CHAPTER 2

REVIEW OF LITERATURE

and

OBJECTIVES

1. REVIEW OF LITERATURE

Millions of people suffer from tissue loss due to catastrophic traumas or surgical interventions every year. Thus, Tissue Engineering and Regenerative Medicine (TERM) has helped in meeting this tremendous demand for tissues (Ikada et.al., 2006). TERM is an interdisciplinary area that involves the concepts of life sciences and engineering for the restoration, replacement and regeneration of tissue. It helps in maintaining and improving a defective tissue or a whole organ. It involves the application of scaffolds, cells and growth factors. The essential requirement for the development of a tissue-engineered construct comprises supporting uniform cell growth, nutrient and waste transport and exchange of gases. This can be achieved by the development of requirement specific cell-scaffold construct. These scaffolds are manufactured by material scientists to meet the concurring demands of clinicians (O'Brien et.al., 2011). The scaffolds are made from different kinds of materials like synthetic materials, composites, biomaterials and nanocomposites, etc. Polymers like collagen, silk, chitosan, polyvinyl alcohol, polyethene glycol, alginate, hyaluronic acid, etc. are well-versed in their utilization in the construction of scaffolds for tissue engineering and regenerative medicine. Fig.2.1 shows the steps involved in the bone tissue engineering process. The selection of material for a particular type of tissue is an essential step while designing a cell-scaffold construct. The interaction of these materials with the cells is supported by different factors like the composition, structure, and function of the developed biomimetic matrix. Natural polymers possess good biocompatibility whereas synthetic materials exhibit good mechanical strength; ceramics have their own intrinsic properties. All these characteristics of different materials make them unique and challenging for researchers. Scaffolds are also one of these materials used for the preparation of artificial tissue-engineered constructs for TERM. Scaffolds have been used for biomedical applications in diverse fashions for the past decade (Shimura et al., 1976).

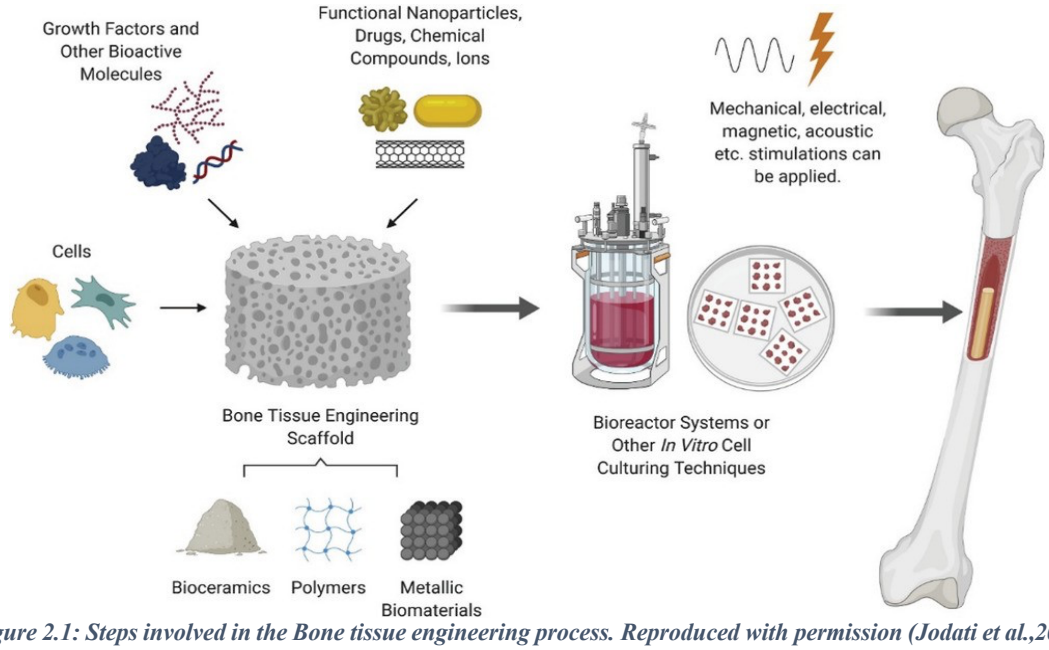

Figure 2.1: Steps involved in the Bone tissue engineering process. Reproduced with permission (Jodati et al.,2020)

1.1 Bone

Bone is a live, developing tissue primarily composed of collagen. Calcium phosphate is a mineral that gives the structure strength and hardens it, and collagen is a protein that offers a flexible framework to it. Collagen and calcium work together to produce bone flexible and strong enough to endure stress. The bones and teeth hold more than 99 per cent of the calcium in the body. The final 1% is located in the blood. The body contains two different types of bones: cortical and trabecular. The cortical bone is compact and dense. It creates the bone's outer layer. Its primary job is to fortify the bones and safeguard the spongy tissue underneath. The innermost layer of the bone is made up of trabecular bone, which has a spongy, honeycomb-like structure. Fig.2.2 shows the different layers of bone tissue. Sometimes it is also known as cancellous bone tissue. It serves to give the bones strength and flexibility as well as serve as a shock absorber in the event of trauma. It is lighter and less dense than compact bone tissue. The spongy layer also helps to lighten the skeleton's total weight because it is lighter than compact bone tissue.

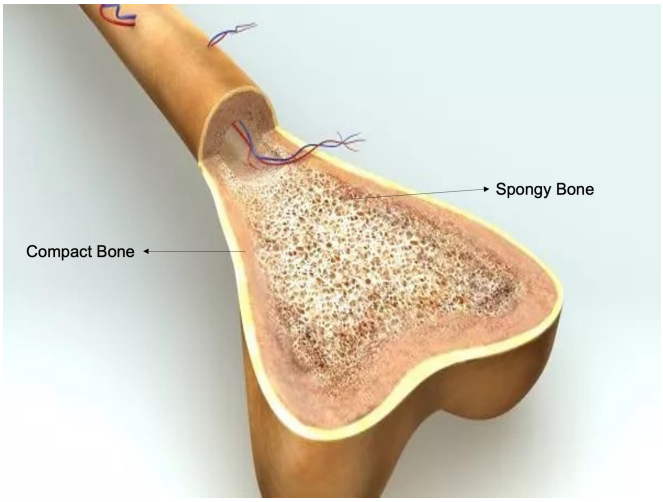

Figure 2.2: Spongy and compact layers of Bone tissue

2.1.1 Types of Bone cells

Osteoblasts, osteoclasts, osteocytes, and osteoprogenitor (or osteogenic) cells are the four different cell types that make up bone (Mohamed et al.,2008). Each type of cell in bones is present in various places and has a specific purpose. Fig.2.3 shows the different types of bone cells involved in the formation of a bone.

a. Osteoblasts

Osteoblasts are the fibroblast like cells that are present in the periosteum and endosteum of bones, which are growing parts of the bone (Chen et al.,2018). They are formed from bone marrow-derived pluripotent mesenchymal stem cells and are in charge of creating new bones. The collagen matrix and calcium salts are synthesized and secreted by osteoblasts, which do not divide. The matrix contains various proteoglycans, non-collagenous proteins, and cell attachment proteins in addition to structural macromolecules like type I collagen, which makes up roughly 90% of the organic matrix. The osteoblast is imprisoned within the secreted matrix that surrounds it as it calcifies. They also promote the mineralization of the organic matrix through vesicles present in the matrix and extracellular organelles.

b. Osteoclasts

Each osteoclast is housed in a cavity (lacuna) that is lined with bone tissue. By secreting enzymes, osteoclasts keep the matrix's mineral concentration stable. In reaction to bone remodelling caused by growth or shifting mechanical loads on the skeleton, osteoclasts are involved in bone resorption. Osteoclasts are involved in long-term blood calcium balance maintenance as well (Boyce et al.,2009). Osteoclasts reshape the surface of the bone during bone resorption, creating depressions called Howship's lacunae. The creation and operation of

osteoclasts are significantly influenced by the local signals from neighbouring cells and the growth factors that are locked up in the bone matrix.

c. Osteocytes

The osteoblast undergoes structural change and develops into an osteocyte, which is the main cell type in adult bone and the most prevalent type of bone cell. Osteocytes do not undergo mitosis, just like osteoblasts (Schaffler et al.,2012). They are "entrapped" by osteoblasts and are found inside the bone itself. They are post-proliferative and represent the osteoblast lineage's most developed differentiation state. Per mm^3 of bone, there are around 25,000 osteocytes. Regularly spaced out lacunae are occupied by osteocytes, and from these lacunae, numerous tiny canals known as canaliculi shoot outward. The bone's canaliculi allow for the diffusion of chemicals. In the canaliculi, many cell processes from the osteocytes travel in all directions. Osteocytes' canaliculi are organised more perpendicularly than parallel to the bone surface direction. Through long cytoplasmic processes that extend through canaliculi channels within the bone matrix, they are able to communicate with one another and receive nutrients.

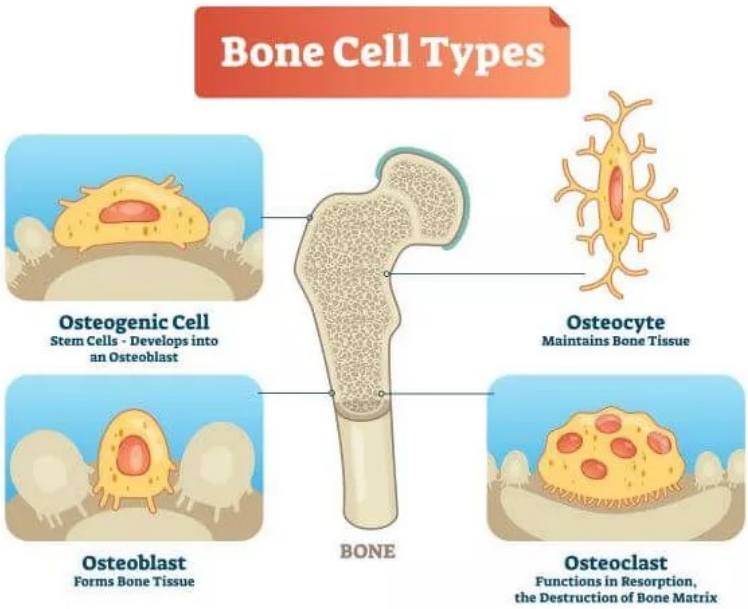

Figure 2.3: Different types of bone cells involved in the formation of a bone

1.2 Diseases and Injuries of Bone

1.2.1 Osteoporosis

Numerous people suffer from osteoporosis. Osteoporosis patients are at a significant risk of experiencing one or more fractures, which can be physically disabling and possibly trigger a

downward spiral in both physical and mental health (Peterson et al.,2001). "Primary osteoporosis" is the name given to the most prevalent type of osteoporosis. It is the end outcome of the cumulative effects of bone loss and bone structural degeneration with ageing. Through a healthy diet, regular exercise, and, if necessary, the right therapy, this bone loss can be reduced and osteoporosis prevented. Osteoporosis can be brought on or exacerbated by a wide range of illnesses, drugs, and harmful substances (Tu et al.,2018). This type of condition, known as secondary osteoporosis, is often preventable through healthy eating and regular exercise, as well as appropriate therapy if necessary if it is identified as a potential threat. Rickets is a syndrome brought on by a delay in the deposition of calcium phosphate minerals in developing bones. It is caused by a number of paediatric illnesses. Skeletal abnormalities are caused by this delay, particularly bowed legs. Osteomalacia is the term used to describe the same condition in adults. While these disorders may normally be avoided by ensuring proper vitamin D levels, they can have extremely negative effects on those who are affected (Florenzano et al.,2021).

1.2.2 Renal Osteodystrophy

Renal osteodystrophy is a complicated bone condition that can occur in people with chronic renal failure. While transplantation and dialysis have increased these patients' life expectancies, they may not be able to stop the bone disease from progressing further. The spine, pelvis, legs, or skull are frequently affected by Paget's disease of the bone, a progressive and frequently disabling illness of bone remodelling (although any bone can be affected) (Elder et al.,2002; Martin et al.,2004). Its effects can be reduced if detected in the early stages. The skeleton is impacted by many hereditary and developmental abnormalities. Osteogenesis imperfecta is one of these that is more prevalent (OI). Bones in those who have this illness are brittle (Rauch et al.,2004).

1.2.3 Traumatic Injuries

Bone injuries can also occur due to horrible accidents leading to mechanical damage or dynamic mechanical degeneration of bones. Limit injuries, entering damages, surgical procedures to treat existing fractures, etc, can also lead to intense bone damage thereby affecting the cellular framework of bone. These injuries can be severe and they require utmost care and priority.

2.2.4. Bone Cancer

Some bone conditions typically manifest later in life. Bone cancer is one of the most prevalent of these acquired skeletal illnesses. These cancers can develop from primary tumours in the bone or, far more frequently, from tumours outside of the skeleton seeding the bone (metastatic tumours). Children might develop primary bone cancer as well (Ferguson et al.,2018).

2.3. Healing of fracture: Mechanism

A fracture is a break in the internal structure of the bone cortex that causes damage to soft tissue. After the fracture, secondary healing starts, and it entails four stages: the development of a hematoma, a fibrocartilaginous callus, a bony callus, and bone remodelling. Up to 10% of all fractures may experience failed or delayed healing, which may be brought on by a variety of conditions, including comminution, infection, malignancy, and interrupted vascular supply. Hematoma Formation starts right after the fracture from day 1 to day 5. A hematoma forms surrounding the fracture site as a result of the blood arteries supplying the bone and the periosteum being torn during the fracture. The hematoma clots create a framework that is eventually used during healing. It is followed by fibro-cartilaginous callus formation. When VEGF is released, angiogenesis occurs and fibrin-rich granulation tissue begins to form within the hematoma. More mesenchymal stem cells are brought in, where they start to develop into fibroblasts, chondroblasts, and osteoblasts under the control of BMPs between day 5 and day 11. Chondrogenesis begins, resulting in the formation of a collagen-rich fibro-cartilaginous network spanning the fatigue crack ends and a sleeve of hyaline cartilage all around it.

After which bony callus formation occurs due to endochondral ossification which starts to take place in the cartilaginous callus between day 11 to day 28. When RANK-L is expressed, it encourages chondroblasts, chondroclasts, osteoblasts, and osteoclasts to differentiate further. As a result, the cartilaginous callus is resorbed and begins to calcify and an immature bone callus that is firm and calcified forms after this stage. Lastly, bone remodelling occurs as a result of the osteoblasts' and osteoclasts' ongoing migration after day 18 and it lasts for months. Osteoclast-driven bone resorption and osteoblast-driven bone formation coexist in this "coupled remodelling" process. Compact bone eventually replaces the callus's centre, while lamellar bone eventually replaces the callus' margins. Alongside these changes, the vasculature undergoes significant remodelling. The typical bone structure eventually regenerates after a protracted, months-long process of bone remodelling (Ripamonti et al.,2009; Kostenuik et al.,2017; Frost et al.,1989; Marsell et al.,2011; Parvizi et al.,2010). The whole process from fracture to the complete healing of bone is explained in Fig.2.4. Fig.2.5 explains the factors involved in fracture healing including both local and systemic factors.

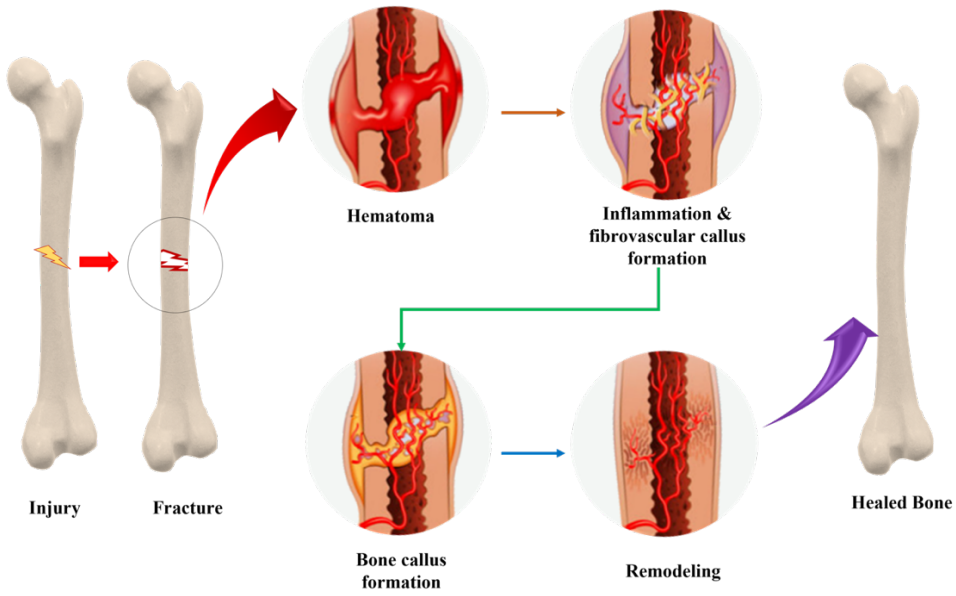

Figure 2.4: Process of fracture healing and mechanism involved in complete bone healing. Reproduced with permission (Kumari et al.,2022)

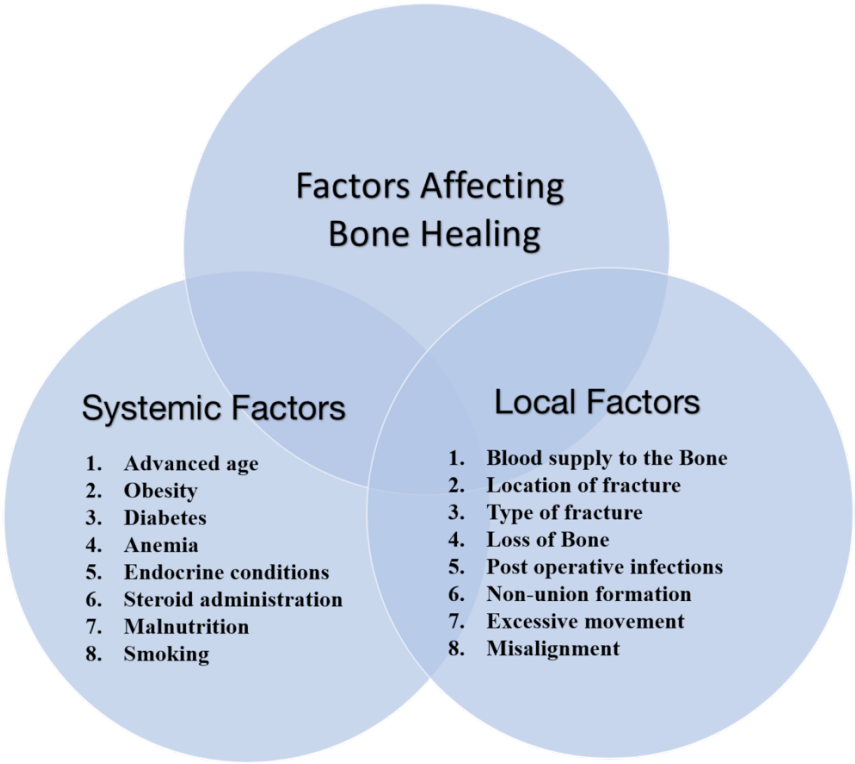

Figure 2.5: Factors responsible for fracture healing, including local and systemic factors. Reproduced with permission (Kumari et al.,2022)

2.4. Current Bone Repair Technology

Bone regeneration, which may be observed during typical fracture healing and is involved in continual remodelling throughout adult life, is a complicated, well-orchestrated physiological

process of bone creation. Avascular necrosis, atrophic non-unions, and osteoporosis are a few complex clinical conditions in which large amounts of bone regeneration are needed, such as for the skeletal reconstruction of large bone defects caused by trauma, infection, tumour resection, and skeletal abnormalities, or situations in which the regenerative process is impaired. The "gold standard" autologous bone graft, free fibula vascularized graft, allograft implantation, and use of growth factors, osteoconductive scaffolds, osteoprogenitor cells, and distraction osteogenesis are just a few of the many current methods used to improve the impaired or "insufficient" bone-regeneration process (Pape et al.,2010; Adani et al.,2004; Stollsteimer et al.,2000; McCarthy et al.,2001). In order to overcome the drawbacks of the current approaches, to develop bone-graft substitutes with biomechanical properties as similar to the normal bone as possible, to speed up the overall regeneration process, or even to address systemic conditions like skeletal disorders and osteoporosis, improved "local" strategies in terms of tissue engineering and gene therapy, or even "systemic" enhancement of bone repair, are under intense investigation.

2.4.1 Distraction osteogenesis

A longer bone can be created from a shorter one by distraction osteogenesis. During surgery, a tool known as a distractor progressively pulls two pieces of broken bone apart. Bones slowly separating from one another do not hurt. Children claim that it hurts less than their tooth-straightening braces. Osteogenesis occurs to produce new bone to fill the void. It takes a few months to complete the process. Greater adjustments in bone position are feasible with distraction osteogenesis than with a single standard surgery (Hariri et al.,2018). This enhances the outcomes and might lessen the number of surgeries a child requires during their lives.

2.4.2 Autologous Bone grafts

Almost all reconstructive orthopaedic surgery procedures require bone grafting. Despite the superior biological and mechanical qualities of autologous bone grafts, significant donor site morbidity and the restricted volume available must be taken into account. There are currently no improved biological or mechanical heterologous or synthetic bone substitutes on the market. The gold standard for treating bone abnormalities or nonunions continues to be autologous bone grafting. Its superior combination of osteogenic, osteoinductive, and osteoconductive capabilities gives it physiological benefits over heterologous and synthetic bone replacements. Tricortical grafts can also be utilised to "bioplate" constructions to increase their strength right

away (Ranalletta et al.,2019). Heterologous or synthetic bone substitutes have not yet reached this blend of biological and mechanical qualities (Fernandez de Grado et al.,2018).

2.4.3 Allografts

Allogeneic bone grafting, which uses bone taken from human cadavers or living donors as an alternative, avoids the issues with harvesting and quantity of graft material (Tournier et al.,2021). Depending on the needs of the recipient location, allogeneic bone is available in a variety of preparations, including demineralized bone matrix (DBM), morcellised and cancellous chips, corticocancellous and cortical grafts, osteochondral and whole-bone segments (Drosos et al.,2015; Kligman et al.,2003; Wei et al.,2021). Because donor grafts are rendered inert by the use of radiation or freeze-drying processes, their biological characteristics vary, but generally speaking, they have diminished osteoinductive qualities and no cellular component. Cost, the likelihood of infection transmission, and immunogenicity and rejection reactions are also challenges.

2.4.4. Ideal Scaffold Materials for Tissue Engineering

To facilitate cell adhesion, proliferation, and differentiation that results in tissue regeneration, the ideal scaffold material should resemble the extracellular matrix (ECM) found in the body. A key factor in the design and development of suitable functioning 3D artificial ECMs is the scaffolding material and manufacturing method. A scaffold that has been developed ought to possess several qualities, including high porosity, suitable pore size, biodegradability, biocompatibility, and bioactivity. It should also be able to degrade in tandem with the regeneration of damaged tissue, offer a large surface area for cell adhesion, proliferation, and migration, have adequate mechanical strength, and be able to deliver active molecules that facilitate tissue-specific differentiation and cell proliferation. The main scaffolding material is either natural or synthetic biopolymer alone or in combination, as these may be easily moulded into the right architecture, permit surface modification to promote cell contact, and aid in biomineralization for BTE application. For successful bone tissue regeneration polymeric materials are reported to be reinforced with bioceramics and nanoparticles to achieve scaffold with enhanced osteogenic potential or other desirable properties.

The creation of scaffolds from bioactive, biodegradable materials that offer chemical and physical cues to direct cell adhesion, proliferation, differentiation, and assembly into suitable 3D structures resembling original tissue is crucial for tissue engineering applications. The primary prerequisites for biomaterials utilized in scaffold development are bioactivity,

appropriate mechanical strength, and biocompatibility. Nevertheless, in order to satisfy this criterion, a single polymeric substance is unable to provide the ideal conditions for tissue regeneration. Consequently, the design and fabrication of functional biomaterials may benefit from the use of a multi-polymeric material system. In recent decades, a variety of possible biopolymers have been investigated for use in TE applications, such as gelatin, chitosan, collagen, and others. In contrast, bioceramics such as bioglass, beta-tricalcium phosphate, and hydroxyapatite have been used as suitable bioceramics with these polymers.

The composition and qualities of these materials determine the strength of the material/bone interface, the rate of bone growth, and the degree of bone attachment. DBM, autogenous and allogenic grafts, and other materials that are osteoconductive act as scaffolds for the development of new bone while being osteogenic or osteoinductive, causing the synthesis of new bone (Roberts et al.,2012). An extremely strong material/bone interface is created by the chemical bonding of bioactive materials with the bone, such as calcium phosphate compounds and glass ceramics. Ceramic oxides, metals and alloys, and polymers, which are bioinert materials, do not adhere to the bone directly, causing the material/bone interaction to be significantly weaker (Wang et al.,2017). A HAP coating on metal implants has been demonstrated to speed up bone attachment, increase skeletal fixation, and, in some instances, appear to have lessened metal ion leakage. But occasionally, there can be a breakdown between the coating and the metal substrate, leading to implant loosening and failure. Below is Table 2.1 which represents the materials utilized for bone tissue repair, restoration and substitution and Table 2.2 which shows the bioceramics and their properties that are used in this research work.

Table 2.1 Materials Used in Bone Repair, Restoration and Substitution

S No.	Materials	Advantages	Disadvantages
1.	Autogenous, freeze-dried or banked bones and bovine bone-derived materials	Osteoconductive and Osteoinductive	Additional surgery, Risks of infection, and Limited accessibility
2.	Demineralized Bone Matrix (DBM)	Structural support and Tissue remodeling	Low immunogenicity, Instability of osteoinductive potential
3.	Coral (CaCO3)	Highly porous, Interconnected and Biocompatible	Limited availability, Variable graft quality

4.	Polymers	Superior structure–property correlation, *In-vivo* stability	Poor degradation rate, Low mechanical properties
5.	Inert ceramics (alumina, zirconia)	Biocompatible, Wear resistance, and Mechanical properties	Time-consuming fabrication, Lack of organic phase, Non-homogenous particle size and shape
6.	Bioactive glass ceramics	Improved mineralization, Biocompatible, Osteoinductive and Osteoconductive	High dissolution of silica, Low mechanical strength
7.	Calcium phosphate materials such as hydroxyapatite (HAP), tricalcium phosphate (TCP), coralline HAP, and non-sintered apatite	Osteoblast adhesion, Enhanced mineralization and Osteoconductive	Poor mechanical strength, Brittleness
8.	Composites	Structural integrity, Enhanced porosity, Tunable mechanical properties	Brittleness, Low chemical stability
9.	Coated and uncoated metal implants	Biomechanical properties, Sterilizable, Osteogenic	Expensive, Chances of amorphous coatings, Requires controlled environment

Table 2.2 Bioceramics, utilized in the research study with their properties and applications

Bioceramic	Description	Advantages	Disadvantages	Applications
Hydroxyapatite (HA)	A calcium phosphate ceramic that closely resembles the mineral component of bone	Excellent biocompatibility, promotes bone formation, and integrates well with bone tissue	Brittle, limited mechanical strength, and sometimes poor interfacial bonding with polymers	Bone repair, bone graft substitutes
Beta-Tricalcium Phosphate (β-TCP)	A resorbable calcium phosphate ceramic that is used for its high porosity and biodegradability	Good biocompatibility, promotes osteoconduction, and resorbs over time	Fast resorption rate can limit long-term support, and can be brittle.	Bone defect repair, implant coatings

Bioglass	A bioactive glass that bonds with bone and stimulates bone formation	Stimulates bone growth, promotes osteogenesis, and has good biocompatibility	Fast resorption rate can limit long-term support, and can be brittle.	Bone repair, coating for implants

2.4.5 Chitosan/gelatin-based polymer-ceramic nanocomposite scaffold

Chitosan/gelatin scaffolds, while promising for bone tissue engineering, have limitations including lower mechanical strength, challenges in controlling degradation rates, variability in biological responses, and potential difficulties with cell infiltration and vascularization. Processing complexities, potential allergic reactions, and limited long-term data further contribute to the challenges associated with these materials. Addressing these limitations often requires additional strategies, such as incorporating reinforcement materials, optimizing processing techniques, and conducting further research to ensure the effectiveness and safety of chitosan/gelatin scaffolds in clinical applications. Therefore, the use of chitosan/gelatin-based polymer with ceramics and nanomaterials offers significant advantages over polymer-polymer scaffolds, particularly for bone tissue engineering applications. The inclusion of ceramics enhances osteoconductivity, mechanical properties, and bioactivity, making the scaffold more suitable for supporting bone regeneration, especially in load-bearing situations (Echeverria Molina et al.,2021; Lakhdar et al.,2021; Rahaman et al.,2011). These advantages make the chitosan-gelatin nanocomposite scaffold a more effective and reliable option for bone repair and regeneration in clinical settings. The choice of bioceramics depends on specific application needs, such as mechanical strength, resorption rate, and bioactivity (Vaiani et al.,2023). Combining these bioceramics with chitosan-gelatin scaffolds can create composite materials that optimize the scaffold's performance for bone tissue engineering. Presently bioceramics like hydroxyapatite, nanobioglass, beta tricalcium phosphate are the most popular bioceramics for BTE applications (Habibovic et al., 2008; Wu et al.,2013; I.R. Serra et al.,2015). HAP and β-TCP lay vital role in osseointegration and promoting osteoconductivity, whereas nanobioglass offers bioactivity that stimulates bone growth and enhances integration (Pilipchuk et al,2015; Yuan et al.,2019; Cañaveral et al.,2019).

Chitosan/gelatin-based nanohydroxyapatite infused porous scaffold developed by freeze-drying process showed better cell attachment and cell growth after the addition of hydroxyapatite in comparison to regular chitosan/gelatin scaffold (Dan et al.,2016). It has also been observed that the production of a scaffold with osteogenic and angiogenic potential can benefit from the use of trace metal ions like copper, cobalt, strontium, etc.(Wu et al.,2013;

Gentleman et al.,2010). Copper-containing mesoporous bioactive glass scaffolds with improved osteogenic and angiogenic potential were developed for bone tissue engineering application by Wu et al. Likewise, it was shown that cobalt-doped mesoporous bioglass scaffold is also a good option with improved osteogenic and angiogenic potential for applications involving bone tissue engineering (Wu et al.,2012).

Bozorgi et al. developed a Chitosan/gelatin-based scaffold with Cu-substituted hydroxyapatite which showed enhanced compressive strength of 88.869 ± 19.574 MPa with prolonged degradation (Bozorgi et al.,2022). Chitosan/gelatin scaffold was developed in another study via freeze drying technique where, it was observed that after the addition of 10-30% bioglass, the scaffold attained more than 80% porosity with mean pore size between 100-300μm and compressive strength of 2.2 ± 0.1 MPa. They also exhibited excellent hydrophilicity and biodegradability properties (Maji et al.,2016). A β-tricalcium phosphate/gelatin composite scaffold was developed which was incorporated with gentamycin-loaded chitosan microsphere by Yu Liu and his team for treating bone defects. They showed that the combination of β-TCP and gelatin mimicked bone composition and were able to provide the requisite mechanical strength of 0.82 ± 0.05 MPa during the first phase of bone generation. They also noticed sustained release of nanoparticles of 41.86% which resulted in neo-formation of bone tissue (Liu et al.,2022). Similarly, in another study, chitosan/gelatin scaffold were prepared with graphene oxide using freeze-drying technique which showed better osteoblast differentiation and increased collagen deposition *in-vivo* in rat model (Saravanan et al.,2017). However, Chitosan /gelatin based graphene oxide and nanobioglass infused scaffold has not been developed and investigated uptil now for BTE applications. Neither the Ch/G based TCP and NBG infused scaffold has been developed and studied. More detailed discussion has been done on the different combinations of metal ions and additives with chitosan and gelatin and other bioceramics in the Doping section.

2.4.6 Bone graft substitutes

Additionally, replacements for autologous or allogeneic bone grafts have been produced. They are composed of biomaterial scaffolds, either synthetic or natural, that support bone cell migration, proliferation, and differentiation for bone regeneration. Collagen, hydroxyapatite (HAP), tricalcium phosphate (TCP), calcium-phosphate cements, glass ceramics, and many other biomaterials and artificial bone substitutes are currently employed as scaffolds. Research in this area is ongoing. The use of cylindrical metallic or titanium mesh cages as a scaffold in

combination with cancellous bone allograft, DBM, or autologous bone is an alternative to massive cortical autografts or allografts, particularly for the reconstruction of large bone defects for which there is a need for a substantial structural scaffold (Wang et al.,2017).

2.4.7 Growth factors

A number of important molecules that control this intricate physiological process have been identified and are currently being used in clinical settings or are the subject of research to improve bone repair, thanks to advances in our molecular understanding of fracture healing and bone regeneration. BMPs have received the most research attention since they are strong osteoinductive agents (Chen et al.,2004). They stimulate mesenchymal stem cells' (MSCs') and other osteoprogenitors' (OPCs') mitogenesis and osteoblast differentiation. In addition to BMPs, other growth factors with different roles in cell proliferation, chemotaxis, and angiogenesis, including platelet-derived growth factor, transforming growth factor-, insulin-like growth factor-1, vascular endothelial growth factor, and fibroblast growth factor, among others, are also being studied or are being used to enhance bone repair (Oliveira et al.,2021).

2.5 Bone Tissue: ECM

Bone regeneration is the process of replacing bone tissue that has been damaged or lost due to trauma, injuries, cancer, or congenital defects. The extracellular space of the bone is filled with the extracellular matrix (ECM), a non-cellular, 3D material that cells release. Particular proteins and carbohydrates make up its structure. It is a complex, dynamic bio-environment with carefully controlled mechanical and biochemical properties. ECMs play a role in controlling cell attachment, proliferation, and responses to growth hormones in bone. They also significantly affect differentiation and, subsequently, the structural and functional attributes of the mature bone. Osteoblast-lineage cells, including mesenchymal stem cells (MSCs), osteoblasts, and osteocytes, as well as osteoclasts, can both produce new bone and absorb existing bone when exposed to bone ECM. As bone regenerative medicine has advanced, researchers have become more interested in the osteoinductive and osteoconductive potential of ECM-based polymeric scaffolds.

Each type of tissue's ECM develops with distinct composition and architecture (Frantz et al.,2010). The ECM provides consistency and flexibility to the body tissues and organs in terms of controlling their growth, activity, and homeostasis. It is constantly restructured as the wide range of receptors, growth regulators, and the pH of the native surroundings changes (Bonnans et al.,2014; Mouw et al.,2014). The fourth component in the evolution of BTE is thought to be

the ECM (Ravindran et al.,2012). 60% of the bone matrix is made up of inorganic, and 40% of it is organic substances.

2.5.1. Major Components of Bone ECM

2.5.1.1. Organic ECM

Collagenous proteins are found in collagen. The organic ECM in bones is mostly composed of collagen types I, III, and V. The principal function of collagens is to supply mechanical stability and support but to also act as a framework for osteocytes (Saito et al.,2015). 90% of the collagen in bone tissue type I collagen, which assembles into triple helices of polypeptides to create collagen fibrils. In order to create higher-order fibril bundles and fibres, these fibrils engage in interactions with other collagenous and non-collagenous proteins (Varma et al.,2016). Less common collagen types III and V control the size of type I collagen fibres and the process of fibrillogenesis (Garnero et al.,2015). The mechanical characteristics of collagen keep the polypeptide chains in a neatly structured fibril framework, depending on the inter and intra-chain crosslinks. Bone strength is significantly influenced by collagen. The ECM is altered by type I collagen deficiency or collagen structural mutations, which significantly raises the risk of fracture (Fonseca et al.,2014).

2.5.1.2. Non-Collagenous protein: Proteoglycans

Proteoglycans are defined as glycosaminoglycan (GAG) residues that have been covalently attached to the core of the protein molecule. Keratan sulfate, chondroitin sulfate, heparan sulfate, and dermatan sulfate are among the six varieties of GAG residues discovered in proteoglycans (Walimbe & Panitch et al.,2020). The bone has a significant family of small leucine-rich proteoglycans including biglycan, decorin, keratocan, and asporin. Small leucine rich proteoglycans (SLRPs) are extracellular proteins secreted by cells that collaborate with cell-surface receptors and cytokines to regulate both healthy and unhealthy cellular functions. SLRPs are involved in all phases of bone development, such as cellular multiplication, osteogenesis, mineral deposition, and bone remodelling (Kirby & Young et al., 2018). SLRPs also control the collagen fibrillogenesis process. The dysregulation of SLRPs results in structural flaws and the production of collagen that causes fibrosis. This might occur either by orthopaedic traumas or genetic deficiencies (Moorehead et al., 2019). Both the class I SLRPs biglycan and decorin each comprises of either dermatan or chondroitin sulfate GAG chains. Biglycan is expressed throughout the growth and mineralization of cells, whereas decorin is continually expressed once the bone matrix has begun to form. Keratocan plays a role in

controlling the pace of mineral deposition and bone formation. It is mostly expressed in osteoblasts (Coulson-Thomas et al., 2015). It has been demonstrated that type I collagen and asporin, another SLRP component, bind together to induce collagen mineralization (Kalamajski et al., 2009). As a result, SLRPs are crucial for preserving bone homeostasis.

2.5.1.3. Inorganic ECM

Hydroxyapatite (HAP) is a substance that promotes the production of bone tissue and is simple to combine with polymeric materials (Amini et al., 2012). Since the 1950s, HAP has been employed as an unreactive scaffold for filling and processing bone abnormalities in regenerative scientific knowledge (Dubok, 2000). Due to its strong osteoconductivity and biocompatibility, the calcium phosphate bioceramic [Ca10(PO4)6(OH)2] has been ubiquitously used as a scaffold in bone tissue engineering (BTE). It is a well-known bioceramic that is present in significant amounts in bone and teeth (In et al., 2020). HAP serves as one of the most extensively utilized bio-ceramics in BTE because it exhibits physiochemical characteristics that are remarkably similar to carbonate apatite, the major inorganic constituent of bone tissue (Pepla et al., 2014; Ripamonti et al., 2009; H. Yuan et al., 1999). Due to its advantageous biological characteristics, such as biological compatibility, bio-affinity, bio-activity, and osteoconduction (Habibovic et al., 2008), HAP bioceramics are frequently employed as artificial bone substitutes.

2.6 Scaffold Characteristics for BTE

a. Porosity

Porosity is a morphological feature that is not dependent on the material and is defined as the percentage of void space in a solid. Because they permit cell migration and proliferation as well as vascularization, pores are essential for the development of bone tissue. Porosity can be measured by a mercury porosity meter or methods like liquid displacement method which are the most widely adopted method in tissue engineering applications for testing the porosity of the developed product. A porous surface also promotes the mechanical interlocking between the implanted scaffold and the surrounding natural bone, resulting in better mechanical strength at this crucial phase (Loh et al.,2013). Depending on the material used to make the scaffold, the most popular methods for introducing porosity into a biomaterial are salt leaching, gas foaming, phase separation, freeze-drying, and sintering (Costantini et al.,2018; Fereshteh et al.,2018; Henderson et al.,2005).

b. Biocompatibility

Any scaffold used in tissue engineering must first and foremost be biocompatible for cells to adhere, operate correctly, move to its surface, finally pass through it, and start to multiply before the formation of a newer matrix. Biocompatibility of any material can be tested using *in-vitro* cell assays like MTT assay which shows the compatibility of the developed product with the specific cell or tissue for which it is developed. Post implantation, the tissue construct must only generate a minor immune response to avoid inducing a strong inflammatory response that could hinder recovery or result in the body rejecting the structure.

c. Pore size

The scaffold's mean pore size is another important factor (Abbasi et al.,2020). Ligands on the scaffold's surface are the primary means by which cells communicate with them. In contrast to scaffolds constructed from synthetic materials, which may require purposeful integration of these ligands through, for example, protein adsorption, natural extracellular materials like collagen naturally contain these ligands in the form of Arg-Gly-Asp (RGD) binding sequences (Ruoslahti et al.,1996). The particular surface area, or the accessible surface inside a pore to which cells can cling, affects the ligand density. This is dependent on the scaffold's mean pore size. Therefore, pores must be both big enough to permit migration of cells inside the structure and should be optimally small to establish a higher surface area, which results in a minimal ligand density to enable specific binding of certain cells to the matrix surface. The pore sizes of scaffolds vary depending on the specific application which can be visualized using SEM. For BTE applications, the ideal pore size ranges from 50 to 300 μm but to achieve significant bone tissue regeneration, the minimum pore size required is typically between 75 and 100 μm. Hence, a crucial range of pore diameters exists for every scaffold which may differ based on the cell type employed and the tissue being developed (Murphy et al.,2010).

d. Biodegradability

The main objective of tissue engineering is to gradually replace the implanted scaffold or tissue-engineered construct with the body's cells over time, therefore, while selecting a material the rate of degradation and resorption should also be considered (Hutmacher et al.,2000). The water permeability and the degradation behaviour are influenced by the structural porosity parameter. A reduction in hydrophilicity for bone-regenerating scaffolds guarantees a prolonged rate of disintegration. Because more severe *in-vivo* conditions result in a decrease in the scaffold lifetime, great caution must be exercised in *in-vitro* degradative investigations. In

addition, the byproducts of this breakdown must be non-toxic and can leave the body without harming other organs (Echeverria Molina et al.,2021). The main enzyme that breaks down chitosan *in vivo* by hydrolyzing its N-acetyl glucosamine groups is called lysozyme. Amino sugars are released during its breakdown and can be eliminated or added to glycosaminoglycans and glycoprotein metabolic pathways. When chitosan is hydrolytically cleaved, oligomeric units are generated on the scaffold surface that dissolve in PBS. In water, gelatin also hydrolyses rapidly. A controlled infusion of cells, such as macrophages, along with an inflammatory response is necessary to allow breakdown to happen concurrently with tissue development.

e. Mechanical Property

The scaffold should ideally have mechanical characteristics that match the tissue and should ideally be sturdy enough to allow surgical manipulation during implantation and be compatible with the anatomical site into which it is to be implanted. One of the biggest obstacles in trying to engineer bone or cartilage is creating scaffolds with acceptable mechanical characteristics (Echeverria Molina et al.,2021). A typical trabecular bone has a mechanical strength of 0.1 to 30MPa. The ultimate compressive strength of bone along the short axis is 131 MPa, which is about 36% less than the 205 MPa longitudinal value (Morgan et al.,2018). Universal compressive testing is the main method utilized to test the compressive strength of the developed product. Age-related differences in healing rates present another difficulty; for instance, in young people, fractures typically recover to the point of weight-bearing in about six weeks, whereas in the elderly, the rate of repair slows down (Clark et al.,2017) to one year. In addition, numerous materials that showed promise in *in-vitro* testing failed badly when they were transplanted *in vivo* due to poor vascularization. Therefore, scientists are creating more such scaffolds but with higher mechanical qualities and enhanced porosity to achieve better vascularization. Hence, it is clearly stated that the success of any scaffold depends on striking a balance between mechanical characteristics and a porous architecture sufficient to permit cell infiltration and vascularization (Bartnikowski et al.,2014).

f. Swelling Behaviour

The scaffold's ability to swell can influence its interactions with body fluids, affecting nutrient absorption and drug delivery. Within the cell-seeded construct, the wetting affinity of the scaffold material affects cell adherence, spreading, proliferation, differentiation, and nutrient diffusion. It can be measured by soaking the scaffolds in phosphate buffer saline which has a

physiological pH of the human body therefore it provides the knowledge about how the scaffold is going to behave under *in-vivo* conditions and about its hydrophilicity too.

g. Bioactivity

The material should support biochemical interactions with cells, enhancing cell adhesion, proliferation, and differentiation. The inclusion of bioactive molecules can stimulate osteogenesis (Emadi et al.,2010). When extremely porous scaffolds made of calcium phosphate are submerged in SBF in physiological circumstances. Ions had leaked out as a result of their increased decay. In the immediate proximity, this release causes an increase in Ca^{2+} and PO_4^{3-} ions. It renders them easily accessible for the scaffolds' biological apatite precipitation. According to earlier research, other scaffolds can also develop apatites on their surface when exposed to biological bodily fluids. Coordination bonds between negatively charged non-collagenous bone extracellular matrix proteins and exposed cations are uncommon. The differentiation of planted MSCs into osteoblasts is triggered by coprecipitation with these deposited apatites. The scaffold should facilitate the attachment and growth of bone-forming cells (osteoblasts) and promote the formation of new bone tissue. Therefore, the main objective in the application of bone tissue engineering is the development of scaffolds with intrinsic osteoconductive and osteoinductive properties.

h. **Nanostructure**

Natural extracellular matrices (ECM) with a diameter range of 50–500 nm is made up of collagen fibres, which make up the biomimetic architecture of the human body. Incorporating a nanostructured surface can mimic the natural extracellular matrix and improve cell interactions, potentially enhancing cell behaviour and tissue integration. The scaffold's uneven fibrous features may promote cell adherence and penetration.

There are several other characteristics also that are now essential to tailor a scaffold for achieving the regenerative properties of bone. Table no.2.3. discussed the acceptance criteria for any scaffold for Bone tissue engineering applications.

a. Morphological studies

The surface behaviour of scaffold materials is crucial in the initial interaction between bone cells and the scaffold. Surface modifications can immediately alter cell attachment properties. Two key factors influencing cellular functions are the scaffold's 'average surface roughness' and 'surface free energy.' Increased surface roughness enhances cell adhesion by promoting

interfacial bonding between the material and the cells, while reduced surface roughness or a smoother surface tends to promote increased cell proliferation studied the effect of surface properties on scaffold microporosity(Zhang et al.). They found that the amount and distribution of void volume significantly influenced cell penetration within the scaffold. The core morphology of the scaffold material also plays a key role in determining mineral adsorption. The growth of bone-like apatite particles on the scaffold was evaluated using SEM-EDX analysis.

b. Elemental analysis

Although carbon, nitrogen, oxygen, and chloride were detectable and quantifiable, calcium and phosphorus were of particular interest. As we know the inorganic component of bone is primarily composed of calcium and phosphate in the form of biological apatite therefore, incorporating calcium and phosphate deposits is a crucial step in scaffold synthesis for bone bioengineering applications. These elements can be added either as a surface coating or integrated into the bulk material during scaffold preparation. Calcium and phosphorus ions were deliberately introduced to form calcium phosphate mineral crystals. Using a semiquantitative method, EDX determines the calcium-to-phosphorus (Ca/P) ratio, providing insights into the nature of the resulting crystal phase. The Ca/P ratio for nanobioglass (NBG), commercial hydroxyapatite (HA), and tricalcium phosphate (TCP) was 1.44, 2.33, and 2.00, respectively. In contrast, the in-situ calcium phosphate had a Ca/P ratio of 2.33. Limin Sun et al. reported that materials with a high Ca/P molar ratio are more stable in biological systems. The theoretical Ca/P value for inorganic HA in bone extracellular matrix (ECM) is 1.67. The crystals formed in this study can be characterized as calcium-rich, making them less soluble in biological media. This reduced solubility could be advantageous, as more soluble materials might induce a stronger response due to their degradation products.

c. Crystal nucleation

Both TEM and Selected Area Diffraction (SAED) patterns revealed crystalline HA nucleation, . It was generally observed that mineralization in calcified tissue resulted from heterogeneous nucleation.

d. Crystallinity and chemical structure

The electron diffraction pattern of HA crystallite arrangement was analyzed using **SAED**. In this arrangement, the mineral phase, formed from fused minerals, mirrors the charge

distribution and stereochemical arrangement of the natural bone matrix Like collagen, gelatin macromolecules play a regulatory role in crystal formation. **XRD** was used to identify the structural phases present in the scaffolds, particularly their crystalline nature. The mineral phase deposited by osteoblasts after seeding in the scaffolds could be quantitatively analyzed through XRD patterns, as studied by Dasgupta and his team (Dasgupta et al 2017).

FTIR analysis of the distinctive peaks in the fingerprint and functional group regions revealed the chemical nature of the scaffold components. Spectral alterations following crosslinking during scaffold formation were also seen in the secondary structures of HA, gelatin, and chitosan. The narrowing of amide I found near 1630 cm^{-1} and decrease in the intensity of amide I component, found around 1660 cm^{-1}. These were indicative of collagen denaturation, as observed from gelatin peaks. For amide I, the peak broadening and peak shifting to lower wave numbers has been found to be associated with the collagen fibre self-assembly.

e. Thermal study (Differential Scanning Colorimetry)

The differential scanning calorimetry was used to determine the glass transition temperature (T_g). At the halfway point of the heating scan, T_g was determined. The scaffold material's "product" shelf life was ascertained by its DSC scan. The sample's potential responses are interpreted by the DSC curve. It helps in determining the shelf life of the product as well. The scaffold material's mechanical properties, biodegradability, morphology, and cytocompatibility have all been significantly impacted by its thermal stability.

Table No. 2.3 Selection criteria for scaffold for BTE applications.

Parameters	Standard/Criteria	Testing Method	References
Pore Size	100-200 μm to facilitate osteogenesis and vascularization	Scanning Electron Microscopy (SEM)	O'Brien et al.,2004 ; Murphy et al.,2010
Porosity	70-90% porosity for optimal cell infiltration and nutrient flow	Liquid Displacement Method	Loh et al.,2013

Mechanical Strength	Compressive strength within 2-12 MPa (similar to cancellous bone)	Compression Testing (UTM)	Echeverria Molina et al.,2021; Morgan et al.,2018
Biocompatibility	No cytotoxic effects on cells	*In vitro* cell culture assay (e.g., MTT assay)	Bhisham et al.,2019; Kumari et al.,2022
Biodegradation	Scaffold degrades over 6-12 months, matching tissue regeneration rate	*In vitro* degradation test	Hutmacher et al.,2000; Echeverria Molina et al.,2021
Osteoinductivity	Scaffold promotes osteogenic differentiation	*In vitro* osteoblast differentiation assay (ALP, mineralization)	X. Wang et al., 2017
Surface Morphology	Surface roughness to promote cell attachment and proliferation	SEM	Chocholata et al.,2019; Kumari et al.,2022
Chemical Composition	Composition matches the specified biomaterial (e.g., HA, TCP)	X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR)	Maji et al.,2016
Water Absorption Capacity	High enough to support cell seeding and nutrient delivery	Gravimetric analysis	Basu et al.,2020; Kumari et al.,2019

2.7 Scaffolding Approaches for Bone Tissue Regeneration

Tissue Engineering is a multi-disciplinary field that develops bio-mimetic equivalents that reinstate, maintain, or enhance tissue functioning using precepts and developments from the biomedical sciences and technology (Montoya et al., 2021a). Biomaterials for tissue engineering applications can be defined as a material designed to take a form that can direct, through interactions with living systems, the course of any therapeutic or diagnostic procedure

(N. Zhang & Kohn et al., 2012). Polymers are still the most commonly used class of biomaterials in scaffold designing and construction for biomedical applications. They are excellent contenders for the growth of artificial bone and other body tissue scaffolds owing to their structural and mechanical properties, appropriate biodegradation rates, and biocompatibility that perfectly approximates those of protein molecules in both soft and hard tissues (Z. Mao et al., 2021). Scaffolding frameworks for BTE are porous materials employed to promote cell attachment, growth, and proliferation while supporting the regeneration of new bone tissue (Lei et al., 2019). The selection of biomaterials or their hybrids used as scaffolds is critical for BTE applications. The usage of different materials and their composites in biomedical treatments has recently grown rapidly. Therapies for a variety of bone-related ailments and disorders using biodegradable materials composites is one domain that is currently expecting to receive substantial research attention from the scientific community.

Biopolymers, the most widely accepted component of tissue engineering used to restore, replace or regenerate damaged human body parts, are divided into natural or synthetic polymers (Taghipour et al., 2020). Polymers synthesized by biological systems, including microbial cells, plants, and animals, are classified as natural polymeric materials. Natural polymers have numerous applications, including adhesive bandages, absorbent materials, primed cosmetic products, therapeutic drug delivery, and clinical scaffolds (Titorencu et al., 2017). Natural polymers can further be classified as protein- or polysaccharide-based polymers. The most intensively investigated naturally derived polymeric materials for BTE are collagen (Col), gelatin (Gel), chitosan (Ch), hyaluronic acid (HAP), silk fibroin (SF), and many more (Kim et al., 2017). These natural polymers have their sorts of advantages and disadvantages during the bone regeneration process and are briefly discussed in Table 2.4. Due to all these limitations mentioned in table 2.3, only multicomponent systems are more popular among researchers. These biopolymers have been blended in a plethora of forms, such as 3D porous scaffolds, hydrogels, nanofibrous scaffolds, films, microspheres, sponges, and composites (Filippi et al., 2020; Han et al., 2017; Ikono et al., 2019; H. D. Kim et al., 2017; Yao et al., 2020).

Protein-based polymers, unlike polysaccharides, contain amino acid (AA) sequences that are traditionally coupled with cell adhesion via integrin-binding domains or RGD sequences (Guo et al., 2021). As a result, cell attachment and osteoconductivity in polysaccharide-based polymeric scaffolds must be improved through surface chemical functionalization, blending with osteoconductive components, and integration of cell adhesion protein sequences or by blending them with protein-based polymers (Yang et al., 2021). Biocomposites are created by blending two or more biomaterials to improve the cytocompatibility and mechanical

characteristics of scaffolds for various applications (Basha et al., 2015; Goonoo et al., 2013; Mao et al., 2018). Table 2.5 shows the synthetic polymers used for the development of biocomposite scaffolds for bone tissue engineering and it discusses their advantages and challenges also.

Various bioceramics, such as hydroxyapatite (HAP), tricalcium phosphate (TCP), biphasic calcium phosphate (BCP), bioglass, and wollastonite, have been widely used by researchers to develop 3D structures in combination with biopolymers for BTE applications (Kalaiselvi et al.,2017; Maji et al.,2016; Maji & Dasgupta, 2014). Despite their potential, matching the mechanical properties of ceramic-based scaffolds with those of native bone remains a significant challenge due to the inherent brittleness of these materials, which limits their clinical applications. Currently, several ceramic-based products are available for clinical use in areas such as dental, maxillofacial, and orthopaedic applications. However, there is a critical need to develop advanced biomaterials that can address the limitations of existing solutions, such as the brittleness of ceramic products, the lack of bioactivity in polymeric scaffolds, the mechanical strength of polymers, the toxic degradation products of synthetic polymers, and the insufficient osteoconductivity of polymeric scaffolds. Hence, the development of ceramic based polymeric scaffolds are seeking great interest in bone tissue engineering applications.

Traditional polymer scaffolds often suffer from poor mechanical strength and also lack bioactive properties, which limit their effectiveness in tissue regeneration. To address these limitations, recent studies have focused on developing smart scaffolds using both basic and advanced material-based approaches to enhance the physical and chemical properties of the scaffolds. One such approach involves integrating metal nanoparticles (NPs), such as gold (Au) and silver (Ag) NPs, into the polymer solution, creating a hybrid material known as a nanocomposite scaffold. These metal NPs not only improve the polymer's physical and chemical properties but also offer bioactive benefits, including natural antibacterial, antiviral, and anti-inflammatory effects, making the composite particularly advantageous for tissue regeneration. Several other metals and metal oxides like Cu, Sr, Zn, Fe and GO have also found great application in BTE application as a doping agent due to their vast physicochemical properties (Ryan et al., 2019; Yuan et al., 2019). Therefore, the development of smart scaffolds using metal nanoparticles has become popular in BTE applications. It is discussed in detail further in the doping section.

Table 2.4 Advantages and Challenges of Natural Polymers used in BTE (Kumari et al.,2022)

Polymer	Advantages	Challenges	References
1. Collagen (Col)	Excellent biocompatible & biodegradable; natural component of ECM; non- toxic; bio-adhesive; highly hydrophilic; mimic bone ECM topography; bio- functional; hemostatic; low immunogenicity; good permeability	Limited osteoinductive ability; poor mechanical strength; poor structural stability; high biodegradability; costly.	(Alvarez Echazú et al., 2022; K. Lin et al., 2019a, 2019b; Xing et al., 2021; D. Zhang et al., 2018)
2. Gelatin (Gel)	Highly biocompatible & biodegradable; high water solubility; low antigenicity; possesses anti-thrombogenic properties; the presence of RGD sequences allows better cell adherence.	Poor mechanical properties; less stable; highly biodegradable; brittleness; lack thermal stability	(Gautam et al., 2021; Raucci et al., 2019; Thomas & Bera, 2019)
3. Silk Fibroin (SF)	Biocompatible; shows slow degradation; biodegradable; excellent mechanical properties; water-based processing; good toughness and ductility.	Limited availability; highly brittle; the presence of residue contaminants.	(J. Chen et al., 2022; D. Ma et al., 2018; X. Zhang et al., 2019)
4. Chitosan (Ch)	Biocompatible; good biodegradability; possesses anti-microbial properties; non-toxic; cationic nature; owns the hemostatic property; less costly; abundantly available	Water insolubility; immunogenic nature; slow osteoconductive property; low mechanical strength and stability	(LogithKumar et al., 2016; Lu et al., 2022; Ribeiro et al., 2022; B. N. Singh et al., 2020a)
5. Chondroitin sulfate (CS)	Biocompatible; non-toxic by-products; non-immunogenic; possess anti-inflammatory & anti-oxidant properties; better bone regenerating properties; easily available	Water insolubility	(Kwon & Han, 2016; R. Sharma et al., 2022; B. N. Singh et al., 2019; Xu et al., 2021)

6. Alginate (Alg)	Highly biocompatible; biodegradable; easy to functionalize; simple gelation methods; chelating ability; excellent encapsulation capacity; easy to mold in different forms, including fibers, sponges, etc.	Poor mechanical properties; leaching of encapsulated drugs; difficult to handle and sterilize.	(Abhinandan et al., 2021; Iglesias-Mejuto & García-González, 2021; Sahoo & Biswal, 2021)
7. Cellulose (CL)	Biocompatible; non-toxicity; easily available; inexpensive; bio-degradability.	Longer renewal time, poor osteointegration; Low in biodegradability.	(Hickey & Pelling, 2019; Janmohammadi et al., 2023; Maharjan et al., 2021; Sharmila et al., 2020)
8. Hyaluronic acid (HA)	Highly biocompatible; biodegradable; high water solubility; natural component of ECM; excellent viscoelasticity; promotes cellular activities and growth; easy to functionalize	Poor mechanical strength; difficult to form fibers; costly	(Gilarska et al., 2020; H. Li et al., 2019; Moztarzadeh et al., 2021; Xing et al., 2020)

Table 2.5 Advantages and Challenges of Synthetic Polymers used in BTE (Kumari et al.,2022)

Polymer	Advantages	Challenges	References
1. Polylactic acid (PLA)	Biocompatible; biodegradable; non-toxic; non-inflammatory; stimulates cellular activities; FDA approved	Biological inertness; slow degradation rate; hydrophobic property	(Farzamfar et al., 2019; Idumah et al., 2021; Kareem et al., 2019; Narayanan et al., 2016)
2. Polyglycolic acid (PGA)	Biocompatible, possess faster biodegradation rates; non-toxic by- products; stable thermal and mechanical properties; supports cell adherence; FDA approved	Induce inflammatory response; high cost	(Budak et al., 2020; B. N. Kim et al., 2019; Yeo et al., 2021)
3. Poly (lactic-co-glycolic acid) (PLGA)	Biodegradable; promotes cell adhesion; FDA approved; mechanical properties can be adjusted	Exhibit immunogenicity; the presence of contaminants	(Babilotte et al., 2021; Kong et al., 2017; Martins et al., 2018; Wei et al., 2022)

4. Polyethylene glycol (PEG)	Biocompatible; low immunogenicity; high water solubility; non- toxic; easy to mold in different forms; FDA approved	High degradation rates; lack of cell adhesion ability	(Bai et al., 2020; Kong et al., 2017; Sreekumaran et al., 2021)
5. Polyvinyl alcohol (PVA)	Excellent biocompatibility; non- toxic; chemical and thermal stability; biodegradation; good mechanical stability and flexibility; high water solubility; possess adhesive properties; wide applications; cost-effective	Inadequate elasticity, poor hydrophilicity, poor cell adhesion properties	(Baker et al., 2012; Christy et al., 2022; Kumar & Han, 2017; Mallakpour et al., 2022; Thangprasert et al., 2019)
6. Poly ε-caprolactone (PCL)	Biodegradable, non-toxic by-products, FDA approved	Hydrophobic; limits cell attachment	(de Souza & Moraes, 2022; Dwivedi et al., 2020; Ghorbani et al., 2021; Saveleva et al., 2021; Surmenev et al., 2019)

2.8 Scaffold Fabrication Methods

Several techniques are used for the development of bone tissue scaffolds (Collins et al.,2021). Some of them are discussed below:

2.8.1 Solvent casting

The classic process of making scaffolds known as "solvent casting/particulate leaching" starts with the dissolution of a polymer in an organic solvent (Siemann et al.,2005). The method makes use of porogens, substances that can be mixed into a formed object and then removed after the object has hardened, creating pores. To construct a network of polymer-porogen, the porogen is incorporated into the polymeric solution. The porogen is further dissolved by adding sufficient water amount, and the porogen is usually a salt like sodium chloride which is obtained after the polymer has solidified and the solvent has evaporated. However, it is challenging to manage the pore geometry and porosity produced by this approach, yet a firm polymeric porous scaffold is formed in this.

2.8.2 Electrospinning Technique

An electrically charged flow of polymer in a viscous condition or solution is pulled into a fibrous structure during the electrospinning process by electrostatic forces. Syringe pump, power source, metallic needle to allow electricity to pass into polymeric solution, and metallic collector for fibre collection make up the four major components of a typical electrospinning system. Typically, a matrix is built by attaching the spinneret and fibre collector to terminals with opposing ends. The material is dragged out and deposited onto a collector as a result of the potential difference between the terminals, which makes it easier to create nanoscale threads (Ghosal et al.,2018). Electrospinning is commonly used to create high porosity, high surface area collagen and gelatin nanofibers (Muthukrishnan et al.,2022).

2.8.3 Lyophilization (Freeze drying)

Drying of polymeric solutions is the foundation of freeze drying, also known as lyophilization. Three steps can be outlined: (1) solution preparation, (2) moulding of the polymeric solution, and (3) freezing and drying at lower pressure. The third process involves extracting ice and liquid water through sublimation and desorption, respectively (Fereshteh et al.,2018). With pore diameters ranging from 20 to 200 μm and a porosity of about 90%, freeze-drying can create scaffolds. Freeze rate, polymer content, and temperature all affect pore size (O'Brien et al.,2004). A high, strong vacuum is necessary to create a scaffold with high porosity and interconnectivity.

2.8.4 3D bioprinting

By using 3D digital models created with CAD software, bioprinting is exceptional in that it produces layered, complicated, and adaptable geometries. This cutting-edge technology can create geometries with tunable structural and mechanical properties, controlled architecture, and porosity—all while being dependable and reasonably priced. Additionally, to improve cellular responsiveness, the structures can integrate cells, bioactive chemicals, and/or medications (Murphy et al.,2014). For the manufacture of bioink, a variety of biological and synthetic polymers have been utilized in combination with bioceramics, although collagen and its derivatives are the most utilized materials for cell-laden fluids. Pre-processing, processing, and post-processing are the three phases that makeup bioprinting. Pre- processing is the process of using computerised tomography (CT) or magnetic resonance imaging (MRI) scans to image the target tissue's anatomy and convert those pictures into sliced 3D models. The creation of

the scaffold and the development of the bioink are both included in the processing step, which includes everything involved in creating the bioprinted tissue (Mandrycky et al., 2016). Post-processing, which usually takes place in a bioreactor, is the maturing of the final printed tissue until it is appropriate for *in vivo* use.

2.8.5 Gas foaming

Gas foaming removes the need for solvents used in particle leaching and solvent casting technique. It creates a porous structure via the formation and expansion of gas bubbles scattered on the overall structure of the polymer (Costantini et al.,2018). In a heated mould, compression moulding is initially used to form solid discs of a scaffold material, such as poly-y (lactic-co-glycolic acid). After that, the discs are exposed to 5.5 MPa of carbon dioxide pressure after 72 h at room temperature, after which the gas's solubility is steeply reduced by lowering the CO_2 pressure to atmospheric levels. This carbon dioxide gas clumps together, as a result, forming pores (Dehghani et al.,2011). Using this method, pore sizes and porosities up to 100 mm and 93%, respectively, can be achieved. However, maintaining pore control is challenging.

2.8.6 Decellularization

For simulating the natural bone microenvironment, decellularized bone matrix (DBM) is employed widely in the field of BTE as matrix information of bioinks for the fabrication of biological substitutes. Decellularization entails the complete extraction of cells from the tissue while saving the original extracellular matrix's structural integrity, composition, and potential for stimulating cellular proliferation and differentiation (Crapo et al.,2011). Surfactants and enzymatic processes (such as sodium dodecyl sulphate (SDS) with ammonium hydroxide, Triton X-100, sodium deoxycholate, nucleases, and proteases), as well as heat shock, sonication, and hydrostatic pressure, are some of the processing methods used to generate DBM. Decellularization offers the advantage of using no hazardous chemicals and minimising protein denaturation, allowing for the preservation of a significant amount of ECM material (Gilpin et al.,2017). Nucleases and dehydrated alcohol are used in the last stages to completely remove all cellular remnants. DNA content analysis and cell nuclei staining are used to verify decellularization.

2.8.7 Thermally Induced Phase Separation

In order to induce the separation of a homogeneous polymeric solution, phase separation depends on the modification in heat energy (Henderson et al.,2005). A thermodynamically unstable polymer, like PLLA, can separate easily into a solvent excess phase and a polymer excess phase when it is dissolved in a solvent. Utilizing this phenomenon, phase separation scaffold manufacturing starts with the solubility of a polymer in a higher temperature, lower-molecular-weight solvent typically around the melting point of the polymer that allows the formation of a homogenous blend. Table 2.6 shows all the different methods and their applications along with advantages and disadvantages. For this process, the solution is further cast into the required scaffold shape and frozen under controlled conditions to develop the desired matrix.

Table No. 2.6: Methods of fabrication of scaffolds and their advantages and disadvantages

Method	Description	Advantages	Disadvantages	Applications
Freeze-Drying	Scaffolds are created by freezing a polymer solution and then sublimating the ice.	High porosity, control over pore structure.	Complex process, requires specialized equipment.	Bone regeneration, tissue engineering.
Solvent Casting and Particulate Leaching	Polymer is dissolved in a solvent, cast into a mold, and then particulate leaching creates pores.	Simple process, customizable porosity.	Limited control over pore interconnectivity.	Bone scaffolds, drug delivery systems.
Electrospinning	A polymer solution is spun into nanofibers using an electric field, creating a mat-like scaffold	High surface area, mimics extracellular matrix	Low mechanical strength, difficult to scale up	Bone repair, tissue engineering
3D Printing	Uses computer-controlled	High precision, customizable designs.	High cost, limited material choices	Customized bone

	deposition of materials layer by layer to build complex structures			scaffolds, implants
Bioprinting	Similar to 3D printing but uses living cells and biomaterials to create tissue constructs.	Can create complex, functional tissue structures	Complex, expensive, requires specific conditions	Personalized tissue and organ engineering
Hydrogel Casting	Scaffolds are formed by polymerizing hydrogels into desired shapes, often using molds	High water content, good biocompatibility	Mechanical strength may be low, degradation over time	Soft tissue repair, drug delivery
Electrochemical Deposition	Uses electrical currents to deposit materials onto a substrate to form a scaffold.	Precise control over thickness and composition	Complex setup, limited material range.	Coatings, functionalized scaffolds
Particle Sintering	Powder particles are fused together using heat or lasers to form a solid scaffold	High porosity, precise control over pore structure	Requires high temperatures, can be expensive	Bone implants, orthopedic applications

2.9 Doping and its Advantages

Biomedical sciences (medicine and biology) and materials science have collaborated in recent years to create biomaterials for application in tissue reconstruction and replacement. For this phenomenon, the biomaterials need to be physically and chemically stable, as well as possess certain mechanical qualities. Because of their biocompatibility, biodegradability, bioactivity, and osteoconductivity (Rahaman et al.,2011), various bioceramics and bioactive glasses (BGs) are among the most frequently utilized materials for hard tissue recovery and prosthesis covering. BGs are among the most extensively studied biomaterials for hard tissue replacement and repair (Philippart et al., 2017). But even before the bioactive glasses, phosphate-based

glasses which date back to the early 20th century were discovered by Dr. Jonathan Knowles. Phosphate-based glasses were initially developed for optical applications due to their unique properties, such as high ultraviolet transmission and low melting points. Over time, research into phosphate glasses expanded, leading to the discovery of their bioactive properties, which opened up new avenues for biomedical applications (Knowles et al.,2003). These include calcium phosphates [e.g., hydroxyapatite (HAP), and tricalcium phosphate (TCP)] and calcium silicates (e.g., wollastonite ($CaSiO_3$) and larnite (-Ca_2O_4). By generating apatite, like bone, on the surface, bone-bonding materials can create a chemically stable contact with the surrounding tissues. Apatite production *in vitro*, when exposed to an aqueous media with a chemical composition that mimics physiological liquids, can be used as a predictor of apatite formation *in vivo*. New generations of bioceramics and BGs have been developed because of their capacity to induce bone regeneration and resemble biological tissue, eliciting a response from the body similar to that seen in the presence of natural tissue. Materials that can both anchor the implant in place and attract the cells that regulate their breakdown rate have become increasingly important. Bone forming capability can be achieved by manipulating the release of certain ions during dissolution *in vivo*. The high solubility rate means that the released ions are carried away from the implantation site by bodily fluids, which might disrupt the regeneration of bone tissue during the ingrowth of new bone. Also, the degradation kinetic growth factors presented by calcium phosphate ceramics are typically unsatisfactory, and the materials lack intrinsic osteoinductivity. The low mechanical strength and fracture toughness of BGs also make it challenging to implant them in weight-bearing areas (Baino et al., 2018).
The addition of a trace amount (a few parts per million to a few per cent) of another element into the bulk material is known as doping. It has been found that an increase in the concentration of dopant ions plays a crucial role in facilitating the formation of new crystalline phases. What this means is that the conditions in which the thermal treatment is carried out can affect the pristine material's qualities (Sikder et al., 2020; Vahabzadeh & Bose, 2017). Bioceramics and BGs are now integrated with certain metal ions that possess therapeutic benefits like $Sr^{2+,}$ $Cu^{2+,}$ and Zn^{2+} into their chemical structure to mitigate the drawbacks of virgin materials and promote their utilization in a variety of biomedical settings. It has been studied from past researches that minerals including cations Mg^{2+}, $Cu^{2+,}$ $Zn^{2+,}$ Sr^{2+}, $Fe^{3+/}Fe^{2+,}$ $Co^{2+/3+}$, and Mn^{2+} are also essential for bone growth, development, and maintenance. Therefore, incorporating them into biomaterials has been shown to increase angiogenesis and osteogenesis (Ryan et al., 2019; Y. Yuan et al., 2019; Zhuang et al., 2019). Doping with Ag^{+}

and Cu^{2+} in the structure of bioceramics and BGs has also been extensively studied because of the crucial role of cations in regulating metabolic pathways and shielding against microbial infections in a wide range of biological contexts (El-Rashidy et al., 2018; Hui et al., 2020; Predoi et al., 2019; Ryan et al., 2019; Sikder et al., 2020; R. P. Singh et al., 2020). Some researchers have taken a different tack, looking at these materials as drug delivery systems. Targeted nano-carriers or scaffolding components, doped-bioceramics are functionalized with the drug or biomolecules for this aim (Morsi & Abd Elhamid, 2019; R. P. Singh et al., 2020). Sr^{2+}, Ag^{+}, Mg^{2+}, and Zn^{2+} have been the most investigated doping metal ions in the last five years (from 2017 to 2020), with frequencies of 13.6%, 12.7%, 12.5%, and 11.2%, respectively. Researchers have looked into using rare earth metals as dopant ions in bioceramics and BGs, creating a new promising category of luminescent optical nanomaterials that could replace organic fluorophores and quantum dots in bio-imaging applications ranging from cancer diagnosis to intraoperative guidance and postoperative evaluation (Neacsu et al., 2019). Still, some obstacles need to be conquered before an ideal biocompatible and biodegradable nanoparticle-imaging probe can be used in clinical settings. Different crystallinity, shape, and stoichiometry in the end products of metal-doped bioceramics and BGs have been achieved by the use of several synthesis techniques (Srinivasan et al., 2019). Methods such as sol-gel (El-Rashidy et al., 2018; Predoi et al., 2019; Zhuang et al., 2019), chemical precipitation (J. Chen et al., 2022; Gallo et al., 2019; Luo et al., 2018; Srinivasan et al., 2019), the melt- quenching approach (Deng et al., 2019), magnetron sputtering (Vranceanu et al., 2019), and pulsed laser deposition (Duta et al., 2019) are only a few of the many options. Once bioceramics and BGs have proven to be acceptable as coating materials (Predoi et al., 2019; Vranceanu et al., 2019), cement (G. Li et al., 2018; X. Zhang & Williams, 2019), scaffolds Luo et al., 2019; H. Yin et al., 2018; Y. Yuan et al., 2019; Zhao et al., 2014; Zhuang et al., 2019), and nanoparticles (S. Chen et al., 2019; El-Rashidy et al., 2018; Morsi & Abd Elhamid, 2019; R. P. Singh et al., 2020), the optimum synthesis process will depend on the application. Over the past decade, researchers have analysed the literature to determine the effects of metal ions in various inorganic matrices for application in a wide variety of diseases. Several studies using ion-doped inorganic matrices for bone tissue engineering have risen in the past years, which shows the growing interest in these materials among scientists. As a result, silicon, sodium, and phosphorus, the primary chemical components of silicate-based BGs and bioceramics, have attracted a major group of scientists and clinicians. Physicochemical, structural, and, by extension, biological behaviour *in vitro* and *in vivo* were investigated as a function of magnesium, silver, copper, strontium, iron, zinc, manganese, lithium, and cobalt ion

incorporation. However, bioceramics and other metal ions have attracted major attention from scientists yet their application is associated with several risks and limitations.

Ceramics are inherently brittle materials, making them prone to fracture under tensile or flexural stresses. This brittleness can limit their use in load-bearing applications where mechanical strength and toughness are crucial (Entezari et al.,2016). While ceramics can handle compressive loads well, they have low tensile strength, which limits their ability to withstand stretching or bending forces. The main drawback of ceramic materials like tricalcium phosphate, silicon carbide, hydroxyapatite, etc., is their low fracture toughness, they possess high compression resistance, high humidity degree, and high Young's modulus (Budharaju et al.,2023). If the ceramic scaffold is too stiff compared to the surrounding bone, it can cause stress shielding, where the scaffold takes on most of the load, leading to bone resorption and weakening of the surrounding natural bone (Lakhdar et al.,2021). Similarly, silica carbide which has excellent mechanical qualities, moderate degradation, and good biocompatibility, is a valuable material for load-bearing bones and hip implants (Li et al.,2014). However, over time, these scaffolds are prone to splitting because of their low resilience (Budharaju et al.,2023). The degradation rate of ceramics can be difficult to control and may vary depending on factors such as composition, porosity, and the local physiological environment (Vaiani et al.,2023). This unpredictability can lead to either too rapid degradation (before sufficient new bone is formed) or too slow degradation (impeding natural bone remodeling). Some ceramics, like pure hydroxyapatite, degrade very slowly or not at all *in vivo*, which may necessitate additional surgical intervention for scaffold removal (Ielo et al.,2022). Bioceramics can sometimes trigger an immune response, leading to inflammation, fibrous encapsulation, or rejection of the scaffold. The response can be exacerbated if the ceramic contains impurities or if the degradation byproducts are not well-tolerated by the body (Fillipovic et al.,2020; Fernandes et al.,2017). While porosity is essential for nutrient flow, cell infiltration, and vascularization, achieving the optimal balance between porosity and mechanical strength is challenging. High porosity can weaken the scaffold, making it more prone to fracture (Liu et al.,2023). Then producing the ceramic scaffolds with precise control over composition, porosity, and mechanical properties can be complex and costly. Advanced techniques like 3D printing or sol-gel processing may be required, which can introduce variability and affect reproducibility (Chen et al.,2021). Also, the sintering process, often used in ceramic scaffold fabrication, requires high temperatures that can cause changes in material properties, such as grain growth, which may reduce the bioactivity or mechanical strength of the scaffold (Miri et al.,2023; Budharaju et al.,2023; Ge et al.,2023). It can be challenging to customize ceramic

scaffolds like TCP and hydroxyapatite scaffolds for individual patients, especially for complex bone defects or irregular shapes. The rigid nature of ceramics limits their adaptability to specific anatomical sites like silicon-based ceramics (Guillén-Martínez et al.,2024). Ceramics, particularly those with low porosity, may hinder the formation of new blood vessels within the scaffold, leading to insufficient oxygen and nutrient supply to the regenerating tissue. This can delay or impair bone healing (Zhou et al.,2023).

While, metal ions which improve the bioactivity of a bone scaffold by interaction with stem cells and pre-osteoblasts at the interface also possess certain limitations like instability in ionic form which may cause cytotoxic effects in case of direct ingestion (Safiaghdam et al.,2019). Some of them might even result in neurological and haematological effects due to improper distribution inside the body (Roseti et al.,2017; Apaza-Bedoya et al.,2018; De Jong et al.,2019). It should be mentioned that to avoid local harmful effects and the negative impacts of ions on the metabolism of nearby cells, a scaffold that controls the precise amount of ions released within a given time frame must be designed (Wu et al.,2012). By carefully adjusting the ion release kinetics from the scaffold, one may decrease ion build-up and dose-dependent toxicity while also promoting advantageous cell behaviour (Safiaghdam et al.,2019). Due to the tiny size of nanoparticles, they can either be swallowed by macrophages or spread throughout the body through lymphatics, ending up in the liver, spleen, bone marrow, and lymph nodes (Urban et al.,2000). Metal ions used in scaffolds released by additional corrosion enter the bloodstream and concentrate in the erythrocytes. As a result, metal ions can penetrate cells and stay in nearby tissues or they can travel throughout the body, where they may have effects on the immune system, cytotoxicity, and genotoxicity that can occur close to or far from the implant (Doorn et al.,1998). Aluminum (Al) toxicity has been linked to various neurological disorders, including memory loss, gait disturbances, involuntary movements, and the development of conditions such as amyotrophic lateral sclerosis. Furthermore, its accumulation in the brain is suspected to play a role in the onset of Parkinson's disease, dialysis encephalopathy, and Alzheimer's disease (Yokel et al.,2000; Coleman et al.,1973). Chronic exposure to Al has also been associated with osteomalacia, pathological fractures, disrupted bone remodelling, altered vitamin D metabolism, and proximal myopathy (Jeffery et al.,1996). In one of the studies performed on rats, intra-articular injections of titanium dioxide (TiO_2) nanoparticles have been shown to cause toxic effects in the lungs, including follicular lymphoid hyperplasia and the aggregation of inflammatory cells around the bronchi (Wang et al.,2009).

Likewise, copper (Cu) which is an essential trace element for both humans and animals can promote osteogenesis and increase angiogenesis potential in addition to giving bioceramics and

bioglass antimicrobial qualities that make them a suitable choice in the field of bone tissue engineering (Wu et al.,2013). Nevertheless, a synergy between Cu^{2+} and the matrix in which it is deposited is required for copper particles to be applied successfully. Consequently, several matrices have been investigated for doping, the primary ones being Cu^{2+}, BGs, calcium phosphate, and HAP (Almaguer-Flores et al.,2020) In one such study, researchers synthesized mesoporous nanoparticles of copper-doped hydroxyapatite for drug delivery applications. XRD analysis revealed no peaks corresponding to Cu_2O or CuO phases, indicating that Cu^{2+} was successfully incorporated into the HAP crystal structure. Drug loading and release tests were performed using Vancomycin, which showed fast release in the first fifteen hours which remained constant afterwards at 86% (Bhisham et al.,2020). Similarly, in another study, scientists developed collagen scaffolds incorporating copper-doped bioactive glass (Cu-BG) with a base composition of $60SiO_2$ - 36CaO - $4P_2O_5$ (mol%). The goal was to reduce bone infection while promoting osteogenesis and angiogenesis. The results demonstrated that osteomyelitis can be treated using this scaffold system in the first stage only while on the other hand, conventional antibiotic therapy takes around six months for its complete treatment. Hence, it was inferred that Cu-doped scaffolds can be fruitful as a drug delivery system also (Ryan et al.,2019). Cu-doped scaffolds are very beneficial for osteogenic initiation and antibacterial properties it was reported by a team of scientists, that scaffolds with a molar ratio higher than 0.1 show a cytotoxicity effect due to Cu^{2+} ions (Zhang et al.,2019). Therefore, we have opted concentration of copper nanoparticles below 0.1M concentration for our research work. We have also chosen copper nanoparticles due to their high antibacterial properties, mechanical properties, osteogenic and angiogenic potential. Table no. 2.7 discusses the latest research done on the doping of materials and their application in bone tissue regeneration and Table No. 2.8 discusses the clinical applications of doping agents in different matrices for tissue engineering applications.

Another versatile dopant is graphene oxide used in bone tissue engineering which offers advantages in scaffold design, mechanical reinforcement, drug delivery, and cell interaction. One key advantage of using it in tissue engineering is its ability to create a specialized cellular niche on its nanoparticle surface for stem cells. Additionally, graphene oxide's unique properties, including its surface chemistry and size, make it an ideal carrier for growth factors, which are essential for promoting cellular migration, maturation, proliferation, and the differentiation of precursor cells into functional tissues. Due to its large surface area (890 m^2/g) and extensive π-conjugated structure, graphene oxide has a strong ability to adsorb various proteins and adhere to cells. These properties make it a promising candidate for use in

connective tissue regeneration. However, ongoing research is necessary to fully understand its long-term effects and to optimize its use in clinical applications. In a recent study, it was reported that graphene oxide possesses antibacterial properties against several gram-positive and gram-negative bacteria (Hu et al., 2010; Krishnamoorthy et al., 2012; Tu et al., 2013; Yin et al., 2013). Graphene-based materials are also found to provide enhanced mechanical strength in various studies and since the stiffness and strain of the substrate used in stem cell cultures play a crucial role in influencing stem cell differentiation, therefore we have also utilized graphene oxide in our research (Jang et al., 2011). Graphene's exceptionally high Young's modulus, combined with its flexibility to accommodate out-of-plane deformation, enhances its effectiveness in promoting the osteogenic differentiation of stem cells. In one such study, scientists cultured human osteoblasts and mesenchymal stem cells (MSCs) on graphene oxide (GO) and silica surfaces, respectively. After 48 hours, they observed a significantly higher rate of cell proliferation on the graphene surface compared to silica. Moreover, the MSCs cultured on graphene exhibited a spindle-shaped morphology, in contrast to the irregular polygonal shape of cells on the SiO_2 surface (Jang et al., 2011). Since MSCs with a spindle shape have a greater propensity for osteogenic differentiation, these findings suggest that graphene may promote the differentiation of MSCs into osteoblasts (McBeath et al., 2004). It is also observed that graphene oxide also retains its sp2 bonding lattice, it possesses high electrical conductivity, making it suitable for applications in cardiac and nerve regeneration too. Furthermore, its mechanical strength allows graphene oxide to serve as a robust matrix with high mechanical resistance, ideal for use in bone and cartilage regeneration also (Stobinski et al.,2014; Feng et al.,2020). Graphene-based materials have also shown higher osteogenic properties in various studies which makes them suitable candidates for bone tissue engineering (Kalbacova et al., 2010; Kumari et al.,2022). Hence, keeping in mind its higher mechanical properties and osteogenic potential, we have utilized graphene oxide in our research.

Table No. 2.7 Doping and its advantages in Bone Tissue Engineering (Reproduced with permission) (Kumari et al.,2022)

Scaffold material	Dopant	Positive effect on bone tissue regeneration	References
Single-doped scaffolds			

Hydroxyapatite	La	La^{3+} stimulated macrophage proliferation and activity via activating the Wnt/-catenin signalling pathway, which in turn improved osteogenic proliferation and differentiation. La-HAP/CS scaffolds demonstrated osteoinduction as well as biodegradation capabilities.	(J. Yin et al., 2019)
Mesoporous calcium silicate		Gradual deterioration of scaffolds released La^{3+} ions stimulating the TGF-β signalling pathway, which in turn encouraged the rBMSC proliferation along with osteogenic differentiation.	(Peng et al., 2019)
Whitlockite	Ce	The inclusion of Ce^{3+} to whitlockite decreased its crystallinity, thereby activating the SMAD signalling system, increasing osteogenic activity, upregulating the osteogenic genes expression, as well as accelerating bone repair.	(M. Hu et al., 2019)
Mesoporous calcium silicate	Eu	The luminescent Eu-mesoporous calcium silicate scaffolds could be utilised to mark and identify *in vitro* cultured cells and nascent bone growth *in vivo*. Eu^{3+} can promote bone regrowth as well as enhance osteoporotic development.	(C. Wu et al., 2016)
Bioglass	Gd	Through the Akt/GSK3beta mechanism, Gd3+ increased the development of hBMSCs and accelerated the process of bone induction.	(Zhu et al., 2019)

Bioglass	Ho	Reduced rapid cation leaching as well as stabilized dissolution of glass	(Delpino et al., 2021)
Co-doped scaffolds			
Hydroxyapatite	Yb/Er	When exposed to a 980 nm infrared laser, Yb/Er-hydroxyapatite nanorods displayed significant fluorescence intensity as well as light stability, were able to track the site of the BMP-2 protein translocation, as well as were biocompatible and capable of osteogenesis.	(M. Liu et al., 2019)
	Sm/Eu	It encourages hASC growth and possesses luminous properties.	(Alicka et al., 2019)
	La/Pr	When compared to single as well as stoichiometric hydroxyapatite, co-substitutions shown higher bioactivity, better cell viability, along with higher antibacterial efficacy.	(Chandran & Am et al., 2021)
Fluorapatite	Yb/Ho	To increase the luminous efficacy of up-conversion, the doping ratio of Yb^{3+} and Ho^{3+} was changed. Dextran-modified water-soluble fluorapatite nanoparticles were also used for biological imaging and cell labelling.	(X. Li & Chen, et al.,2016)

Table No. 2.8 Application of metal ions and their biological effect in different systems (Reproduced with permission) (Schatkoski V. M. et al.,2023)

Ion	Type of Matrices	Ion Concentration	Biological Effects	References
Mg^{2+}	CS	5 and 10 wt.%	Promoted angiogenesis and osteogenesis (*in vivo*)	Du Z. et al.,2020
	BGs ($85SiO_2$-15CaO, in mol%)	1 to 10 mol %	Controlled drug release	Tabia Z. et al.,2019
Ag^{2+}	HAP	4.8 mol %	Antibacterial activity and adsorption of blood plasma proteins	Chen K et al.,2019
	BGs ($58SiO_2$-$33CaO9P_2O_5$ and $50SiO_2$-50CaO, in mol%)	5 wt.%	Antibacterial activity	El-Rashidy A. A. et al.,2018
Cu^{2+}	HAP	0.6, 1.2, 1.8 and 2.4 (Cu/Ca molar ratio)	Antibacterial activity	Hui Y. et al.,2020
	β-TCP	5 and 10 mol%	Enhanced osteogenic differentiation and stimulated angiogenesis	Zhang J. et al.,2019
	BGs ($60SiO_2$-$36CaO4P_2O_5$, in mol%)	2 mol%	•Antibacterial activity •Enhanced angiogenesis and osteogenesis (*in vitro* and *in vivo*)	Ryan E.J. et al.,2019
Sr^{2+}	HAP	1:4 (Sr^{2+}: Ca^{2+})	•Improved protein adhesion, cell viability and ALP activity. • Formation of new bone and bone vessels (*in vitro* and *in vivo*)	Ge M. et al.,2018
	BGs ($55SiO_2$-$40CaO5P_2O_5$, in mol%)	10 mol%	•Stimulated the cell proliferation. • Enhanced angiogenesis and osteogenesis	Leite, A. J. et al.,2018
Li^{+}	Ca(H2PO4)2·H2O	2 mol%	Improved proliferation and differentiation of osteoblasts (*in vivo*) and stimulated osteoinduction (*in vivo*)	Yuan Y. etal.,2019
	HAP	•1 wt.% •1.5 mol%	•Antimicrobial activity •Improves osteogenesis (*in vitro* and *in vivo*)	• Duta L.et al.,2019 • Luo Y. et al.,2018
Fe^{2+}/ Fe^{3+}	OCP	0.58, 1.24, and 2.21 %	•Stimulated cell proliferation and	Shi H. et al.,2019

			improved protein adsorption (*in vitro*) • Improved angiogenesis (*in vivo*)	
	HAP	10-30%	•Antibacterial activity •Superparamagnetism	Morsi M.A. et al.,2019
	BGs ($51SiO_2$-$25CaO_20Na_2O$-$4P_2O_5$, in mol%)	7 mol%	•Inhibited the cancerous cell viability (MG-63) •Superparamagnetism	Rahman M.S.U. et al.,2020
Zn^{2+}	HAP	1 mol%	•Antibacterial activity • Stimulated cell proliferation	Predoi D. et al.,2019
	BGs ($70SiO_2$–$30CaO$, in mol%)	8 mol%	Improved protein absorption	Neščáková, Z. et al.,2019
Mn^{2+}	HAP	1, 3 and 5%	•Stimulated cell proliferation • Antibacterial activity	Panneerselvam, R. et al.,2020
	β-TCP	5 mol%	•Stimulated cell proliferation • Improved protein adsorption • Stimulated gene expression • Enhanced osteogenic differentiation	Wu t. et al.,2020
	BGs (58S)	3 and 5 mol%	Improved the bioactivity	Cañaveral, S. et al.,2019
	BGs ($50SiO_2$-$40CaO10P_2O_5$, in mol%)	3,5 and 7%	Antibacterial activity	Nawaz Q. et al.,2018
Co^{2+}	HAP	NA	•Improved osteogenesis • Antibacterial activity	Lin W.C. et al.,2019
	BGs ($36B_2O_3$-$22CaO18SiO_2$-$8MgO$-$8K_2O6Na_2O$-$2P_2O_5$, in mol %)	0.5 to 3.0 wt.%	• Improved protein adsorption • Enhanced angiogenesis and osteogenesis (*in vivo*)	Deng Z et al.,2019

2.10 Future Prospects of Tissue Engineering

Tissue engineering is emerging as a promising field with the potential to restore, maintain, or replace damaged and diseased tissues of various types. However, its scope must expand in the future to not only focus on developing engineered tissues that reduce the need for organ transplantation but also to accelerate the discovery of novel drugs. Some tissue-engineered

products have already received approval from the Food and Drug Administration, and over 70 corporate and government research agencies are investing $600 million annually in the development of new tissue-engineered products. According to the Alliance for Regenerative Medicine (ARM) 2021 report, global financing in this market increased to USD 22.7 billion in 2021, up from USD 19.9 billion in 2020, to support the development of 2,406 products currently under investigation, yet there is still much to be done. Large-scale production of tissue-engineered products still requires efficient techniques for cell isolation, culture, and long-term maintenance, as well as the optimization of smart scaffolds and the development of bioreactors that can mimic the body's tissue environment and support scaling up. As these technologies advance, tissue engineering is likely to become a key clinical treatment method, offering superior solutions compared to traditional approaches for treating not only bone but also other tissue defects and diseases.

SCOPE AND OBJECTIVES

Bone tissue disorders are one of the major global incidences that are increasing rapidly, and it is observed that the current clinical treatments are proving insufficient within the healthcare sector. Bone tissue engineering has evolved as a promising approach in recent years for treating bone tissue defects and diseases through the development of functionalized bioactive scaffolds. This is achieved by combining suitable biomaterials for scaffold development. However, several challenges have hindered the successful implementation of bone tissue engineering at the clinical level. In this context, the fabrication of scaffold matrices from appropriate biomaterials serves as the backbone of tissue engineering. Chitosan derived from chitin, is considered a promising natural biopolymer due to its biocompatibility, biodegradability, and wound healing properties while, gelatin which itself is a form of hydrolyzed collagen is used because of its biocompatibility, hydrophilicity and natural extracellular matrix-like structure. However, chitosan and gelatin scaffolds work well synergistically yet, they possess poor mechanical strength and osteoconductivity, limiting their effectiveness in bone tissue engineering. This limitation highlights the significant potential for developing chitosan/gelatin-based composite scaffolds with the incorporation of bioceramics and other nanoparticles to achieve the desired properties for bone tissue regeneration. Therefore, the present research focuses on developing a novel chitosan/gelatin based nanocomposite scaffold for bone tissue engineering applications, aiming to overcome the current limitations associated with chitosan and gelatin.

2.11 The specific objectives of this research are as follows:

1. To synthesize and characterize Copper nanoparticles and prepare and characterize Copper nanoparticles impregnated Chitosan/Gelatin based scaffolds using lyophilization
2. To synthesize and characterize nano-hydroxyapatite (nHAP) and prepare and characterize nHAP and CN infused Chitosan/Gelatin based scaffolds with improved mechanical strength
3. To synthesize and characterize nanobioglass (NBG) and prepare and characterize NBG and β-TCP infused Chitosan/Gelatin scaffolds with a controlled degradation rate
4. Characterize Graphene oxide (GO) and prepare and characterize NBG and GO embedded Chitosan/Gelatin scaffolds with enhanced mechanical strength and osteoconductivity.
5. Evaluation of biocompatibility, bioactivity and osteogenic potential of developed scaffolds by *in vitro* cell study.
6. Comparative evaluation of all the prepared nanocomposite scaffolds for their suitability towards artificial bone tissue constructs.

Scope of the Research Work

1. **Synthesis and characterization of copper nanoparticles and preparation and characterization of copper nanoparticles impregnated Chitosan/Gelatin based scaffolds**

As discussed earlier, chitosan/gelatin blend is a unique biomaterial with various superior properties such as biocompatibility, biodegradability and hydrophilicity. But despite having all these properties, it lacks good porosity and tunable mechanical strength limits its applications in tissue engineering applications. Copper nanoparticles known for their high antibacterial properties, wound healing tendency and mechanical properties find widespread applicability in wound dressing, skin and bone tissue engineering applications. Therefore, this section of research work focuses on the improvement of porosity and mechanical strength of the Ch-G blend by adding CN using the lyophilization technique. Finally, developed Ch/G/CN nanocomposite scaffolds are characterized for their physicochemical properties and biocompatibility in this phase of work.

2. **Synthesis and characterization of nanohydroxyapatite (nHAP) and preparation and characterization of CN/nHAP infused Chitosan/Gelatin based scaffolds**

It has been reported earlier that nanohydroxyapatite can enhance the bioactivity of biopolymeric scaffolds and can improve the mechanical strength of Chitosan/gelatin scaffolds. Whereas copper nanoparticles which possess high mechanical properties as reported earlier can enhance the overall mechanical strength of the scaffolds. Thus, it is hypothesized that the incorporation of nHAP with CN may improve the mechanical strength and the cell-supportive property of Ch/G scaffold for BTE application. So, this section of research work deals with the synthesis of nano-hydroxyapatite by sol-gel method. Synthesized nHAP was further characterized for particle size, morphology and elemental composition. Furthermore, we prepared nHAP/CN/Ch/G scaffolds with the same concentration of CN as in the previous study and characterized them for their physicochemical properties, bioactivity and *in-vitro* cell study in this phase of work.

3. **Synthesis and characterization of nanobioglass (NBG) and preparation and characterization of NBG/TCP infused Chitosan/Gelatin based scaffolds**

Nanobioglass is reported to possess excellent bioactivity and it also facilitates osseointegration with surrounding bone tissue *in vivo.* β-TCP and NBG both possess good biodegradation and NBG also offers ion-release phenomenon. Therefore, we have hypothesized that adding nanobioglass into β-TCP in different ratios will improve the biodegradation of the Ch/G scaffolds without hindering their mechanical strength and porosity. Thus the present work aims to develop a novel Ch/G/NBG/TCP nanocomposite scaffold matrix as a bioactive template with enhanced osteogenic potential for bone tissue regeneration. The developed scaffolds are evaluated in terms of physicochemical, mechanical properties and biomineralization. The biocompatibility, bioactivity and osteogenic differentiation ability of the developed scaffolds were investigated *in vitro.*

4. Characterization of graphene oxide (GO) and preparation and characterization of NBG/GO-infused Chitosan/Gelatin based scaffolds

Graphene oxide is proven to possess excellent electrical conductivity which influences the osteoconductivity and it also has great mechanical strength as discussed earlier. While nanobioglass proved to possess excellent bioactivity as shown in previous study therefore we hypothesized that the combination of NBG and GO incorporated scaffolds will result in a bone matrix with improved osteoconductivity and mechanical strength. Thus, this phase of research work focused on the development of graphene oxide and nanobioglass embedded Ch/G scaffold. The developed scaffold was further characterized for physicochemical properties, mechanical properties and bioactivity under physiological conditions. In addition, the biocompatibility and osteogenic differentiation of the developed scaffolds was investigated *in vitro*.