CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1. Background and significance of this study

Bone is the second most transplanted tissue globally. Every year millions of people suffer from bone loss due to degenerative bone diseases such as osteoporosis, osteoarthritis, accidental bone damage, etc., which entails surgical intervention to ease the limited ability of bone tissues to repair and regenerate (Abbasi et al.,2020). The frequency of such cases was trending steeply upward. It is projected to double by 2020 due to the prolonged life expectancy and ageing world population, coupled with poor physical activity and increased obesity (Amini et al., 2012). Autologous bone grafts and banked bone allografts came out as remarkable techniques for repairing damaged and diseased bones. Although there is a range of limitations, like another surgical site requirement, the risk of immune rejection and disease transfer too, therefore repair and regeneration of bone continue to be a significant challenge in orthopaedic and reconstructive surgery (Oryan et al., 2014). Bone tissue engineering has emerged as an alternative approach with a concept involving the use of three-dimensional (3D) scaffolds made of synthetic alloplastic materials as templates to promote cell attachment, and allow cell proliferation while maintaining differentiated functions. The alloplastic materials will then gradually degrade and eventually get replaced by new bone tissues (Sheikh et al.,2015; Feng et al.,2009). The extracellular matrix of bone is composed of an inorganic and an organic phase. The organic phase consists of type I and type III collagen and glycosaminoglycans, while the inorganic phase is made of hydroxyapatite which accounts for 69% of bone mass and the remaining 31% is the organic phase (Feng et al.,2009).

The primary challenges in bone tissue engineering involve creating scaffolds with specific physicochemical properties, including biomimetic characteristics, non-immunogenicity, optimal mechanical strength, controlled biodegradation, appropriate porosity, and suitable swelling behaviour to facilitate tissue regeneration. Additionally, modifying the scaffold's surface to create a biologically active matrix is crucial, as it enhances cell adhesion, proliferation, and differentiation of the host cells. To replicate the biomimetic properties of the bone extracellular matrix, there is significant interest in utilizing various biopolymers (like silk fibroin, chitosan and gelatin), inorganic ceramics like β-tricalcium phosphate, hydroxyapatite and bioglass, and their composites also. Notably, chitosan and gelatin have been used for the past two decades in medical and tissue engineering applications due to their remarkable attributes including mechanical strength, biodegradability, biocompatibility, and the desired hydrophilicity. When used together, chitosan and gelatin can synergistically enhance the

properties of bone scaffolds, offering improved mechanical strength, bioactivity, and support for cell growth.

However, chitosan and gelatin alone cannot fulfil all the required properties for tissue-engineered scaffolds, as reported in various studies. Therefore, many researchers have focused on developing chitosan/silk fibroin blends, chitosan/collagen blends, and chitosan/gelatin/hydroxyapatite composites to enhance the properties of the chitosan/gelatin blend. In addition to chitosan/gelatin polymers, bioceramics like β-Tricalcium phosphate, nanobioglass and nanohydroxyapatite have emerged as promising candidates for fabricating scaffolds used in tissue engineering applications for bone regeneration. Moreover, the development of biopolymer/ceramic composite scaffolds has garnered significant interest in the past decade for bone tissue engineering applications, integration of nanoparticles (such as copper, and graphene oxide) for surface modification and enhanced mechanical properties is still an explorable field for scientists in bone tissue engineering. Beyond exploring potential scaffold materials, creating porous scaffolds is crucial, as they mimic the extracellular matrix and provide an appropriate template for cell attachment, proliferation, and differentiation. In this context, lyophilization is recognized as an effective fabrication technique that can produce porous structures from polymeric blends and their composite solutions. Therefore, this research is focussed on the development of chitosan/gelatin-based nanocomposite scaffolds with the integration of bioceramics and nanoparticles as a potential artificial extracellular matrix for bone tissue engineering applications.

1.2 Scaffolds in Bone Tissue Engineering

Since bone is a hybrid composite made up of organic and inorganic components, one of the most promising approaches in fabricating its artificial extracellular matrix is to develop similar porous biomimetic substrates (Nikolova et al.,2019). Biomaterials such as natural polymers, synthetic polymers, bioceramics and biocomposites are widely used to manufacture this artificial matrix that resembles an extracellular matrix (ECM) for bone.

However, ceramic implants can be used temporarily and permanently for such conditions. Yet, polymeric and composite materials have recently been the focus of research and development (Skorulska et al., 2021). Almost all polymeric materials and their composites are biologically compatible, bio-resorbable, and also have Young's modulus interoperable with bone. It implies that once transplanted, they will promote the bone regeneration process and gradually be overtaken by newly formed tissue, leaving no scars once the process of bone healing is

complete. Some other choices are ceramic and bio-polymeric composites, which combine the functions of a polymer network reinforced with biologically active ceramic particles. These materials outperform others in terms of performance and mechanical behaviour, including osteoconductive effects (X. Wang et al., 2017). The scaffold (Do et al., 2015; C. Wang et al., 2020), a three-dimensional biological or synthetic artificial structure used to support bone compressive damages and allow bone tissue repair and re-growth (Roseti et al., 2017; X. Wang et al., 2017), is the principal temporary treatment for BTE. For BTE to be successfully applied, four key scaffold characteristics must be met: biological, structural, related material composition, and regarding production procedures. The resultant materials have outstanding osteoinductivity, osteoconductivity, and biocompatibility as potential benefits, offering a promising new technique for bone healing. Scaffold material composition is essential in creating artificial bone because they serve as the physical foundation for artificial grafts (Noori et al., 2017). The ideal and effective scaffold material should include attributes comparable to native bone, ensuring favourable physiochemical surroundings and biomechanical assistance for seed cell attachment, migration, multiplication, osteogenic differentiation potential, and neo-angiogenesis on the scaffold framework.

Last but not least, it must permit gradual integration into the host tissue throughout the healing process to support regular loads (Mishra et al., 2016; Roseti et al., 2017). To be organically digested, scaffold degradation products also need to be non-toxic and non-immunogenic. Mesenchymal stem cells homing, osteoblast differentiation, extracellular matrix and osteoid mineralization, and the development of terminally differentiated osteocytes all play significant roles in bone formation during the regeneration process (X. Wang et al., 2013).

Several materials are being utilized to fabricate bone tissue scaffolds, including both natural and synthetic polymers. Researchers incorporate various bioactive molecules, inorganic materials like hydroxyapatite, bioactive glass, and metallic and non-metallic nanoparticles to enhance these scaffolds' bioactivity. Since inorganic materials bioglass possesses elastic modulus similar to the cortical bone, they are widely used in the fabrication process. Whereas natural polymers possess less than 70MPa compressive strength, and synthetic polymers exhibit about 10GPa (Gunatillake et al., 2003; Pilipchuk et al., 2015; Chocholata et al., 2019). Therefore, they are combined to form a replicating matrix for bone tissue. Synthetic and natural polymers have been widely used as biomaterials to create these scaffolds, in large part because of their enormous diversity (Stratton et al., 2016).

The natural materials include polysaccharides like chitosan, hyaluronic acid, gelatin, starch, etc. Likewise, collagen, fibrin gels, silk fibroin, etc., also help cell adhesion. The only limitation is their

mechanical strength and pathogenic impurities that can result in immunogenicity. However, synthetic polymers like poly lactic acid, polyvinyl alcohol, polyglycolic acid, etc., and their copolymers, on the other hand, are utilized widely in scaffold development due to their tunable mechanical properties and degradation rate control. Inorganic materials include tricalcium phosphate, metals, HAP, and their combinations replicating the bone mineral phase.

Gelatin is one of the natural biopolymers that is derived from the hydrolysis of collagen protein. Its application is widely known in the field of biomedical engineering because of its similarity to collagen. Therefore, it is applied in several studies for Bone bioengineering. Due to its biocompatible nature, gelatin is combined with different biomaterials for enhanced compressive strength. Similarly, nano- hydroxyapatite and chitosan have been applied in several combinations for Bone tissue regeneration because of their chemical uniqueness to ECM (Nikolova & Chavali et al., 2019).

Chitosan is a linear polysaccharide that is made up of non-ionic N-acetyl- D-glucosamine monomer units and cationic D-glucosamine after being deacetylated from chitin. It is abundantly found polysaccharides in invertebrates like crustaceans and insects (Elieh-Ali-Komi & Hamblin et al., 2016). Apart from its easy accessibility and abundance, it possesses outstanding antimicrobial, biocompatible, biodegradable and nontoxic properties (Elieh-Ali-Komi & Hamblin et al., 2016). Chitosan's backbone is structurally identical to glycosaminoglycans which is an integral constituent of bone's ECM. Therefore, it has gained intense interest from researchers and clinicians working on bone regeneration. Nevertheless, chitosan exhibits some drawbacks like weak mechanical properties and reduced cell adhesion which can be overcome by chemical and physical modification of its molecule. Hence the integration of nano-hydroxyapatite and gelatin with chitosan polymer is supposed to promote mechanical strength and enhance the osteoconductivity of the scaffolds (Maji et al., 2015; Y. Wang et al., 2021).

Hydroxyapatite is majorly used for bone tissue due to its biocompatibility and structural and chemical similarities to bone mineral constituents. Both pure minerals and composites exhibit the unique property that they can directly bond with bone via strong biochemical bonds. Hence, they boost the host bone tissue and graft artificial ECM interaction, ultimately promoting the regeneration of bone tissue (Polo-Corrales et al., 2014). Hydroxyapatite is a bioceramic of calcium phosphate that has been widely used in regenerative medicine as a bone filler since the 1950s. It is highly osteoconductive and biocompatible for bone tissue due to its chemical similarity to natural bone ECM. It can promote bone ingrowth and also support osseointegration. Its microstructure can be controlled to promote pore formation and therefore it can promote vasculogenesis in the tissue. Nano-hydroxyapatite is the nano form of hydroxyapatite that can be synthesized through various methods like sol-gel method, coprecipitation, hydrothermal method, ultrasonic method, etc. It also

possesses non-inflammatory, non-immunogenic, non-toxic and osteoconductive properties that make it a suitable candidate for bone tissue regeneration (Turnbull et al., 2018). Regardless of its exquisite characteristics, a scaffold developed from pure hydroxyapatite with high porosity cannot withstand higher mechanical stress because of its inelastic property. For reducing these limitations, it's mostly integrated inside the polymeric ECM or it is applied as a filler to achieve higher mechanical strength. In the present study, we have presented the developed scaffold biomaterial and its *in-vitro* characterization using osteoblast cells for treating smaller to larger bone defects.

Nanobioglass are surface reactive glass ceramic biomaterials which can be synthesized by the sol-gel method or melt-quench method. They are promising materials for bone tissue engineering due to their controllable degradability and tendency to stimulate new tissue formation (Vafa et al., 2021). It is mainly composed of 45%SiO_2- 32.5% CaO-4%P_2O_5-10%Na_2O (silicate, calcium oxides, phosphorus pentoxide and sodium oxides). 60S Bioglass (60 wt% SiO_2, 4 wt% P_2O_5, 46 wt% CaO) is also a type of bioglass only the composition of above mentioned components is different in case of it. Nanobioglass (60S) is found suitable for bone tissue regeneration because of its bioactive nature. It is absorbed by the bone and finally replaced by bone because all the constituents are found in the human body physiologically (Durgalakshmi et al., 2014). It results in the formation of mechanically strong bone tissues by binding softly with the bone matrix. Therefore, it is considered a good option for using it as a bone filler or matrix for bone tissue engineering (Pajares-Chamorro et al., 2020).

Tri-calcium phosphate (TCP) is also one of the crystalline forms of calcium phosphate which can be easily absorbed by the human body (Wen et al., 2017). Therefore, it is highly biocompatible in nature and leads to the formation of a resorbable interlocking 3D network inside the defect site thereby promoting healing. It is more porous than hydroxyapatite and thus possesses faster resorbability. As soon as TCP resorbs, new bone formation occurs (Bhatia et al., 2010). TCP is osteoconductive in nature also thus it is used where resorption of biomaterial implant is desired. TCP is commercially used in different forms and it is widely used in combination with other polymers for splint injuries and other bone-related injuries. The addition of nanoparticles to these biocomposites can be very significant in enhancing the properties of the biopolymers.

Nanomaterials play an essential role in tissue engineering since achieving the appropriate mechanical strength, delivering bioactive agents and monitoring cell behavioural activities require a nanoscale approach. Therefore, nanoparticles can be highly advantageous in controlling and tuning these properties along with the slow and controlled release of drugs as per desired requirements.

Graphene oxide nanomaterial is also one of the novel and potential nanomaterials for bone tissue engineering applications and other biomedical applications. Due to its exceptional mechanical

properties, high surface area, physicochemical properties and electron transport (Shadjou et al., 2018). From previous studies, it is seen that when the cells were cultured over graphene-based nanomaterials their cell adherence and proliferation were better in comparison to only silicates (Kalbacova et al., 2010). Graphene oxide (GO) nanoparticles help in the construction of osteoconductive scaffolds for promoting cell adhesion and proliferation for the regeneration of bone tissue. GO not only mimics the extracellular matrix of bone but also biomimic the mechanical and chemical properties of bone while promoting cell differentiation and regeneration (Maleki et al., 2020). It also helps transport growth factors that ultimately induce improved cell migration, maturation and proliferation. It also enables the differentiation of immature precursors into functional tissues, thereby playing a vital role in tissue regeneration (Zhou et al., 2019). All these properties make GO a promising choice for application in bone tissue engineering applications.

The incorporation of **copper nanoparticles** enhances the mechanical properties of the developed matrix as seen in several studies (Sehmani et al.,2019; Wang et al.,2021). They have a unique antimicrobial property and they possess a conductive nature which supports the electrical conductivity of the cells. Copper is a crucial trace element and it plays a key role in angiogenesis and in the expression and stabilization of extracellular skin proteins. It also exhibits broad biocidal properties (Borkow et al., 2010). Copper is mobilized to injured sites to enhance host defence and early wound healing mechanisms (Zinc et al., 2013). The proangiogenic property of copper is exhaustively documented (Hu et al.,1998; Sen et al., 2002; Martin et al., 2005; Gerard et al., 2010). Also, it is observed that copper induces vascular endothelial growth factor in wounds possibly by stabilizing hypoxia-inducible factor- 1α (Sen et al., 2002; Martin et al., 2005). There are no data on the consequences of copper deficiency per se on wound healing except for a case report (Liusuwan et al., 2008). Study on copper nanoparticle composite hydrogels was done showing the efficacy of chitosan-based copper nanocomposite in wound healing phenomenon (Gopal et al., 2014). Later in 2015, a study was conducted wherein copper nanoparticles in chitosan scaffolds were shown to promote bone regeneration (D'Mello et al., 2015). Copper nanoparticles (CN) are widely applied in drug delivery, imaging of biomolecules, diagnostic purposes, enzyme immobilization, etc. They can be synthesized by various methods, including co-precipitation, sol-gel, hydrothermal, electrochemical techniques, pyrolysis, etc. (Kudr et al., 2017). High surface area and biocompatibility of these nanoparticles are the two properties due to which they are utilized in bone tissue regeneration along with other biopolymers. Understanding the merits of copper in metal, herein we have hypothesized that the incorporation of copper into scaffolds would aid the wound-healing potential of scaffolds by enhancing the VEGFs and providing enhanced mechanical strength to the artificial bone substitutes.

For manufacturing artificial biomimetic scaffolds, there are several fabrication and processing techniques used like lyophilization, salt leaching, 3D printing, electrospinning, phase separation, etc. that have their own benefits and limitations. But for bone tissue engineering porosity plays an essential role therefore in this context lyophilization has proved to be beneficial for scaffold development. The combination of all the above discussed biomaterials is applied to this study. Since they have found immense scope in bone tissue regeneration thus, they can form a potential scaffold for bone regeneration application.