaterials and scaffolds are developed by BTE methods still there is a need to develop a system for individualized treatment of bone defects which has same extracellular matrix like bone, should be conductive in nature with minimum surgical invasiveness during implantation at effective site.

2.14. Aims and Objectives

In the field of BTE, injectable hydrogels system is one of less explored area although it is one of the advanced method for Individualized treatment of bone defects as this system is capable to provide scaffolds with perfect geometry to the patients for treatment of the specific bone defects.

The fundamental goal of this studies is to fabricate bioceramic containing polymeric injectable conductive hydrogels for various applications such as specified individualized treatment and bone regeneration using different concentrations of whitlockite which have good Injectability and gelation rate. The specific objectives of this thesis are as follow:

- Fabrication of novel composite based injectable conductive hydrogels.
- Characterization of material with available techniques.
- Investigation of viscosity of formulated hydrogels and optimization of Injectability and gelation rate of hydrogels along with exploring ionic conductivity of injectable conductive hydrogels. Measuring the cytotoxicity of prepared hydrogels.

Chapter 3

Experimental

3.1. Synthesis Route:

For the synthesis of whitlockite nanoparticles and hydrogels fabrication, bottom-up approaches were used. Bottom-up approaches are discussed in detail below.Whitlockite Nanoparticles were prepared using liquid precipitation method. It is solution of two or more substances which are ionic in nature are reacted chemically to produce insoluble solid material. The produced solid substances should be denser than liquid material. Precipitates are removed from solution by means of physical methods like filtration Obtained precipitates are than dried and further used. Whitlockite-polypyrrole and alginate composite was prepared using polymerization technique. In this process small building blocks also known as monomer are linked chemically together to produce macromolecules and then large number of these macromolecules combined to synthesize polymer. Finally, whitlockite containing polymeric injectable conductive hydrogels were synthesized and hydrogels were cross-linked using ionic crosslinker.

3.2. Selection of material:

Whitlockite is a biocompatible, biodegradable and bioactive material which helps in osteogenic differentiation of cells required for bone regeneration. Whitlockite Crystals has hexagonal geometry so it shows excellent mechanical stability as hexagonal geometry helps in superior stress distribution. Negatively charged surface of whitlockite aids in good cell adhesion. Whitlockite is a bioactive material so helps in osteoconduction.

Alginate hydrogels are widely used for in fabrication of injectable hydrogel in tissue Engineering. One of its advantages is that gelation rate of hydrogel can be controlled and mechanical stability can be achieved within in desire time. As due to trauma, bones often fractures in irregular manners but Irregular defect morphology is not a problem for injectable scaffold as compare to other conventional static scaffolds used for tissue engineering.

Conductive polypyrrole is excellent electroactive biomaterial which can be used in coatings directly and useful to maintain the cell behavior along with metabolism.

Gelatin is used cause of its biomedical applications as it is biodegradable material have been used in tissue regeneration scaffolds in many studies

3.3. Selected Synthesis Method:

For the synthesis of whitlockite nanoparticles, the liquid precipitation method was employed. For the preparation of WH NPs and PPY composite, the chemical polymerization was used. For preparation of hydrogel chemical crosslinking method was used.

3.4. Materials Required:

- Magnesium Hydroxide (Duksan, Korea)
- Calcium Hydroxide (GPR Rectapur, Belgium)
- Orth phosphoric Acid (Honeywell, Germany)
- Dilute Hydrochloric acid (Sigma-aldrich, Germany)
- Ethanol (Sigma-aldrich, Germany)
- Sodium hydroxide (Emsure, Germany)
- Distilled water
- Pyrrole
- Sodium Alginate (Daejun,Korea)
- Iron Chloride Hexahydrate (Daejung, Korea)
- Gelatin (AppliChem, Spain)
- Calcium Chloride (Duksan, Korea)
- Phosphate Buffer Saline (Invitrogen, USA)

3.5 Fabrication of Injectable Hydrogel:

3.5.1 Synthesis of Whitlockite:

Whitlockite was synthesized by already reported precipitation method in which we used 0.13M magnesium Hydroxide ($Mg(OH)_2$ solution and 0.37M Calcium Hydroxide ($Ca(OH)_2$. Stirring was done for 20 min at 40 ˚C and then 0.5M orthophosphoric acid was added dropwise with feed rate of 10ml/5minute until pH 5 of solution was

obtained. Condensation reaction takes place for 10 hours and then we aged our sample for 14 hours. Then filtration of precipitates with subsequent washing was done and obtained sample was dried overnight at 50 ^{o}C. Dried sample was then converted into fine powder by using mortar pestle.

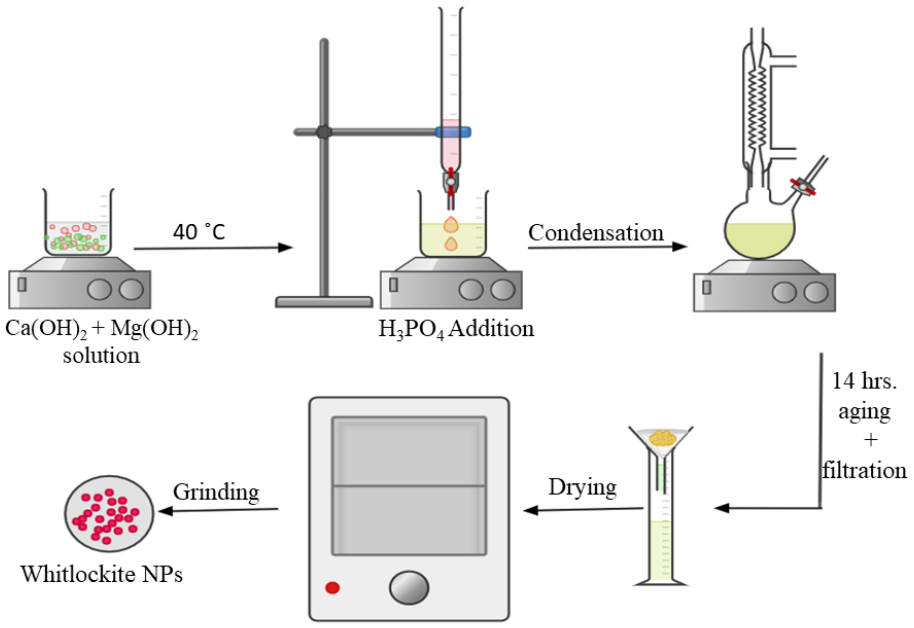

Figure 3. 1 Schematic Representation Whitlockite Synthesis

3.5.2 Synthesis of Whitlockite – Alginate grafted Polypyrrole Composite.

3wt.% solution of sodium alginate was prepared by dissolving 0.6g of sodium alginate in 20ml of water and stirring of about 4 hours. In parallel, 0.1 M pyrrole solution was prepared in 1 N HCl. This pyrrole monomer solution was added dropwise in sodium alginate solution. Whole mixture was stirred for 30 minutes at 90 $^{\circ}$C. The solution was than allowed to cool down to 50 $^{\circ}$C and sonicated for 15 minutes. 1wt% Whitlockite solution was prepared and sonicated for 10 min. this solution was then added pyrrole monomer containing alginate solution and the whole mixture was than sonicated for 15 min. after that solution was placed in ice bath to maintain the temperature for polymerization and 0.2M $FeCl_3$ was added drop by drop until the solution turns black. It was than stirred for 5 hours for complete polymerization. Solution was than filtered, washed and dried in vacuum oven. A fine powder of composite was obtained by using mortar pestle.

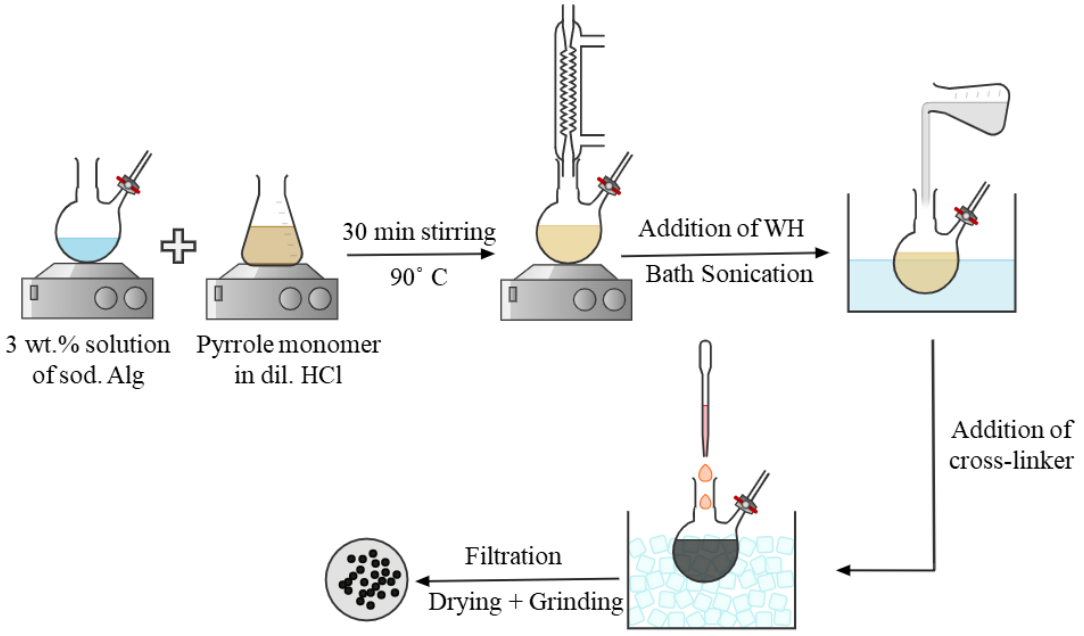

Figure 3. 2 Synthesis WH-ALG-PPY Composite

3.5.3. ALG-WH-ALG grafted Polypyrrole Solution:

2% of sodium alginate solution in phosphate buffer solution was prepared and whitlockite based alginate grafted polypyrrole composite was added to this solution with a ratio of 20:80 and stirred till it become homogenous.

3.5.4. Gelatin Addition in Composite:

A solution of gelatin was prepared by dissolving 5mg of gelatin in 10ml of PBS solution and added into the solution prepared in step 3. The pH of solution was maintained at 7.4 by using 1M NaOH solution.

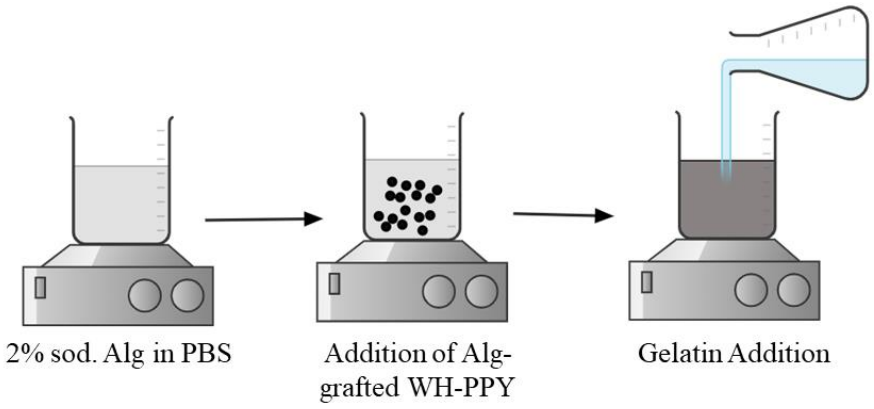

Figure 3. 3 Synthesis of WH-ALG-PPY-ALG-GLT Composite

Here is how gelatin containing ALG grafted PPY-WH soln. appears during experimentation

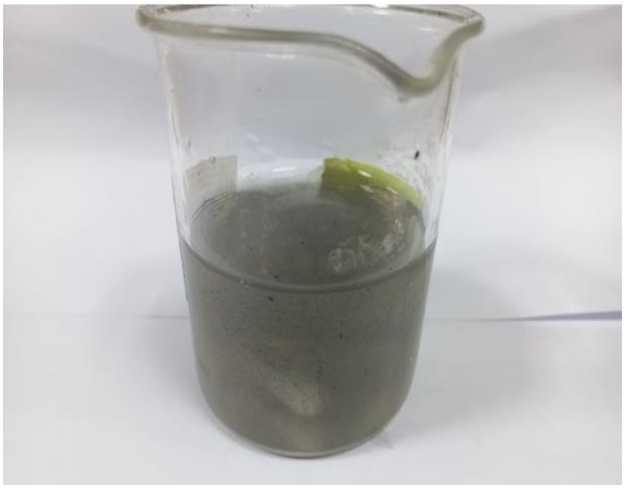

Figure 3. 4 Hydrogel before crosslinking

3.5.5. Crosslinking of Hydrogels.

0.2M solution of calcium chloride was prepared as cross linker and added into hydrogel immediately before injection. It was than stirred till homogenization.

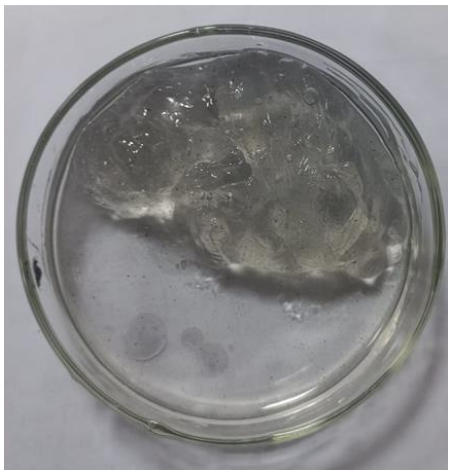

Figure 3. 5 Cross linked Hydrogels

By using the above mentioned method a composite containing four different concentrations of whitlockite (1%, 2%, 3%), were prepared. and a pure sample which do not contain any whitlockite concentration was also prepared.

Chapter 4

Characterization Techniques

In the present research project different characterization techniques were used to analyze the fabricated hydrogels. XRD, FTIR, SEM, Conductometry, Viscometry, Injectability and vial inversion test was used to characterize injectable conductive hydrogels sample.

4.1. Gelation:

For injectable hydrogels fabricated for Bone Tissue Engineering applications, Rate of gelation is their one of the most vital feature as it has to lie somewhere between neither to fast that it causes problems during surgical handling nor to slow as the hydrogel needs to attain *in vivo* stability as well as functionality. Normally gelation time for hydrogels ranges between 5 to 30 minutes.

In alginate based hydrogels concentration of alginate effects the gelation rate of hydrogel. As gelation times decreases with the increase in alginate concentration. In our study whitlockite is also responsible for change in gelation time of hydrogels. Here we are optimizing the conditions to set the gelation time for injectable hydrogels to prepare scaffolds which are appropriate for surgical procedures.

Mechanism that is responsible for conversion of solution into gel also supervise the gelation rate of hydrogels Different methods can be used to study the gelation rate of injectable hydrogels. Vial inversion test is the most widely used method, it is a table top method and based on rheological properties of material. This method is a based on naked eye observation where we noticed that if our gel is able to flow under its weight. The experiment is done by keeping the temperature constant.

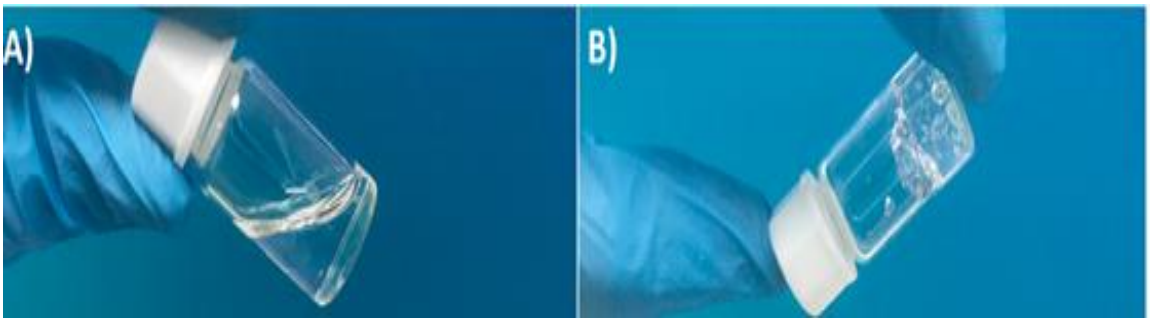

Figure 4. 1(A) hydrogel before gelation (b) Hydrogel after gelation

4.2. Injectability:

During the fabrication of injectable hydrogels, Injectability of hydrogel is the most vital factors and is directly influenced by viscosity of hydrogel. Injectability is referred to hydrogel release from the injection needle.

There is no specific standard is set internationally about performance of Injectability test for injectable hydrogels but scientists used different methods to measure the Injectability of hydrogel. In our study we used 21G and 24G needle and use following formula to measure the Injectability of material.

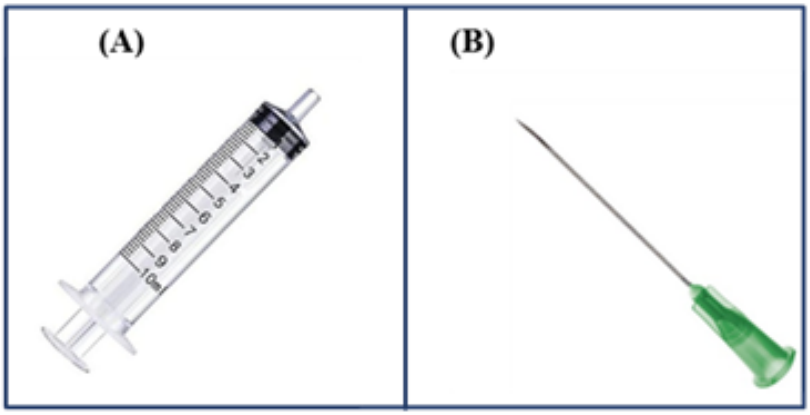

Figure 4. 2 (A) 10ml Syringes (B) 21G Needle

4.3. Fourier Transform Infrared Spectroscopy (FTIR):

It is a physio chemical spectroscopic technique which helps in determining the functional group of non-organic or organic compounds by determining their vibrations. molecules are when exposed to certain IR wavelength they get excited and shows vibrations. In composites FTIR help to determine presence of fillers as well as chemical bonding of materials.

On Electromagnetic Spectrum, Infrared region lies between the range of (14000-10) cm^{-1}. and can be further divided into three regions

- Near IR (400 - 10) cm^{-1}
- Mid IR (4000-400) cm^{-1}
- Far IR (14000-4000) cm^{-1}

THE mid IR region is also known as finger print region. All IR active molecules show their characteristic primary vibrations in this region which helps to identify the functional group.

In FTIR spectroscopy, when analyte is exposed to range of wavelength, molecular bonds shows absorbance at specific wavelength and undergoes excitation which leads to vibrations. These vibrations can be stretching vibrations, bending vibrations or contractions. IR spectrum is plotted between wavenumber on X-axis and transmittance or absorbance on Y-axis. It follows the Beer-lambert law which states that "Absorbance is directly proportional to concentration of material"

$A = l \varepsilon C$

Where ,A= absorbance, l =path length, C= concentration and ε= molar absorptivity

4.4. Scanning Electron Microscopy (SEM):

In this technique, the fine beam of electrons is focused over a specimen's surface. Photons or electrons are knocked off from the material's surface as a result. These knocked-off electrons are then focused on the detector. The output from the detector modulates the brightness of the cathode ray tube (CRT). For each point where the beams interact, a consequent point on CRT is plotted and the material's image is produced [112].

The electron-surface interaction causes the release of secondary electrons (SE), backscattered electrons (BSE), and X-rays [113]. The common SEM mode for detection is via secondary electrons. These electrons are emitted from near the sample surface. So, a pronounced and clear image of the sample is obtained. It can reveal sample detail even less than 1nm in size. Also, elastic scattering of incident electrons also takes place and releases backscattered electrons. They emerge from deeper locations as compared to secondary electrons. So, their resolution is comparatively low. When an inner shell electron knocks off from its shell it emits characteristic x-

rays [114].

We use SEM as it has easy sample preparation and we can figure our sample's morphology, chemistry, crystallography, and orientation of planes. Magnification of SEM can be controlled from 10 to 500,000 times.

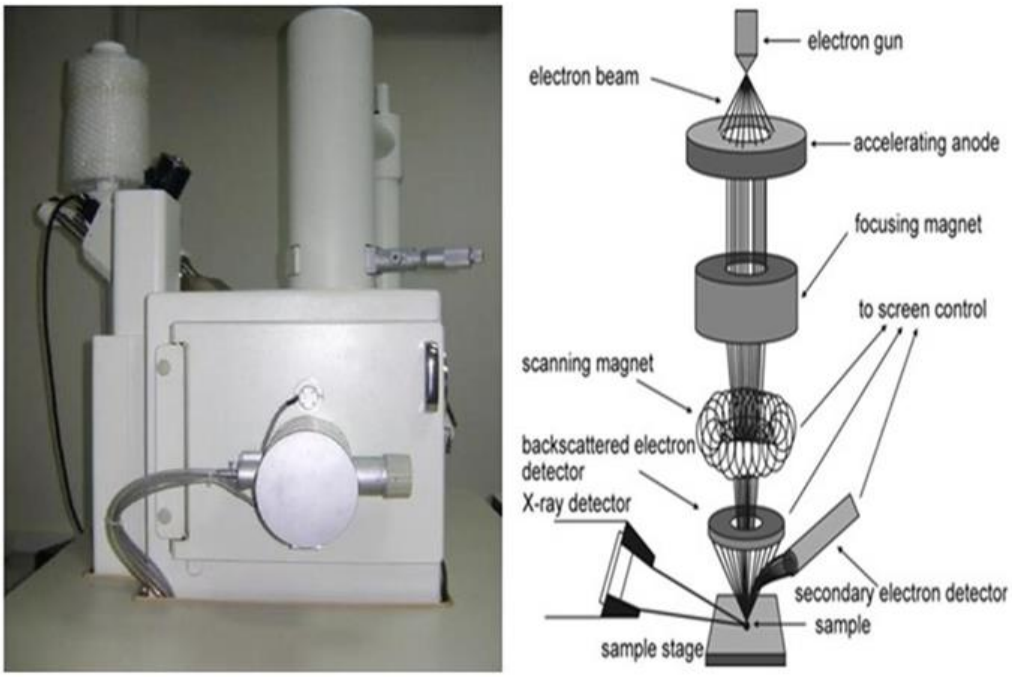

Figure 4. 3 (a) JOEL JSM-6490LA present at SCME; (b) SEM Schematic

4.4 MTT Assay:

This technique is used for evaluating the cytotoxicity if hydrogels by using "MTT (3-(4,5dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide" which is a cell viability assay.

MTT is a calorimetric assay in which metabolic activity of cell was measured by reducing the yellow colored MTT reagent into formazan crystals which are purple in color. MTT reagent is reduced as oxidoreductase enzymes dependent on NAD(P)H present in viable cell. after reducing formazan crystals are solubilized by solubilizing agent. Incubation of sample is done for several minutes till complete solubilization of formazon crystals. Absorbance of resultant solution was done at 550nm at Nanoweel spectrophotometer. The results are than plotted using Microsoft Excel

For cytotoxicity assay following steps were followed

- Culturing of cell lines
- Sub culturing of cell lines
- Cell counting
- Preparation of Hydrogel concentration
- MTT assay
- Counting of cell survival and cell inhibition percentage

Formulae for MTT Assay Calculation

For evaluation of MTT assay following formulas are employed:

% cell Survival = (Sample absorbance – Blank absorbance) / Control absorbance - Blank absorbance) × 100

% Cell Inhibition = 100 – percent cell survived

4.5 X-Ray Diffraction (XRD):

It is used for the crystal structure determination of the material. It is a non-destructive technique, and it provides fingerprints of Bragg's reflections of crystalline materials. It consists of 3 main parts. A cathode tube, sample holder, and detector. X-rays are produced by heating filament element which accelerates electrons towards a target that collide with target material with electrons. Crystal is composed of layers and planes. So, an x-ray which has a wavelength similar to these planes is reflected that that angle of incidence is equal to the angle of reflection. "Diffraction" takes place and it can be described as by Bragg's Law:

$$2d\sin\theta = n\lambda \quad \ldots\ldots. (3)$$

When Bragg's law is satisfied, it means there is constructive interference, and "Bragg's reflections" will be picked up by the detector. These reflection positions tell us about inter-layer spacing-ray diffraction tells us about the phase, crystallinity, and sample purity. By this technique, one can also determine lattice mismatch, dislocations, and unit cell dimensions. X-ray diffractions were performed by STOE diffractometer at SCME-NUST. The scan angle was taken from 10° to 90°.

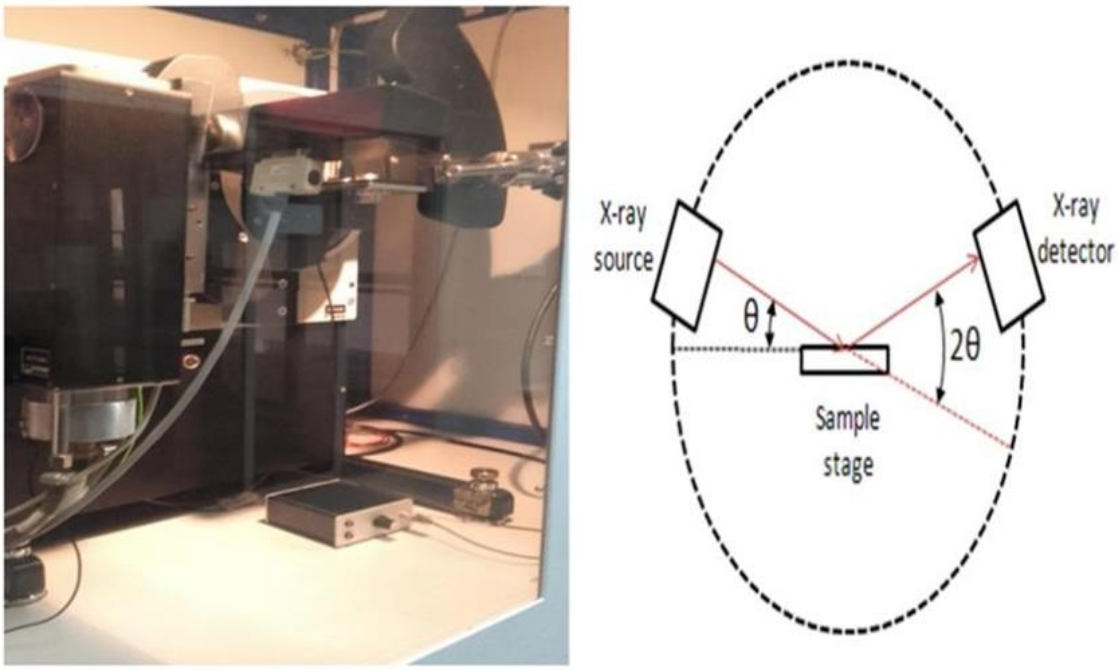

Figure 4. 4 (a) XRD present at SCME- NUST (b) XRD basic schematic

Chapter 5

Results and Discussion

5.1 FTIR:

FTIR spectrum of synthesized bone whitlockite (figure 5.1) confirmed the P-O-H bond of HPO_4^{2-} group at 872 cm^{-1} which is the characteristic peak of bone whitlockite and distinguish the WH from β- TCP while peak at PO_4^{3-} shows its peak at 1029cm^{-1}. Peaks at 606cm^{-1} and 963cm^{-1} are the bending and stretching vibrations of O-P-O bond of PO_4^{3-}. There are no peaks at 650 cm^{-1} and 3570 cm^{-1} which confirms that no secondary phase is present in our sample.

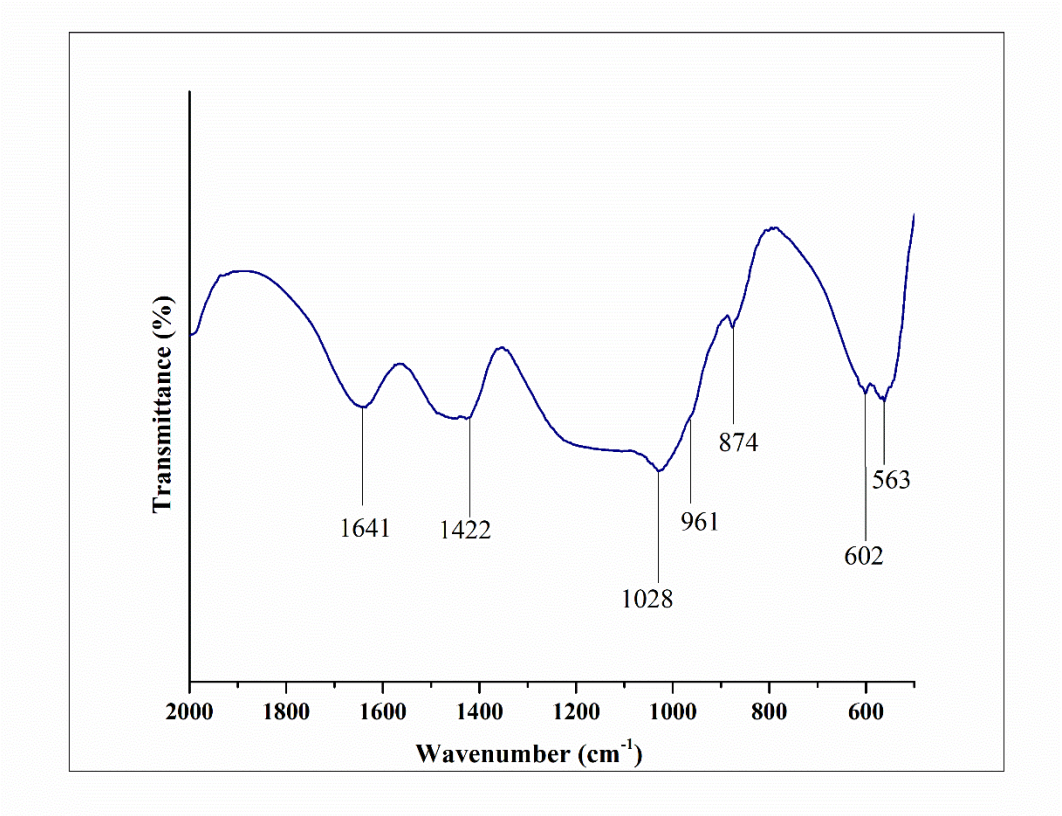

Figure 5. 1 FTIR of Whitlockite

- FTIR Spectra of Sodium Alginate (fig 5.2) shows O-H stretching between 3600 – 3000cm^{-1} and COO^- shows its stretching range between 1615 – 1415cm^{-1}. While CH_2 stretching is at 2918$cm^{-1.}$

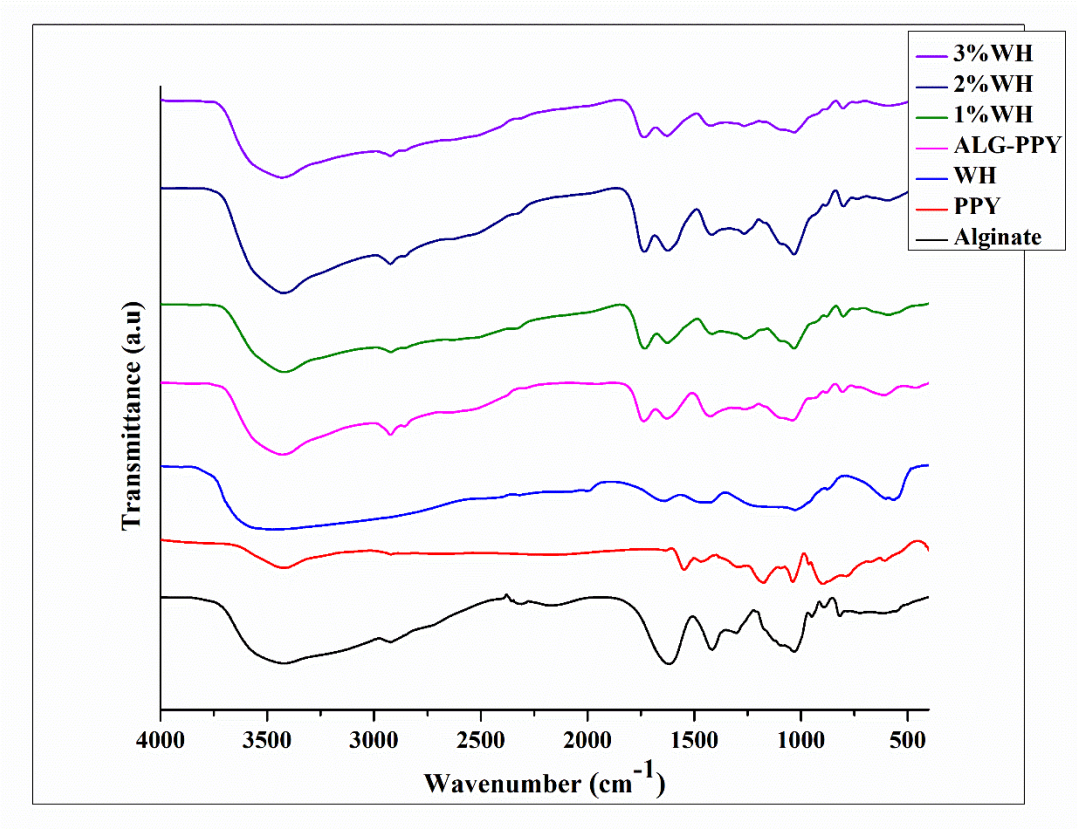

Figure 5. 2 FTIR of Composites

- Polypyrrole represents its characteristics peaks at 1512 and 1438 which is due to C=C stretching. C=N bond shows its peak at 1632cm^{-1} and C-N shows its peak around 1315cm^{-1}.
- In composite material((ALG-PPY), the peaks at 1315cm^{-1} and 1260cm^{-1} shows C-N stretching, the peak at 1735 cm^{-1} is associated with C=O of aromatic ring. A peak at 1735 cm^{-1} represents that pyrrole rings are over oxidized these are the distinctive peaks distinctive peaks which are not found individual spectra of sodium alginate or polypyrrole. Similarly composites of (ALG-PPY-WH) shows these Peak shifting which represents blending and mixing of precursors. As whitlockite is present in small amount and its IR absorption area is in close proximity with ALG-PPY composite so it cannot be distinguished actively however peak sharpening was observed with the increase in concentration of whitlockite in composite material. In composite

material peak shifting takes place which shows blending and mixing of precursors.

5.2. XRD:

XRD results of whitlockite confirms the synthesis of pure bone whitlockite .matches with JCPDS card (01-070-2064 and 00-042-0578). Presence of (0210) peak also confirms the synthesis of pure bone whitlockite and our sample does not show any reflection at (001) face as well as 1t 31.2^0 and 31.8^0 which shows that no mix phases of hydroxyapatite and β-TCP were involved in whitlockite synthesis.

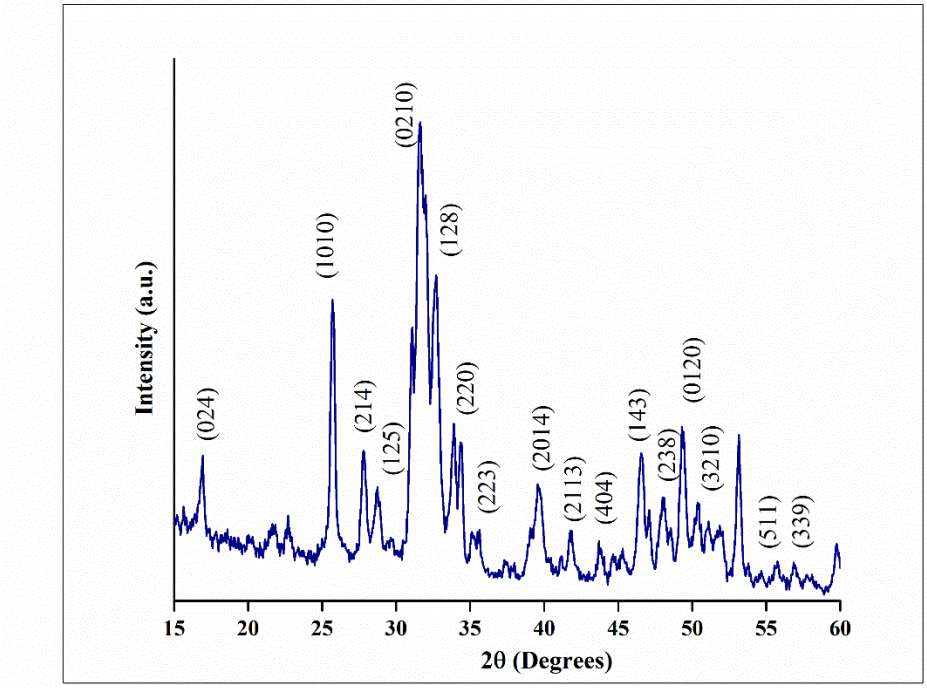

Figure 5. 3 XRD of Pure Whitlockite

5.3. Scanning Electron Microscopy:

Structural morphology of Hydrogels was studied using scanning electron microscope. For this purpose, we freeze dried our hydrogel samples for 52 hours using Labconco freezone plus 6 freeze dryer and investigated the morphology of hydrogels.

The results show that hydrogels are well interconnected in nature. For bone tissue engineering, the porosity of biomaterials and scaffolds is an important factor as significant space is required for adhesion of cells, proliferation and vascularization.

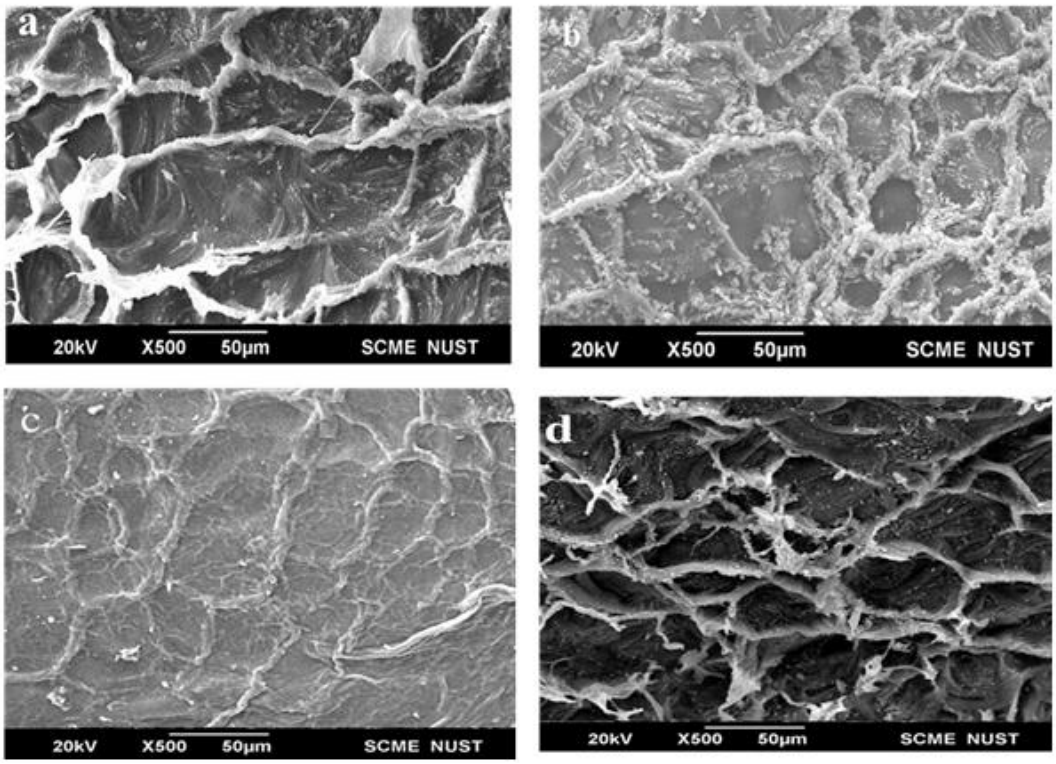

Figure 5. 4 SEM Results of hydrogels (a) Pure sample, (b) composite with 1% whitlockite, (c) composite with 2% whitlockite and (d) composite with 2% whitlockite

5.4. Viscosity:

Viscosity of hydrogels were measured using ROOKFIELD (DV-II + Pro) Viscometer using spindle of S series (S5). Temperature of all samples were maintained at 30 ^{0}C. Results were measure in centipoise (cP). 1 centipoise is equal to 1 millipascal second.

Viscosity of sample was measured AT 5 rpm. 400ml of each sample was prepared and their viscosity was measured. The results show that by increasing the amount of whitlockite in composite viscosity of sample also increases. As he particles of ceramic material causes the resistance in flow of hydrogels. it is also associated with the small particle size of whitlockite. Small particle size provide larger surface and more chances of interactions between polymer- ceramic interaction and eramic ceramic interactions . Result of viscosity of hydrogels are given in following table.

Table 4 Viscosity of Hydrogels

Sample Name	Composition	Viscosity
		(cP)
Pure	Hydrogel without WH	544.6
1%	Hydrogel with 1%WH	601.7
2%	Hydrogel with 2%WH	624.6
3%	Hydrogel with 3%WH	642.1

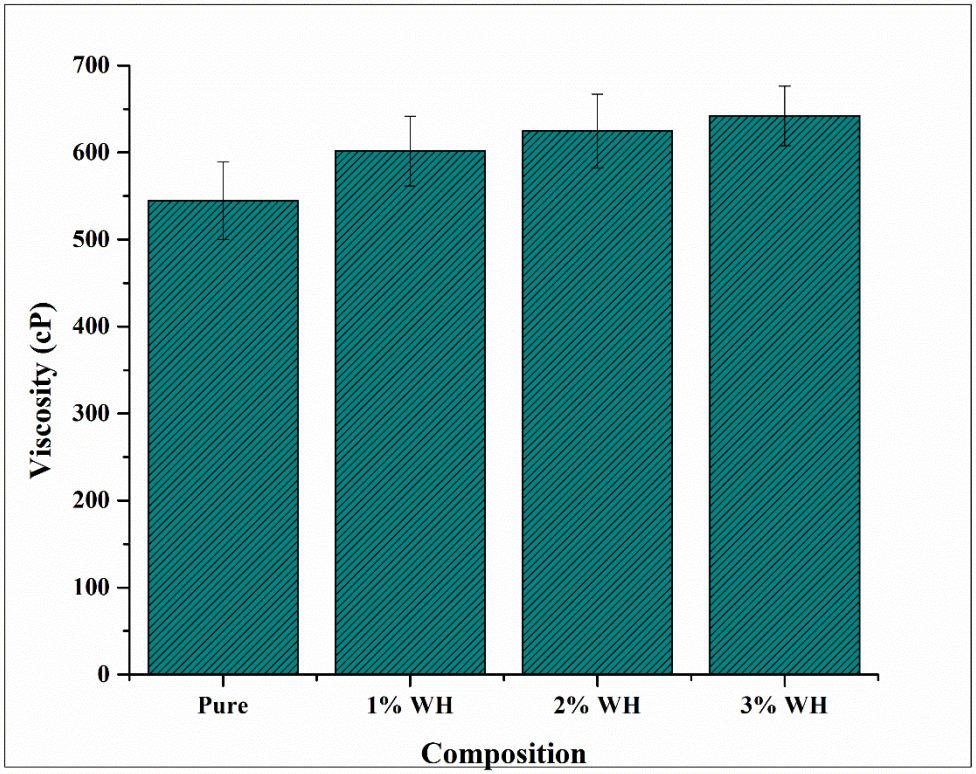

Figure 5. 5 Viscosity of composites; pure represent the composite which do not contain any whitlockite while 1% WH, 2% WH, 3%WH represents composite with 1% whitlockite, composite with 2% whitlockite and composites with 3% whitlockite concentration respectively.

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5.5. Injectability:

Injectability of cross linked hydrogels was checked manually using 21G and 24G needle and hydrogel was injected in air. The results show that whitlockite changes the viscosity of hydrogel as the Injectability time of whitlockite increases with increase in whitlockite concentration. All the hydrogels can be injected in less than 30 secs. The results of Injectability are supported by viscosity of samples. As the viscosity of samples increases with the amount of whitlockite added in sample which results in change in Injectability of hydrogels.

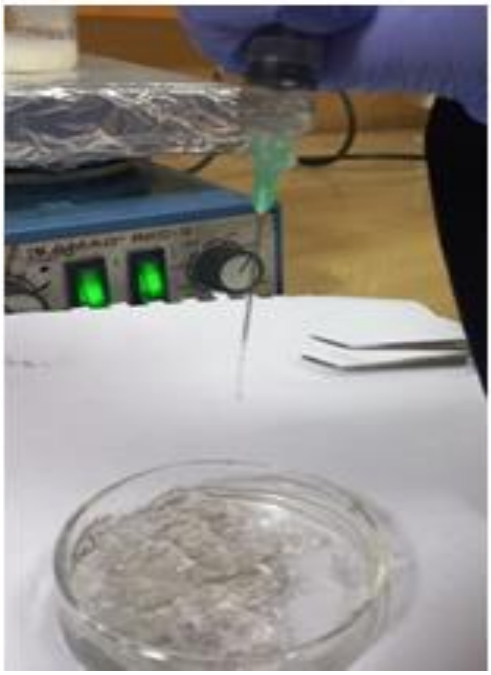

Figure 5. 6 Setup for Injectability measurement of hydrogels

Results of hydrogel Injectability are given in the following table.

Table 5 Injectability of Hydrogels

Sample	**21 G Needle**	**24G Needle**
	Time (sec)	**Time (sec)**
Pure	5.62	10.68
1% WH	6.61	12.67
2% WH	8.05	16.06
3% WH	13.16	22.25

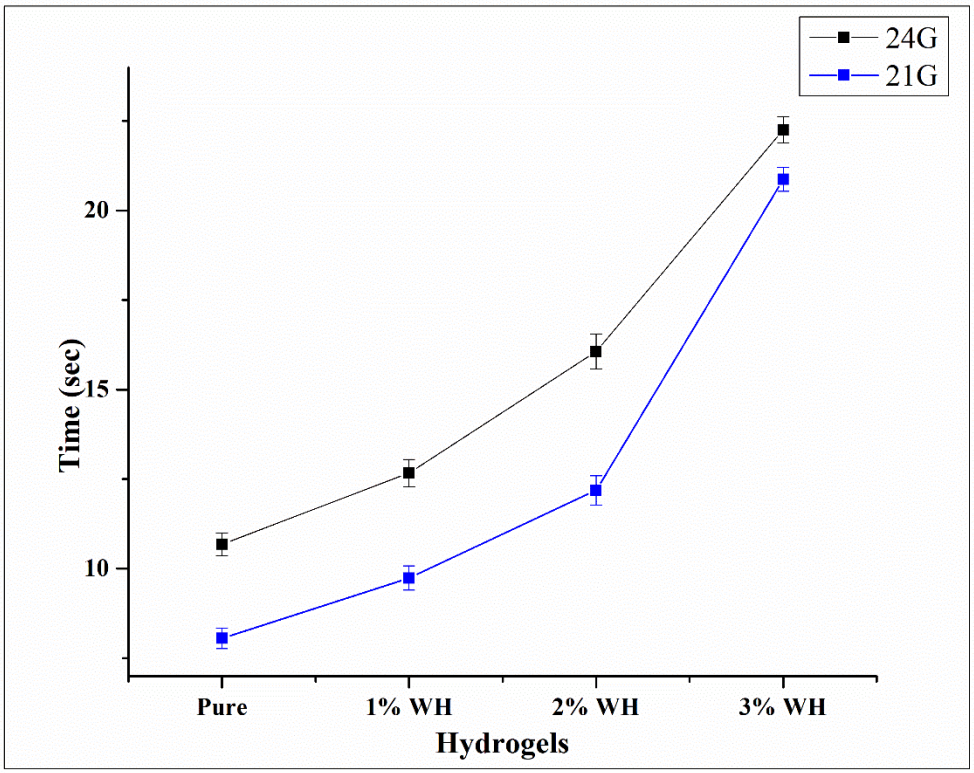

Figure 5. 7 Injectability of Hydrogels from 21G and 24G needles

5.6. Gelation Rate of Hydrogels

For injectable hydrogels, time of gelation plays an important part. As gelation time must not be so fast that it becomes difficult for surgeons to inject in the body as well as it should not be slow so that hydrogels can't achieve their stability inside the body and cause hindrance in the work. The optimum time of gelation for hydrogels ranges from 5 to 30 minutes. In present experiment, gelation of cross-linked hydrogels were done at 28 ˚C. and gelation of all samples were achieve within 30 minutes. Following is the data for gelation of hydrogels.

Table 6 Gelation time of Hydrogels

Sample Name	Composition	Gelation Time
		(minutes)
Pure	Hydrogel without WH	27
1% WH	Hydrogel with 1%WH	24
2% WH	Hydrogel with 2%WH	19
3% WH	Hydrogel with 3%WH	16

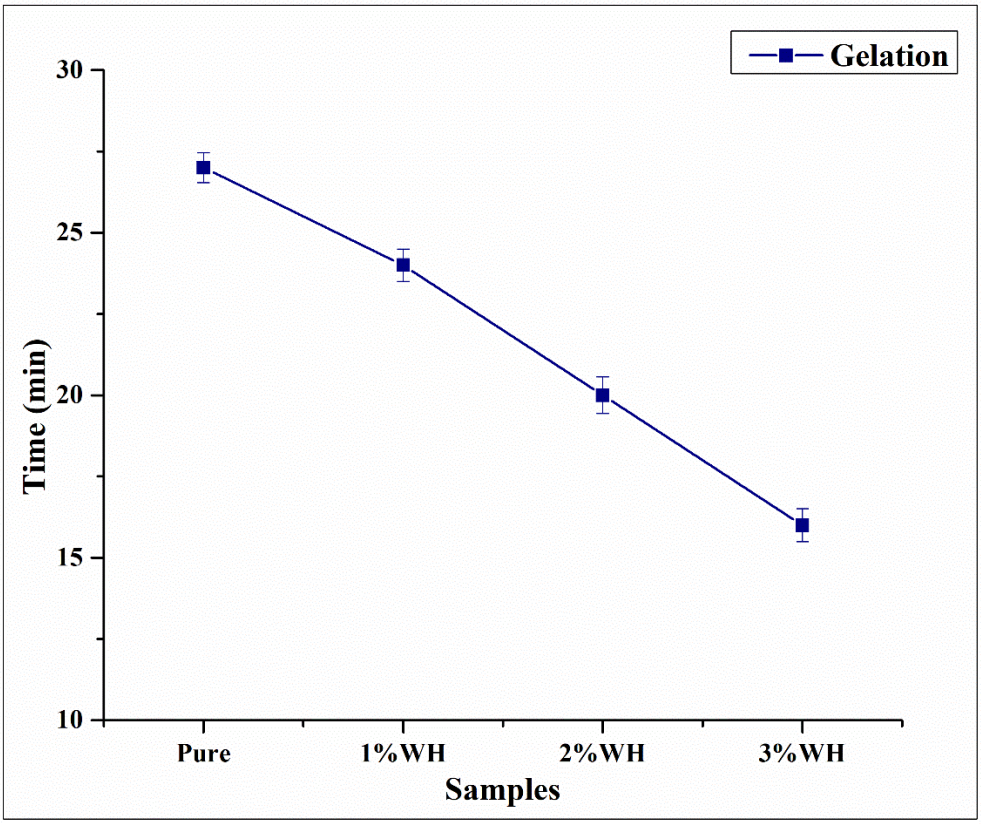

Figure 5. 8 Gelation rate of hydrogels

Gelation rate of hydrogels are measured by the times required for cross-linked hydrogel to become stable like gel and stop flowing like viscous liquid. Following figure we show the images of hydrogels before and after gelation in our experiment.

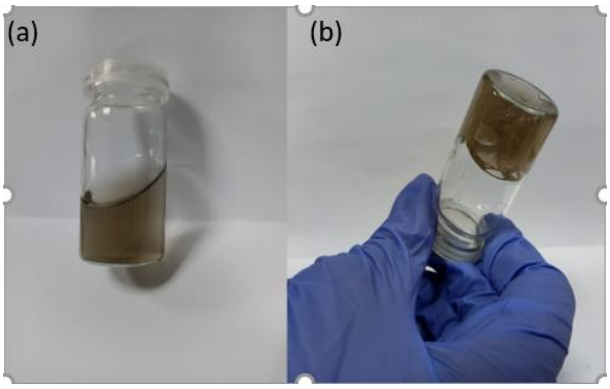

Figure 5. 9 (a) Hydrogel before gelation (b) Hydrogel after Gelation

5.7. Ionic Conductivity:

Ionic conductivity of our samples was measured using JENWAY 4510 Conductivity meter. As polypyrrole is a conductive material when it forms a composite hydrogel

material with piezoelectric whitlockite it produces the ionically conductive hydrogel. Following setup was used to measure the conductivity of hydrogels

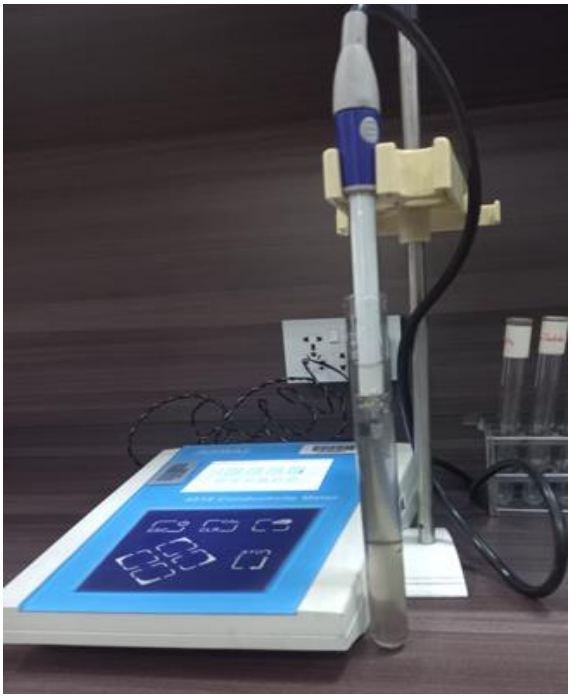

Figure 5. 10 Set up for ionic Conductivity Measurement

In present experiment, ionic conductivity of whitlockite polypyrrole, hydrogels with 1% WH , 2%WH, 3% WH was measured.

Table 7 Ionic conductivity of Hydrogels

Sample	**Name**	**Ionic Conductivity**
		(mS/cm)
a	Whitlockite in PBS	0.199
b	Polypyrrole in PBS	15.46
c	Hydrogel with 1%WH	15.61
d	Hydrogel with 2%WH	15.82
e	Hydrogel with 3%WH	16

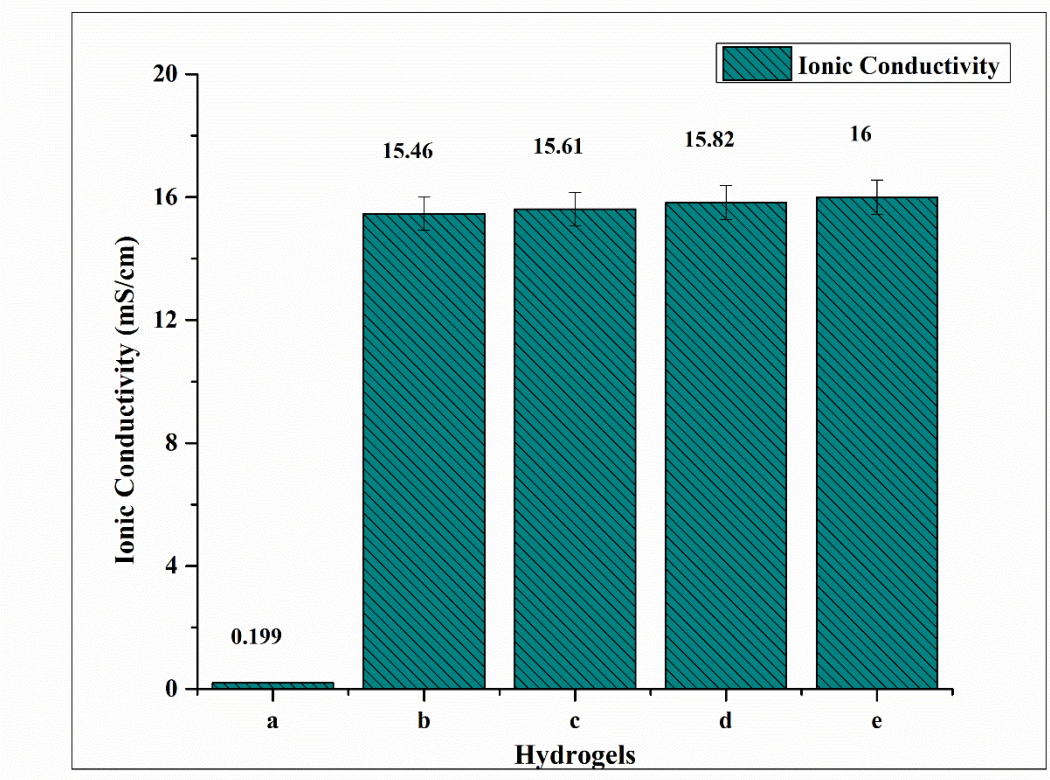

Figure 5. 11 Ionic Conductivity of Hydrogels

For measurement of ionic conductivity, samples were stirred for 48 hours which results to generate piezoelectric effect in whitlockite. the results show that conductivity of sample increase with increase in amount of whitlockite. Which confirms that whitlockite shows conductive properties.

5.8. MTT Assay:

MTT Assay was performed to check the cytotoxicity hydrogels. For this purpose, we cultured two cell lines in culture medium (RPMI-1640) which were supplemented by 10% of FBS solution. (Fetal bovine solution) purchased from sigma Aldrich and streptomycin penicillin antibiotic. (2%). Cell lines were than subculture and counting of cells were done. To evaluate cytotoxicity of hydrogels we used PBS as positive control and media as negative control. Extraction of hydrogels were done by ISO-1093 by incubating our sample in CO_2 incubator at37^{o} C for 24 hours. 96 well plate was used for seeding of the HBMMSCS cells. After 24 hours, culture medium was replaced with hydrogel extract at 37^{o} C and 17ul of MTT dye was introduced to each well and again incubated for 3.5 hours. in final step solution absorbance was measured at 550nm using spectrophotometer.

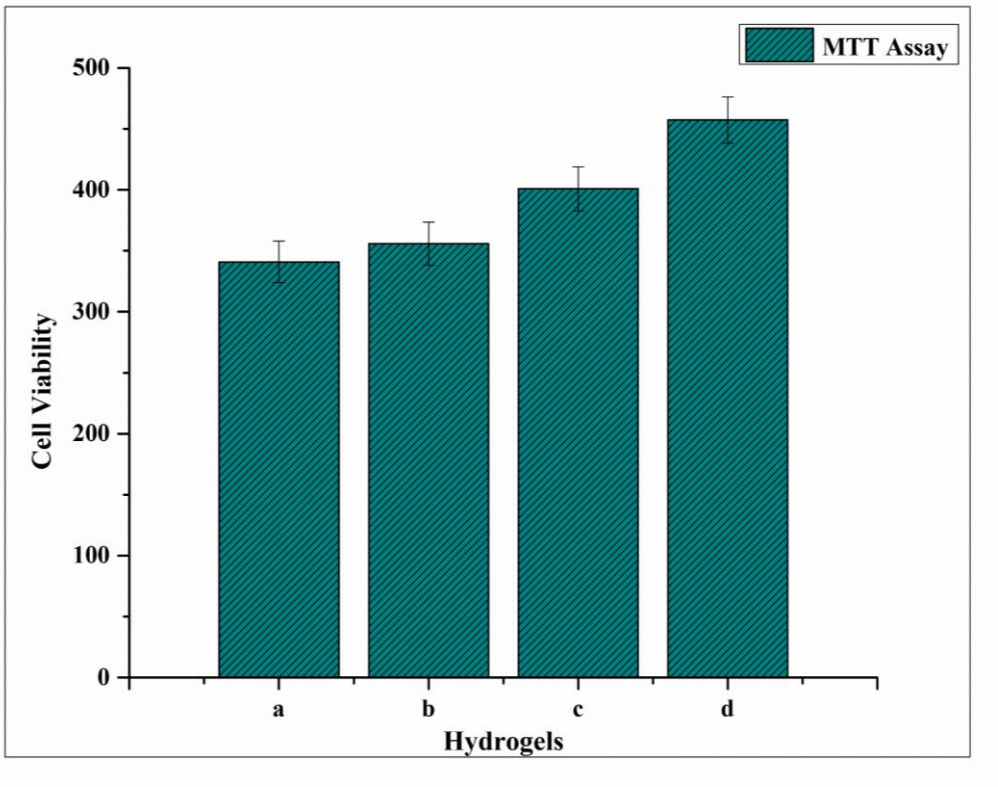

Figure 5. 12 MTT Assay of Hydrogels(a) shows hydrogel sample without WH control which we assume 100 while (b-d) shows MTT results hydrogels with WH concentration 1%, 2%, and 3% respectively

The results show clearly that presence of whitlockite has positive effects on viability of cells. Increase in the concentration of whitlockite in composite material increases the cell viability.

Conclusions:

Synthesis of biomaterials that can fulfill the requirements for bone regeneration is a challenging process. Since most of the scaffolds used in bone tissue engineering are static in nature so their use in case of irregular bone defect is sometimes not possible. there is need to develop BTE scaffolds which can be used at asymmetrical traumatic sites random. Endogenous current in microenvironment of bone plays an important role in bone regeneration. Therefore, in this research, we aim to develop cytocompatible and conductive tissue engineering scaffold that require minimum invasiveness for implant at traumatic site and can be used for individualized treatment of bone defects. For this purpose, we used a piezoelectric bioceramic material (bone Whitlockite) and optimized the method for the fabrication of injectable conductive hydrogels scaffolds. we used a chemical approach to synthesize bioceramic-polymer (WH-ALG-PPY) composite and then used other biomaterials (alginate, gelatin and PBS) to convert these composite into hydrogels. These hydrogels were than optimized in such a way that they can be injected to the system with the help of 21G and 24G needles to reduce surgical invasiveness with the gelation time of less than 30 minutes to attain the in vivo stability as well as provide ease in surgical handling. With the help of SEM, crosslinking in Hydrogels could be see which shows that hydrogels are highly cross-linked with porous structures which is necessary for vascularization of bone tissues. Different characterizations were done confirm that we fabricated ionically conductive hydrogels with controlled viscosity, Injectability and gelation rate.

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