

SUSTAINED RELEASE OF ANDROGRAPHOLIDE FROM FREEZE GELATION SCAFFOLD FOR BONE REGENERATION

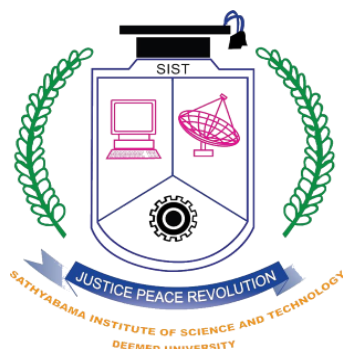
PROJECT WORK (PHASE-II)

Submitted in partial fulfilment of the requirements for the award of
Bachelor of Technology degree in Biotechnology

By

SHERIL TABITHA.Y (Reg.No - 39230042)

SUCHITHRA.L (Reg.No - 39230048)



**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO & CHEMICAL ENGINEERING**

**SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

Accredited with Grade "A" by NAAC
JEPPIAAR NAGAR, RAJIV GANDHI SALAI,
CHENNAI - 600 119

APRIL – 2023



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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of, Ms. Suchithra.L (39230048) who carried out the project "**SUSTAINED RELEASE OF ANDROGRAPHOLIDE FROM FREEZE GELATION SCAFFOLD FOR BONE REGENERATION**" under our supervision from December 2022 to April 2023.



Internal guide

Dr. S. Gracy Jenifer, PhD



External guide

Dr. S. Manjunath Kamath, PhD



Head of the Department

DR. V. RAMESH KUMAR

Submitted for Viva-voce Examination held on 10-05-2023



Internal Examiner



External Examiner

DECLARATION

I, **Ms. SUCHITHRA. L (39230048)** hereby declare that the Project Phase-II Report entitled "**SUSTAINED RELEASE OF ANDROGRAPHOLIDE FROM FREEZE GELATION SCAFFOLD FOR BONE REGENERATION**" done under the guidance of **Dr.S.Gracy Jenifer** at Department of Biotechnology and **Dr.S.Manjunath Kamath** at Makerspace, Sathyabama Centre for Advanced Studies is submitted in partial fulfilment of the requirements for the award of Bachelor of Technology in Biotechnology.

DATE: 10/05/2023

PLACE: Chennai

L. Suchithra

SIGNATURE OF THE CANDIDATE

I am pleased to acknowledge my sincere thanks to **Board of Management of SATHYABAMA** for their kind encouragement in doing this project and for completing it successfully. I am grateful to them.

I convey my thanks to **Dr. V. Ramesh Kumar, Ph.D**, Head of the Department of Biotechnology for providing me necessary support and details at the right time during the progressive reviews.

I would like to express my sincere and deep sense of gratitude to my Internal Project Guide **Dr. S. Gracy Jenifer, Ph.D**, for her valuable guidance, suggestions and constant encouragement paved way for the successful completion of my phase-II project work.

I am extremely grateful to **Dr. S. Manjunath Kamath, Ph.D**, for his support, valuable suggestions and timely guidance that has helped me throughout, for the completion of my phase-II project work.

I wish to express my thanks to all Teaching and Non-teaching staff members of the **Department of Biotechnology** who were helpful in many ways for the completion of the project.

Lastly, I would thank the Almighty for providing me with the strength, knowledge, ability, and opportunity to undertake this research study and to persevere and complete it. Without his positivity and stress handling techniques it wouldn't have been possible to have a wonderful completing of this project.

ABSTRACT:

The incidence of bone illnesses and ailments has trended sharply upward globally and is predicted to increase in the coming years, particularly in societies where ageing is associated with rising obesity and inadequate physical activity. By combining biomaterials, cells, and factor therapy in a synergistic manner, bone tissue engineering seeks to promote new functional bone regeneration. A key component of bone tissue engineering is the use of biomaterials as the building blocks for the creation of scaffolds. To encourage the production of bone, a prospective scaffold should be osteogenic, osteoinductive, and osteoconductive. This study aims at fabricating a natural polymeric scaffold for sustained release of pro-osteogenic compound. By freeze gelation technique, andrographolide-loaded albumin nanoparticles (AND NP) were synthesized and then immobilized into a scaffold at three different concentrations to fabricate CHIT- AND NP. X-ray diffraction investigation of AND NP and CHIT- AND NP, revealed that andrographolide was amorphized, which is highly desirable in drug delivery. Furthermore, release of andrographolide was prolonged, as inferred from drug release analysis. The scaffold was examined for its cytotoxicity and osteogenic potential *in vitro* using murine mesenchymal stem cell for 14 days. On day 14, von Kossa staining for calcium deposits revealed elevated mineralization in CHIT- AND NP groups. According to these findings, CHIT- AND NP enhances mineralization through a regulated, extended release of andrographolide, which raises the therapeutic potential for bone tissue engineering.

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LIST OF ABBREVIATIONS:

	ABBREVIATIONS:
BTE	Bone Tissue Engineering
3D	Three Dimension
BM	Bone Marrow
C-FMS	Colony – stimulating factor – 1 receptor
M-CSF	Macrophage colony – stimulating factor
HSC	Hematopoietic stem cells
SSC	Skeletal stem cells
DDK1	Dickkopf WNT signalling pathway inhibitor 1
ECM	Extracellular matrix
PCL	Polycaprolactone
PEG	Polyethylene glycol
kDA	Kilo Dalton
CS	Chitosan
BSA	Bovine serum albumin
mg	Milligram
mm	Millimetre
ml	Milli litre
nm	Nano metre
µm	Micro metre

μl	Micro litre
μM	Micro molar
NaOH	Sodium hydroxide
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco modified eagle medium
SEM	Scanning electron microscope
XRD	X – ray diffraction
VKS	Von Kossa Staining
CA	Contact Angle
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide
AND-NP	Andrographolide nanoparticle
CHIT	Chitosan
ALB	Albumin
STD DEV	Standard deviation

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1. INTRODUCTION:

Need for bone regeneration is skyrocketing with every year increase in bone defects—tumour, trauma and infection. Bone has the innate ability to regenerate as part of the healing process following an injury, as well as during the development of the skeleton or ongoing remodelling throughout adulthood. The most frequent type of bone regeneration that occurs in a clinical context is fracture healing, which recapitulates the normal embryonic skeletogenesis process, including intramembranous and endochondral ossification. Beyond the normal potential for self-healing, there are additional conditions in orthopaedic surgery and oral and maxillofacial surgery where large amounts of bone regeneration are needed, such as for skeletal reconstruction of large bone defects caused by trauma, infection, tumour resection, and skeletal abnormalities (*Dimitriou et al., 2011*).

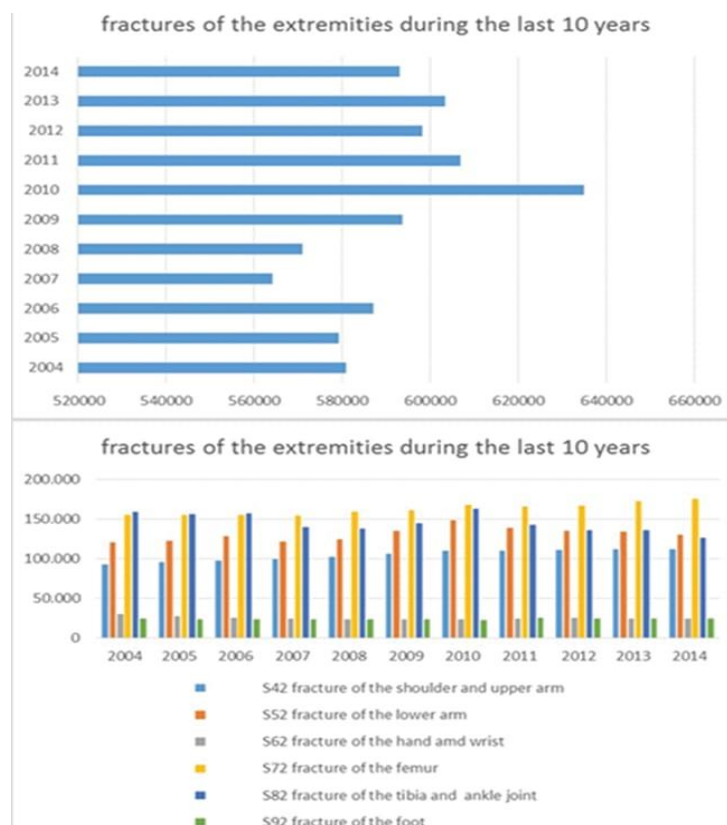


Fig 1.1 Extremity fracture statistics, Source: (Bucher et al., 2016)

During the healing of a fracture, cellular activity, angiogenesis, proliferation, and differentiation must be detected in tactile models. Research into the variables that trigger and regulate responses, the cells that take part in these responses, and the chemicals that are produced by these cells will provide new insights and help focus future efforts in the field of bone regeneration (Oryan *et al.*, 2015).

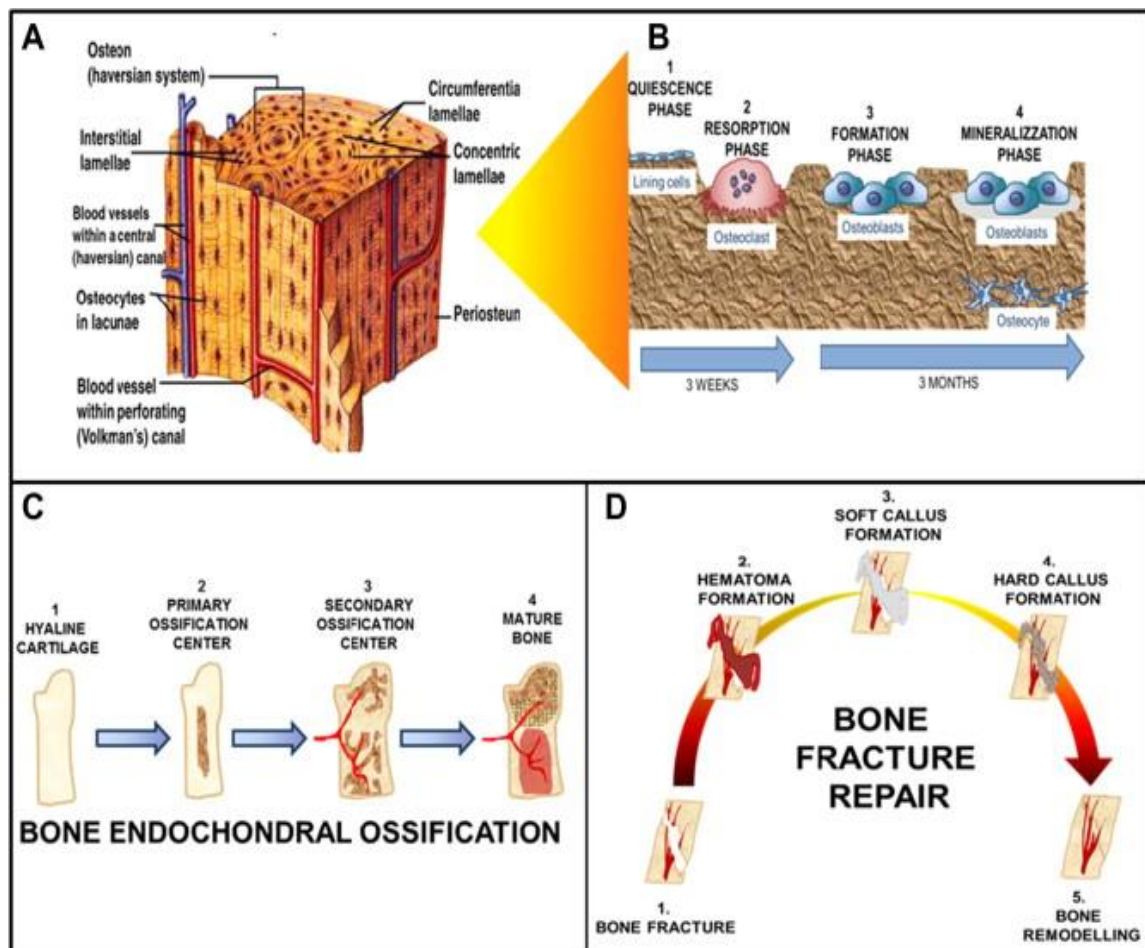


Fig 1.2 Anatomical illustration of bone vasculature, Source: (Saran *et al.*, 2014)

The use of autologous bone grafts is the gold standard for repairing bone deformities among the wide range of techniques. Due to the presence of growth factors and the recruitment of stem cells, bone autografts exhibit osteoinductive and osteoconductive qualities; nonetheless, their use raises issues with donor-site morbidity, poor anatomical fit, insufficient bone volume, and bone graft resorption. Alternatives to autologous bone that are most frequently employed are allogeneic and xenogeneic bone graft materials. Clinical use of patient-specific titanium and

synthetic implants for substantial bone defect repairs was made possible by the development of computer-aided 3D design and prototyping techniques. Despite perfect anatomical fit and high-precision manufacture, there are still a lot of complications with implants. Fractures, instability, implant extrusion, and rejection are the most common complications. Distraction osteogenesis has gained popularity in treating patients with extensive bone defects, but due to the intricate three-dimensional design of the defects and the high likelihood of surgical complications, it is technically difficult and not frequently employed in craniofacial bone restoration. Therefore, modern bone defect repair techniques are desperately needed. Reducing functional donor-site morbidity, overcoming bone graft volume restrictions, and enhancing the geometric match of the graft for the recipient site are current advancements in bone reconstructive surgery. . (Tsiklin et al., 2022)

Nearly three decades ago, the field of bone tissue engineering (BTE) was established. Over the years, there has been a tremendous increase in interest and progress in the BTE. The field of BTE focuses on alternative treatment options that, ideally, eliminate the previously mentioned issues linked to current diagnostically utilised treatment methods (i.e., donor site morbidity, limited availability, immune rejection, and pathogen transfer). To accomplish the ultimate objective of creating bone grafts that improve bone repair and regeneration, BTE necessitate the close collaboration of scientists, engineers, and surgeons.(Amini et al., 2012). Tissue engineering is the process of combining cells with scaffolding materials and growth factors to regenerate or replace damaged or degenerated tissues and organs. Scaffold materials act as frameworks for tissue formation and are critical in tissue engineering. (Li & Sun, 2022).

The understanding of each intervenient's individual and coordinated role on bone tissue physiology, has inspired the development of a variety of biomimetic and/or bioinspired bone regeneration approaches. In fact, new, effective, and compatible treatments for bone-related injuries are desperately needed.(Lopes et al., 2018). Understanding the composition and structure of native bone tissue, as well as the appropriate selection of biomimetic natural or tunable synthetic materials (biomaterials) such as polymers, bio ceramics, metals, and composites, is required for successful materials design for bone tissue engineering. Scalable fabrication

technologies, such as three-dimensional printing and electric-field- assisted techniques, that enable control over construct architecture at multiple length scales, can then be used to sequence these biomaterials into suitable forms for bone- tissue engineering.(Koons et al., 2020)

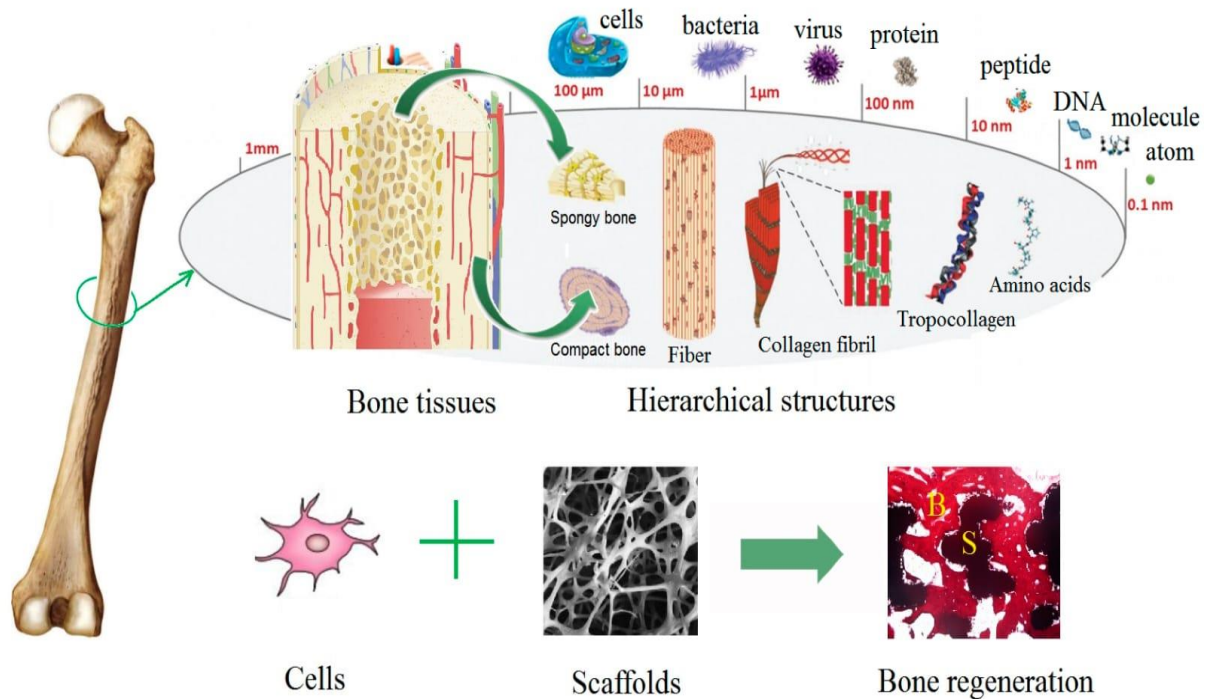


Fig 1.3 The hierarchical anatomy structure of bone tissue,

Source: (Chen et al., 2018)

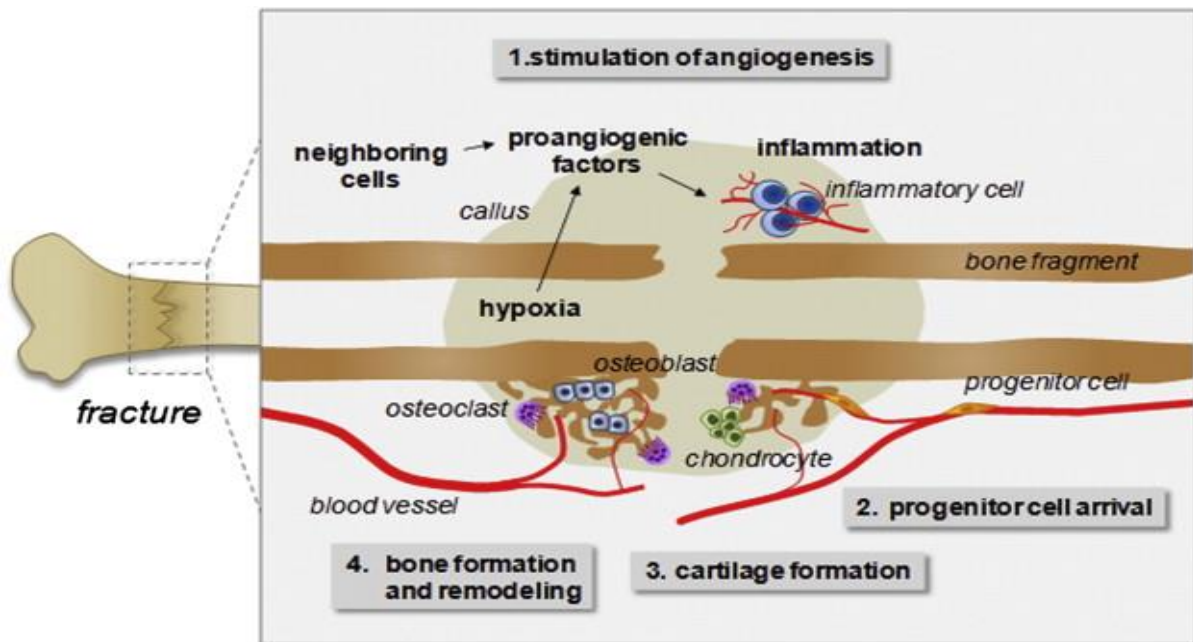


Fig 1.4 Angiogenic response during normal fracture healing,

Source: (Stegen et al., 2015)

disorders are connected to any imbalance between the processes of bone production and bone. Several key players are highlighted in the classic BTE paradigm: (1) a biocompatible scaffold that closely mimics the natural bone extracellular matrix niche, (2) osteogenic cells to lay down the bone tissue matrix, (3) morphogenic signals to help direct the cells to the phenotypically desirable type, and (4) adequate vascularization to meet the growing tissue's nutrient supply and clearance needs. (Amini et al., 2012). Antibiotics must penetrate through the rigid bone structure and into the synovial space to treat bone and joint infections. Several pharmacokinetic studies were conducted to assess the extent to which various antibiotics penetrated bone and joint tissues. Most antibiotics showed good penetration into bone and joint tissues, including amoxicillin, piperacillin/tazobactam, cloxacillin, cephalosporins, carbapenems, aztreonam, aminoglycosides, fluoroquinolones, doxycycline, vancomycin, linezolid, daptomycin, clindamycin, trimethoprim/sulfamethoxazole (Thabit et al., 2019). Although much progress has been made, many crucial hurdles remain to be cleared on the way to BTE becoming a true clinical reality. (Amini et al., 2012)

2. LITERATURE SURVEY:

2.1 FUNDAMENTALS OF BONE:

2.1.1 BONE:

Bone is an adaptable tissue that can constantly remodel and model itself to retain its functionality. This tissue has a number of important roles in the body, including supporting the body's soft tissues structurally, serving as the adult human haematopoiesis' core region, and assisting with mineral homeostasis. When harmed, it possesses a natural ability to regenerate itself. It is exceptionally dynamic, vascularized, active, and capable of promoting self-repair in a way that doesn't leave scars (*Manzini et al., 2021*). It is capable of carrying out a diverse range of tasks, and it reacts to a range of metabolic, physical, and endocrine stimuli. It serve as the basis for our physical movement, support our skeleton and protect our internal organs, store the biological components needed for haematopoiesis, trap harmful metals like lead, and maintain the homeostasis of important electrolytes by storing calcium and phosphate ions (*Amini et al., 2012*).

Bone is a living tissue that is regularly rebuilt in healthy people with significant immune system involvement. Numerous inflammatory bone resorption. The immune system is crucial for both tissue growth and bone resorption. In addition to immune cells and cytokines, bone cells such as osteoblasts, osteoclasts, and osteocytes also have an impact on how immune cells function on a cellular level. In addition to coexisting in the bone marrow (BM) cavity, bone and immune cells also share a range of regulatory molecules and progenitor cells. The regulation of bone homeostasis by immune cells is influenced by bone cells, and the proliferation and differentiation of immune cells is also influenced by bone cells (*Yang & Liu, 2021*).

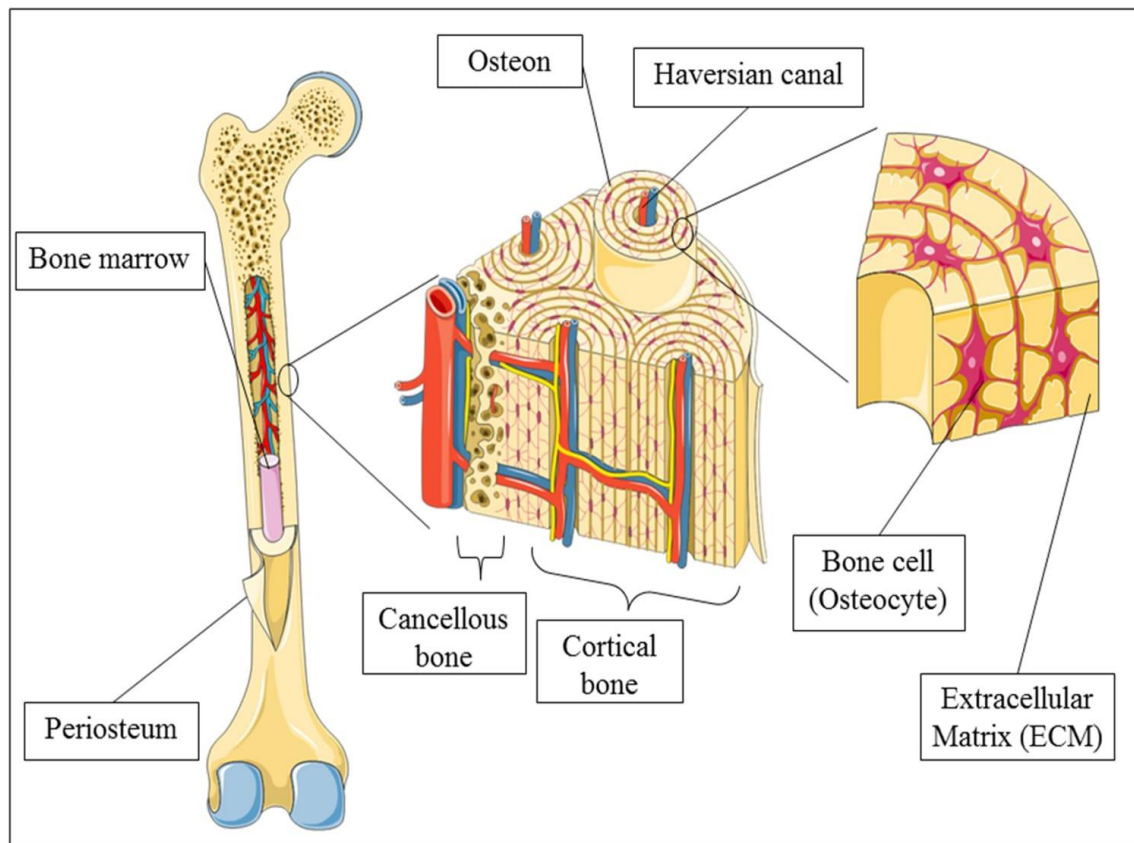


Fig 2.1 Schematic overview of bone, Source: (Le et al., 2013)

2.1.2 BONE GROWTH:

More than 200 distinct types of bones in the human body can serve a structural purpose and shield important organs. Bone is modelled and remodelled during life to accommodate growth or shape change. Bone remodelling occurs so that bone can retain its strength and mineral balance while bone modelling occurs when bones change in response to physiologic stimuli or mechanical forces that the skeleton encounters. Both bone remodelling (the replacement or renewal of old bone) and bone modelling (the development and contouring of bone) take place during early childhood. Bone remodelling occurs more frequently in adults than it does in children, who primarily model their bones at this time. The epiphyseal and metaphyseal regions of long bones are the first places where cartilage grows before it is mineralized to generate new bone (Katsimbri, 2017).

The close connection between the immune system and the skeletal system is made clear by osteoimmunology. Numerous shared cytokines and signalling molecules between the two systems imply that immune cells are essential for bone development. By balancing the production of osteogenesis and osteoclasts, optimising the bone immunomodulatory functions can lead to a mild inflammatory response that promotes bone regeneration. As a result, research on the relationship between bone tissue engineering and the immune system has increased (Ouyang et al., 2021).

Osteogenesis and ossification are frequently used interchangeably to refer to the process of bone formation. In the first few weeks following conception, the skeleton begins to take shape in parts. The skeletal pattern is generated in the cartilage and connective tissue membranes by the end of the eighth week following conception, and ossification starts. Bone growth continues into adulthood. Even after reaching adult height, bone formation continues for fracture repair and remodelling to accommodate changing lifestyles. Three different cell types osteoblasts, osteocytes, and osteoclasts are involved in the formation, expansion, and remodelling of bones. Bone is formed by osteoblasts, matured by osteocytes, and broken down and reabsorbed by osteoclasts (*Training seer, Sept. 2008*). Additionally, cartilage cells (chondrocytes) play a role in the development and growth of bones. These cells do not work independently; rather, they collaborate with other cell types in the bone microenvironment as well as with other lineages (*Ansari & Sims, 2020*).

2.1.3 BONE CELLS:

Three cell types are the fundamental components for the structural integrity of the bone at the microscopic level. The survival and activity of this tissue with a dynamic shape that changes constantly throughout life requires the cooperation of osteoblasts, osteocytes, and osteoclasts. These three cells are essential for this tissue's survival (*Manzini et al., 2021*).

2.1.3.1 OSTEOLAST:

Osteoblasts are cells that produce the bone matrix and are arranged in layers before mineralization. The lamellar resource of bone is produced by these cuboidal-shaped cells. Osteoblasts can generate bone tissue and have receptors and proteins that are involved in mineralization and bone remodelling. Additionally, these cells have the capacity to create additional proteins, including those involved in the catabolic process of regenerating bone tissue as well as the proteins that make up the bone matrix (*Manzini et al., 2021*). Osteoblasts are cuboidal cells that make up 4-6% of all resident bone cells and are found along the surface of the bone. They are primarily recognised for their role in the formation of new bone. These cells exhibit the physical traits of protein-synthesizing cells, such as a profusion of secretory vesicles, a conspicuous Golgi apparatus, and an abundance of rough endoplasmic reticulum (*Florencio-Silva et al., 2015*). Osteoblasts control the differentiation of immune cells. Osteoprogenitors helps in hematopoietic stem cell (HSC) maintenance. To support lymphopoiesis and T cell differentiation, respectively, osteoblasts secrete IL-7 and DLL4 (*Terashima & Takayanagi, 2019*).

The osteoblast lineage consists of matrix-producing osteoblasts, they are pluripotent and lineage-committed precursors, bone lining cells, and matrix-embedded osteocytes. Each stage has a unique function, morphology, location in relation to the surface of the bone, and a growing number of well-defined markers. Pluripotent skeletal stem cells (SSC), which also can be differentiated into stromal cells that promote haematopoiesis, chondrocytes, and adipocytes, give rise to the osteoblast lineage (*Ansari & Sims, 2020*). Osteoblasts do not make "bone," but rather osteoid matrix, which is eventually mineralized to form bone. Osteoid is the collagen-containing matrix component prior to mineralization. Osteoid can be deposited in either a woven or lamellar form. During bone growth and fracture repair, woven bone is deposited; it is made up of osteocytes and collagen fibres that are disorganised and appear to be arranged randomly. Contrarily, lamellar bone is highly ordered, and the parallel collagen fibres that contribute strength to nearby lamellae are arranged in perpendicular planes. Additionally, intermolecular and intramolecular cross-links fortify and reinforce the collagen fibres. Following the deposition of osteoid, osteoblasts might undergo apoptosis, become entrapped

inside the bone matrix and undergo osteocyte differentiation, or stay on the surface of the bone as bone lining cells (*Ansari & Sims, 2020*).

Type I collagen is produced and secreted by the osteoblast to create bone. The main protein in the bone matrix is type I collagen. The mineralization of this newly created bone osteoid is also aided by the osteoblast. The osteoid's matrix vesicles, which are descended from osteoblasts, release phosphates locally to cause mineralization. Hydroxyapatite crystals form together with the calcium from the extracellular fluid. For the skeleton to be as rigid and flexible as it should be, the ratio of organic matrix to mineral must be just right. 40% water, 20% organic material, and 60% minerals make up the adult human cortical bone (*Katsimbri, 2017*). Mature osteoblasts are visible as a single layer of cuboidal cells with a substantial Golgi complex and an abundance of rough endoplasmic reticulum (*Florencio-Silva et al., 2015*).

2.1.3.2 OSTEOCLAST:

Osteoclasts are large multinucleated cells whose primary function is bone resorption (*Salhotra et al., 2020*). Osteoclasts secrete two substances that cause bone resorption: cathepsin K, an enzyme that breaks down the insoluble portion of the bone matrix, and hydrogen ions, which cause the resorption compartment to become acidic and dissolve the mineral bone matrix (mainly type I collagen) (*Lopes et al., 2018*). Circulating mononuclear hematopoietic progenitor cells connect to the surface of the bone to produce active osteoclasts by fusing with each other after migrating there to form osteoclasts. By splitting into smaller pieces, the released mononuclear or multinucleated osteoclasts join up with other osteoclasts or osteoclast precursors to continue the resorptive process. Osteoclasts are also capable of producing new osteoclasts through fusion (*Ansari & Sims, 2020*). Functionally, osteoclasts can reabsorb the mineralized bone quickly by controlling the production of matrix enzymes. In this regard, the existence of an apical membrane facilitates the establishment of a seal with the calcified matrix, activating lytic enzymes. The discovery of new therapeutics to stop the bone loss can be aided by knowing the mechanisms involved in this process (*Manzini et al.,*

2021). Osteoclast differentiation depends on signalling through c- fms (the receptor for macrophage colony- stimulating factor; M- CSF) in mononuclear precursor cells which in turn up- regulate RANK expression and regulate essential osteoclast genes (Katsimbri, 2017). While RANKL promotes the differentiation of osteoclast precursors into mature osteoclasts, M-CSF promotes the proliferation of osteoclast precursors. Osteoclast precursors begin to differentiate into osteoclasts when RANKL and M-CSF are expressed in the bone marrow compartment, which marks the beginning of bone remodelling. To maintain constancy in bone mass, the ratio of bone production to bone resorption is strictly maintained during homeostasis (Salhotra et al., 2020). Some bone illnesses, including osteoporosis, where resorption outpaces creation and results in decreased bone density and an increase in bone fractures, are caused by an abnormal increase in osteoclast development and activity. Abnormal osteoclast activation causes painful osteolytic lesions and periarticular erosions in several pathologic situations, such as bone metastases and inflammatory arthritis (Florencio-Silva et al., 2015).

2.1.3.3 OSTEOCYTE:

The primary and most numerous cells in mature bone are osteoclasts (Manzini et al., 2021). When osteoblasts attach themselves within the osteoid matrix during bone formation, osteocytes are created. Osteocytes do not divide and can exist for many years in the bone matrix; the length of their life depends on how quickly the bone is changing. Although the exact method by which some osteoblasts become caught in the matrix is still unknown, they undergo morphological changes as they develop into osteocytes, including an increase in dendritic length and a decrease in cell body size (Ansari & Sims, 2020).

Ninety-five percent of the cells in bone are osteoclasts. They come from osteoblasts that went through terminal differentiation during forming bones and were then absorbed by the unmineralized osteoid. Following mineralization, these cells become osteocytes. They are located within the mineralized bone's in lacunae (Katsimbri, 2017). They are in charge of creating a network inside the mineralized bone. They are developed osteoblasts that are derived from MSCs and are

specialised, but they differ physically and functionally. Morphologically, they are more compact, have a larger nucleus/cytoplasm ratio, and have fewer organelles. Numerous cephapods are present on osteocytes, aiding in their interaction with the cells that make up the lining of the bone. Osteocytes carry out mechanotransduction and mechanosensitivity in a functional manner. Mechanotransduction is the process of converting physical forces into biochemical signals that cause cellular reactions. The main and most numerous cells in mature bone are called osteoclasts.(Amini et al., 2012) (Manzini et al., 2021).Osteocytes are produced through osteoblast differentiation from MSC lineage. Osteoid-osteocyte, preosteocyte, young osteocyte, and mature osteocyte are four distinguishable stages in this process. A subpopulation of osteoblasts turns into osteocytes and is integrated into the bone matrix at the end of a cycle of bone formation. Conspicuous morphological and ultrastructural alterations are seen in conjunction with this process, one of which is a reduction in the size of the round osteoblast. Protein synthesis and secretion are reduced as a result of a reduction in the number of organelles such the rough endoplasmic reticulum and Golgi apparatus and an increase in the nucleus to cytoplasm ratio(Florencio-Silva et al., 2015).

Osteocytes regulate bone-forming and bone-destroying cells to manage bone remodelling. Sclerostin and Dickkopf Wingless-related integration site signalling pathway inhibitor 1 (DKK1), two wingless-related integration site signalling antagonists, are released by osteocytes to reduce osteoblast differentiation and activity during bone demineralization (67). The primary bone cell type that produces sclerostin is the osteocyte, and this protein prevents osteocytes and osteoblasts from joining wingless-related integration site ligands with their receptors. As a result, sclerostin, a crucial bone formation inhibitor, has received a lot of interest as a focused therapy strategy for low bone density. (Riquelme et al., 2020).

2.1.4 BONE TISSUE:

Bone tissue has a built-in potential for regeneration that is enough for repairing minor wounds like cracks and some forms of fractures. Like any biological tissue,

bone is made up of distinct cell types that are encased in an extracellular matrix (ECM) that contains or is formed by biologically active molecules. Organic proteins (mostly collagen type I) (*Florencio-Silva et al., 2015*), inorganic minerals (primarily calcium phosphate), and many cell types. The crosslinked collagen fibrils that make up the bone ECM, which is comparable to a bone- tissue- engineered scaffold, self-assemble into fibres and eventually into sheets or bundles of fibres. As a crucial component of most bone tissue, Membranes and three-dimensional scaffolds are just two examples of how engineering techniques, materials, and combinations, such as polymers and ceramics, might be used. Typically, throughout the tissue regeneration process, these materials act as an ECM mimic by providing mechanical support and an environment that is favourable for cell attachment, proliferation, and differentiation (*Koons et al., 2020*). A collagen-rich matrix incorporating non-collagenous organic substances and mineral crystals strengthens the bone tissue. Bone quality, quantity, and structure are all reflected in bone strength. Osteoblasts (bone-forming cells), osteoclasts (bone-resorbing cells), and osteocytes embedded in the bone matrix determine and maintain these characteristics (*Ansari & Sims, 2020*).

2.2 BONE TISSUE ENGINEERING:

In conditions when bone does not function effectively, including osteogenesis imperfecta, osteoarthritis, osteomyelitis, and osteoporosis, the significance of bone becomes apparent. These conditions, combined with trauma, orthopedic procedures (such as total joint replacement, spine arthrodesis, implant insertion, etc.), and primary tumor removal, cause or contribute to bone defects or voids (*Porter et al., 2009*).

A wounded or infected bone may not be able to mend itself with mechanical fixation alone for a variety of reasons (including the extent of the bone defect, infection, and numerous others), leading to a non-union. The bone defect must be filled with a substitute material in non-union or crucial defect situations. Autogenous bone (*Witzler et al., 2018*) grafting is currently the gold standard treatment for critical-sized bone lesions. Autogenous bone grafting does have a high rate of complications, which can reach 30%. These complications can include donor site

mortality, discomfort, paresthesia, extended hospital stays and rehabilitation, a higher risk of deep infection, inflammation, hematoma and limited availability. The utilization of bone tissue from other people (usually cadavers) is known as an allograft, and it is a feasible alternative for patients and surgeons. However, orthopedic allografts come with dangers of infection and disease transfer from donor to recipient as well as host immune reactions. Bone xenografts are now widely regarded as being unsuitable for transplantation due to actual and perceived threat of disease or virus transmission, infection, toxicity related with sterilization, immunogenicity, and ultimately host rejection. Patients needing bone repair or replacement may also consider them as a last resort. Concerns about the use of autogenous cancellous bone grafts as the standard conventional treatments for critical-sized bone lesions, such as the ones indicated above and others, have spurred the creation of a wide range of sophisticated synthetic (tissue-engineered) bone scaffolds in latest years.(Porter *et al.*, 2009)

The idea of BTE entails integrating a number of coordinated elements, including stem cells hosted together by a tridimensional biomaterial structure that gives the form and preliminary mechanical strength and molecular signals that cause progenitor cells to differentiate into the osteoblastic phenotype.(Perez *et al.*, 2018) The absence of disease transmission potential, fewer surgical operations, a decreased risk of infection or immunogenicity, and the abundance of artificial scaffold materials are benefits of using synthetic bone scaffolds.(Hollinger *et al.*, 2004) Ideal synthetic scaffolds should be able to offer a physiochemically biomimetic environment while disintegrating as native tissue integrates, and actively encourage or prevent both favorable and unwanted physiological reactions.(Chan & Leong, 2008)

The ambiguous success criteria for therapeutic design is a significant barrier to the creation of successful tissue-engineering products. The ability to create *ex vivo* tissues is now a reality because to recent developments in the carefully regulated regulation of the bioreactor environment.(Shrivats *et al.*, 2014)

2.3 SCAFFOLD:

Three-dimensional (3D) biomaterial that offers a favourable environment for cells to rebuild tissues and organs is referred to as a "scaffold". The goal is to create scaffolds that can mimic natural functioning and send cells regeneration messages. (*Chocholata et al., 2019*) Enhancing cell adhesion, proliferation, osteogenic differentiation, host integration, vascularization, and load bearing, as needed, are qualities that a perfect

scaffold should have. Additionally, biodegradability is another crucial scaffolding characteristic since it enables simultaneous tissue development and scaffold decomposition. In conclusion, products from the decomposition of scaffolds must also be harmless and safe for the body to eliminate. It is crucial to follow mechanical strength principles while building bone scaffolds because load-bearing implants must convert mechanical strength during deterioration so that the load is softly transmitted to the regenerating tissue. (*Tariverdian et al., 2019*)

The characteristics of bone scaffolds are greatly influenced by the choice of matrix material. (*Qu et al., 2019*) Bone repair and regeneration are based on scaffolds that are non-toxic and have strong biocompatibility, and biomaterials significantly contribute to the scaffolds' great performance in this process. (*Qu et al., 2019*) More control over physiochemical properties of scaffolds, such as pore size, solubility, porosity, biocompatibility, enzymatic reactions, and allergic reactivity, is possible with polymeric materials. (*Ghassemi et al., 2018*)

It has been proposed to use bioactive composite materials to combine the benefits of two or more distinct materials (ceramic, metallic, and polymeric materials) (*Ghassemi et al., 2018*) Biomaterials called composites are made to blend two or more substances. The major goal of employing composite materials is to enhance the scaffolds' processability, printing efficiency, mechanical characteristics, and bioactivity. (*Qu et al., 2019*) Only a small number of outstanding biocompatible, osteocompatible, osteoconducting, osteoinducing, and neovasculogenic principles are thoroughly proven for biomaterials and scaffold fabricating technology, despite the advancements made in the production of bone scaffolds. (*Tariverdian et al., 2019*)

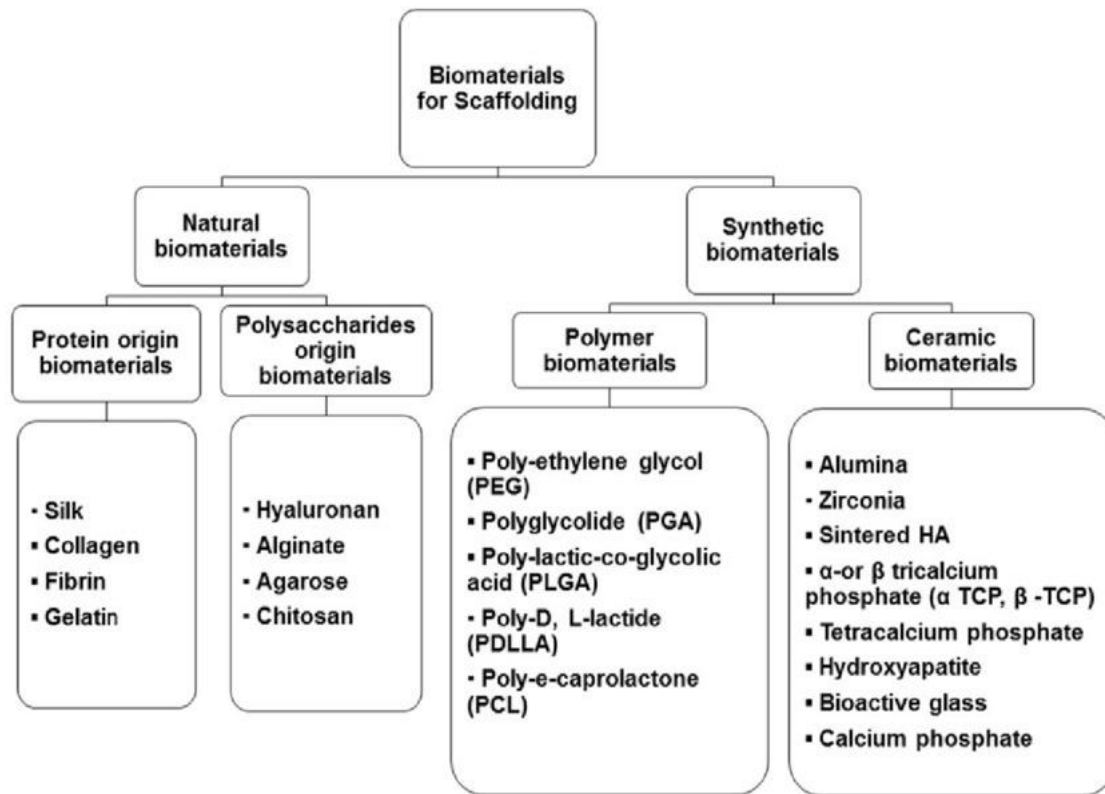


Fig 2.2 Biomaterials for scaffolding, Source: (Alaribe et al., 2016)

2.3.1 NATURAL SCAFFOLD:

Protein or carbohydrates with certain mechanical, biochemical, and structural features make up natural scaffolds. They are often both biocompatible and biodegradable and can come from either plant or animal origins. Due to the existence of multifunctional group over scaffold surfaces, which may be customised based on individual applications, these scaffolds offer an edge over competing ones. (Patel & Fisher, 2008) Biopolymers are essentially divided into three kinds based on their structure and monomeric units. Fibrin, Collagen, fibrinogen, gelatin, elastin, silk, myosin, keratin, and actin are polypeptide- and protein-based substances. Chitosan, chitin, alginate, hyaluronic acid, agarose, cellulose, dextran, and glycosaminoglycans are polysaccharide-based substances. RNA, DNA, and linear plasmid DNA are all polynucleotide-based. (Bhargava, n.d.) (Sell et al., 2010) Proteins, as opposed to polysaccharides, display the amino acid sequences commonly linked to cell attachment via integrin-binding domains. In order to

increase cell adherence and osteoconductivity in polysaccharidic scaffolds, osteoconductive materials must be mixed with chemical surface modifications, and integrin-binding sequences or cell adhesion proteins must be added. (Kowalczewski and Saul, 2018) (Engineering et al., n.d.) (Filippi et al., 2020) Presently, biomimetic natural biopolymer scaffolds have acquired significance as tissue scaffolds for tissue regeneration because of their superior biological response compared to synthetic scaffolds. It is possible to acquire these naturally occurring biomaterials from their sources and process them to create porous scaffolds. The benefits of natural biomaterials include their biodegradability, biocompatibility, and availability from renewable agricultural food supplies. (Alaribe et al., 2016) The mechanical characteristics, immunogenic potential, and supply of natural polymers are frequently constrained. (Filippi et al., 2020) Despite their lower repeatability, natural-derived polymer scaffolds have superior biological responsiveness and ecological safety versus synthetic scaffolds. (Negrini et al., 2020)

2.3.2 SYNTHETIC SCAFFOLD:

In order to serve as scaffolds for the cultivation of stem cells, synthetic biomaterials seem to be a viable alternative to natural biomaterials. Biomaterials' synthetic biomaterial guidance could make it easier to restore the structure and function of harmed or diseased tissues. Since their properties may be customised for certain uses, synthetic polymers are very helpful in the biomedical area. (Alaribe et al., 2016) Synthetic polymers are a significant category of biodegradable polymers that can be created under controlled conditions. Strength, Young's modulus, and degradation rate are a few examples of mechanical and physical qualities that are predictable and repeatable throughout a wide spectrum. The most extensively researched synthetic materials that degrade include poly (hydroxy esters), which include PGA, PCL, PLA, and their copolymer, PLGA, and poly(ethers), which include PEO and PEG, PVA, and PU. Although there are many additional synthetic materials being studied at the moment, these are presumably the most prevalent examples. (Bhargava, n.d.) Over natural polymers, synthetic polymers have a few advantages, including customizable properties, infinite shapes, and pre-existing structures. Synthetic biomaterials' support can help restore the structure and

function of diseased or damaged tissue. They are more easily produced than naturally occurring polymers due to their polymerization, interlinkage, and functionality of their molecular weight, molecular structure, physical properties, and chemical properties. (*Bhargava, n.d.*) Because they can be generated in identical and significant quantities and because their physical and biologic qualities can be altered, synthetic biomaterials are preferred as scaffolds. Lactic acid derivatives, Glycolic acid derivatives, and various polyester derivatives are some of the main groups of synthetic biomaterials. (*Patel & Fisher, 2008*)

2.4 SCAFFOLD EMPLOYED IN DRUG RELEASE:

A review of the literature reveals that a variety of three-dimensional functional scaffolds have been created, some of which may be employed to deliver therapeutic medications important for bone regeneration processes. (*Han et al., 2021*).

Additionally, biomaterials provide hope for avoiding the discomfort connected with due to bone trauma. Non-steroidal anti-inflammatory medications, opioids, and local anaesthetics are frequently used for pain management during bone regeneration procedures. The problem in treating patients with osteosarcoma is to successfully regenerate bone while also pharmacologically controlling the proliferation of cancer cells. (*L. Y. Zhang et al., 2020*) By concentrating chemotherapy medications in bone tissues, bone-targeted drug delivery devices reduce the side effects of neoadjuvant chemotherapy and address the challenge of reaching the desired foci. (*Ferracini et al., 2018*). Problematic obstacles in bone regeneration that arise after a severe injury or during tissue restoration are infection and inflammation. Notable studies on the local administration of anti-inflammatory medicines such as gentamycin, sodium diclofenac and vancomycin have been conducted to address these problems. (*Janmohammadi et al., 2023*)

In order to overcome the drawbacks of systemic therapy and effectively manage osteomyelitis, a new therapeutic method was envisioned: local delivery of antibiotics paired with a biopolymers scaffold for bone regeneration. Delivering antibiotics locally has various benefits, including a considerably higher concentration of the drug at the site of action while maintaining a low level of systemic antibiotic

concentration and the avoidance of side effects that are frequently associated with large systemic doses of antibiotics (*Ferracini et al., 2018*).

To facilitate the regulated release of medications from scaffolds (or materials) to specific target areas, a number of techniques can be used. The method that might be regarded as being the simplest and most directly involves drug-impregnating scaffolds. A scaffold with sufficient adsorptive qualities can be immersed in a drug solution to accomplish this operation, which results in the creation of a drug covering over the surfaces of the scaffold or substance. Another typical technique for creating drug-delivery scaffolds is to incorporate the medication into the scaffold's material as it is being made using temperature- or solvent-based techniques. (*Costa, 2015*) It is crucial to emphasize that while numerous in vitro tests have been carried out to examine if bone scaffolds are suitable as drug delivery carriers, there is still no in vitro systematic procedure available for this purpose.

2.4.1 CHITOSAN

Scaffolds are constructed using components from a variety of classes, such as ceramics and polymers. Composite materials made of natural polymers are receiving more interest since they can be used in BTE. Natural polymer chitosan (CS), employed as one of the scaffolding materials, offers a wide range of applications. (*Saravanan et al., 2016*) One of the most prevalent polymers in nature, chitin is found in the walls of fungi and the shells of crustaceans. Chitosan is a partly deacetylated derivative of chitin. . (*Fourie et al., 2022*) Similar in structure to glycosaminoglycan, a crucial component of the bone matrix and cell surface that regulates the bioavailability and activity of various osteoclastic and osteogenic factors, chitosan is made up of randomly distributed -(1-4)-linked D-glucosamine (glucosamine) and N-acetyl-D-glucosamine (N-acetylglucosamine) structure units. (*Ressler, 2022*) The physical characteristics of CS have enabled it to be formed into a wide range of structures, including porous scaffolds , sponges, fibres, and membranes for BTE. (*Saravanan et al., 2016*)

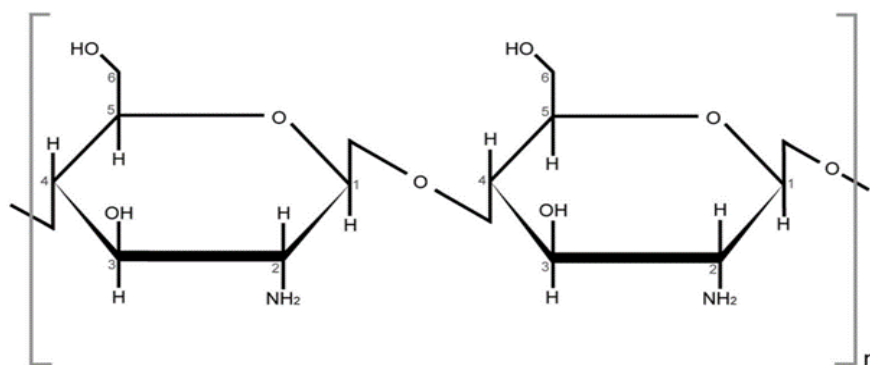


Fig 2.3 Chemical structure of chitosan, Source: (Fourie et al., 2022)

2.4.1.1 PROPERTIES OF CHITOSAN:

Chitosan has a hydrophilic surface that is absent in many synthetic polymers and has the attributes of being bioactive, biodegradable, anti-bacterial, and biocompatible. (Saravanan et al., 2016)

i. PHYSICO CHEMICAL PROPERTIES:

Based on their molecular weights (300 to 1000 kDa) and the amount of acetylation (ranging from 50 to 95%) there are various forms of CS. Moreover, the level of acetylation and molecular weights affect the physicochemical characteristics of CS, including degradation, solubility, and crystallinity. As a result, 0% deacetylated chitin and 100% deacetylated CS are extremely crystalline in nature, and other intermediate degrees of deacetylated CS are semi-crystalline in nature. (Saravanan et al., 2016)

Chitosan's solubility is influenced by pH, the degree of acetylation, and molecular weight. Due to its strong intermolecular hydrogen bonding and main amino groups, chitosan has a low solubility in physiological solvents (such as water) and is regarded as a strong base with a pKa value of 6.3. (Ressler, 2022) Chitosan is soluble in acidic solvents like acetic acid, formic acid, and lactic acid below pH 6, with 1% acetic acid being the most popular solvent. (Fourie et al., 2022)

ii. BIOLOGICAL PROPERTIES:

Chitosan is a crucial biomaterial because of its numerous biological characteristics. (Levengood & Zhang, 2014) Due to its cationic character, chitosan has been employed extensively in accelerating the ability for wound healing and antibacterial activity. Chitosan is a powerful wound healing accelerator because it stimulates and controls inflammatory cells, which promotes the development of granular tissue. It is a crucial part of wound dressing because it binds to anionic red blood cells to encourage coagulation. (Saravanan et al., 2016). For the chitosan chain to have antibacterial activity, it must be polycationic. The most likely mechanism by which chitosan exerts its antibacterial effect is by attaching to the negatively charged bacterial cell wall, disrupting the cell membrane, inhibiting DNA replication, and ultimately causing cell death. (Ressler, 2022)

2.4.1.2 CHITOSAN SCAFFOLD PROPERTIES:

In terms of cell adhesion, spreading, proliferation, differentiation, and other biological processes, biomaterials offer significant benefits over traditional metallic implants. The materials' physicochemical and biological characteristics are what determine whether BTE scaffolds are successful. The scaffolds must possess a variety of qualities appropriate for BTE applications. These are porosity, biocompatibility, water retention, protein adsorption, mineralization through biomineralization, biodegradability, mechanical characteristics, and water retention. (Saravanan et al., 2016)

i. POROSITY:

Infiltration of cells, adhesion, the production of ECM components, and the development of bone tissue are all supported by porosity. The size of the pores affects cell adhesion to the scaffold. (Saravanan et al., 2016). Interconnected pores in the scaffold are necessary for the effective diffusion of nutrients, oxygen, and metabolic waste during bone regeneration. Together with porosity in the range of 50–90%, micro- (20 µm) and macroporosity (>100–400µm) need to be taken into account while designing a functioning scaffold. For cell seeding and retention,

capillary growth, vascularization, and interactions between cells and the matrix, microporosity is essential. Through improving cell migration, cell-cell network formation, vascularization, nutrition supply, and metabolic waste transport, macroporosity encourages osteogenesis. (Ressler, 2022)

ii. WATER RETENTION ABILITY:

Water retention ability, commonly referred to as swelling ability, describes the capacity of tissue engineering scaffolds to hold water. When scaffolds are implanted in living organisms, water from the surrounding tissues are absorbed, increasing pore size, and allowing cellular infiltration deep inside the scaffolds' internal architecture. (Saravanan *et al.*, 2016)

iii. BIODEGRADATION:

The implanted graft materials need to degrade over time in a manner that coincides with the growth of new bone. While designing any BTE scaffolds, it is crucial to take biodegradation into account for long-term success. (Saravanan *et al.*, 2016) Chitosan degrades physically and chemically under physiological conditions, including oxidation, non-enzymatic and enzymatic hydrolysis, swelling, cracking, and dissolving. The degradation products are non-toxic, mainly composed of glucosamine and saccharide, which can then be easily extracted from the body without interference with organs. (Ressler, 2022)

iv. MECHANICAL PROPERTIES:

A crucial aspect of bone regeneration is mechanical strength, which is primarily governed by porous structure and the properties of the materials utilised (Upadhyaya *et al.*, 2013) The development of the scaffold still faces a significant problem in finding the ideal balance between the needs for porosity, pore size distribution, and mechanical qualities. (Ressler, 2022) For effective load transfer to the surrounding tissues, the mechanical characteristics of the scaffolds should match the host bone. (Saravanan *et al.*, 2016) As chitosan has mild to moderate

mechanical characteristics, its applicability in load-bearing applications is constrained. (Przekora et al., 2016)(Rogina et al., 2016)

V. **BIOCOMPATIBLE:**

One of the key considerations for materials utilised in tissue engineering applications is biocompatibility. The human body does not react toxically or immunologically to biocompatible materials. Almost all articles that have been published characterise chitosan as a non-toxic, biocompatible biopolymer that is suitable for usage in the human body as a scaffold or drug carrier. (Ressler, 2022)

2.5 ALBUMIN – NANOCARRIER:

A globular plasma protein with a molecular weight of 66,500 Dalton (Da), human serum albumin (HSA) is made up of 585 amino acids. HSA is a heart-shaped molecule made up of three homologous domains. It is resistant to many different conditions, such as temperature, pH (4–9), and organic solvents. The molecular weight of bovine serum albumin (BSA) is 69,323 Da. BSA has drawn a lot of interest in the pharmaceutical sector due to its inexpensive cost, large availability, and simplicity of purification. BSA may, however, cause less favourable immunogenic reactions than HSA. (Elzoghby et al., 2012)

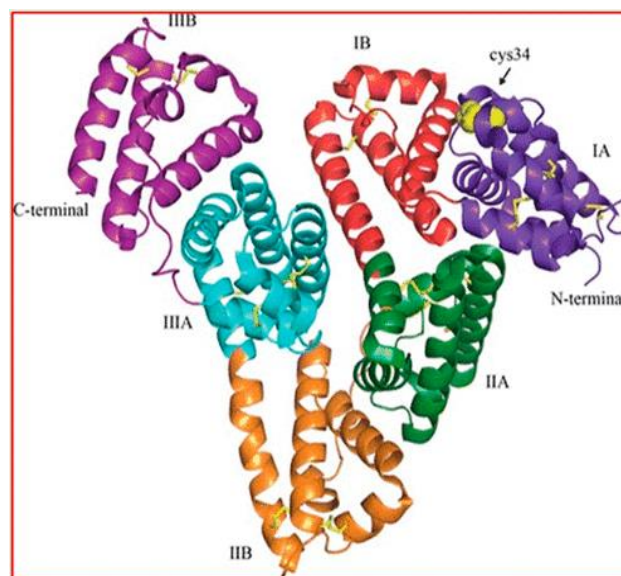


Fig 2.4 Structure of human serum albumin, Source: (Spada et al., 2021)

It has been established that albumin, a versatile protein carrier for drug administration, is nontoxic, non-immunogenic, biocompatible, and biodegradable. As a result, it makes for the best material to create drug delivery nanoparticles. Albumin nanoparticles have drawn a lot of attention since they have a high drug-binding capacity and are well-tolerated without producing any negative side effects. Albumin-based nanoparticles have several distinct benefits, including biodegradability, simplicity of preparation, and reproducibility. The matrix of albumin nanoparticles can be used for effective incorporation of these substances due to the high protein binding of different medicines. Because the surfaces of the albumin nanoparticles have functional groups (such as amino and carboxylic groups), covalent derivatization of the nanoparticles with drug targeting ligands is feasible (Yu *et al.*, 2014). Albumin-based nanocarriers are a versatile choice, implemented to overcome drug resistance mechanisms for multiple types of tumours. Albumin nanoparticles can be synthesized utilizing various approaches, and they offer significant implications including passive and active targeting and controlled drug release. (Hassanin & Elzoghby, 2020)

2.6 DRUG – ANDROGRAPHOLIDE:

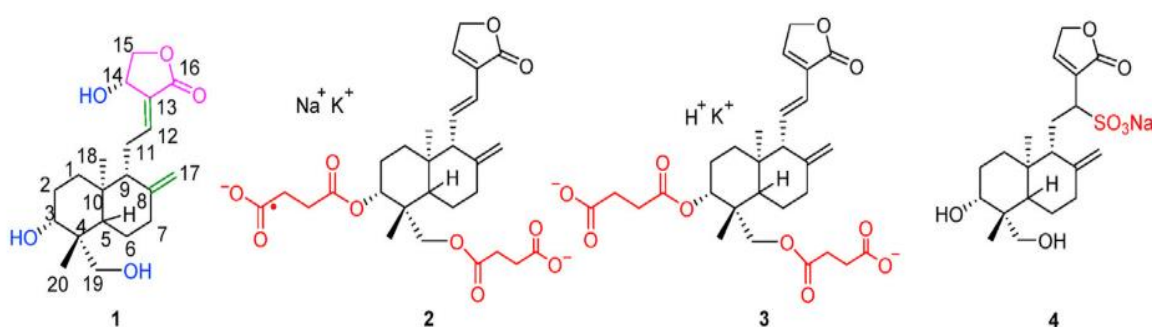


Fig 2.5 Chemical structures of andrographolide,

Source: (H. Zhang *et al.*, 2021)

Andrographolide is a labdane diterpene that has been isolated from the herb *Andrographis paniculata*. Andrographolide has a wide range of biological effects, including anti-inflammatory, antioxidant, and anti-cancer properties. Clinical trials have shown that andrographolide may be useful in the treatment of a variety of diseases, including osteoarthritis, upper respiratory diseases, and multiple sclerosis. (Agust *et al.*, 2021) *Andrographis paniculata* (*A. paniculata*) is a herbal medicine that has traditionally been utilized to treat inflammation and bacteria. Andrographolide, the principal active component of *A. paniculata*, has a wide range of pharmacological activities, including anti-inflammation, anti-cancer, anti-obesity, anti-diabetes, and others. (Dai *et al.*, 2019) *Andrographis paniculata*, a member of the *acanthaceae* family, has been widely used in China, India, and Southeast Asia for the clinical treatment of viral and bacterial infections since ancient times. Intensive phytochemical research on its constituents began in 1911, with the identification of various compounds such as lactones, diterpenoids, and flavonoids. Diterpene lactone andrographolide and its derivatives were the most well-known bioactive chemical constituents of *Andrographis paniculata*. The majority of research in this field has concentrated on the synthesis, pharmacological effects, and potential therapeutical efficacy. (H. Zhang *et al.*, 2021) Human plasma, urine, rat plasma, tissue, urine and feces samples, rabbit plasma and fowl serum have been analyzed for the andrographolide by the abovementioned methods. These methods can be used for the routine analysis of andrographolide. These methods have been used for the separation and quantification of andrographolide in various biological samples and pharmaceutical preparations. (Patil & Jain, 2021)

3. AIM AND SCOPE:

- To encapsulate drug (andrographolide) in albumin nanoparticle.
- To fabricate a chitosan scaffold for sustained release of andrographolide via freeze gelation route.
- To evaluate physicochemical characteristics of the fabricated scaffold and ANDNP,
 - a) Morphological characteristics analysis – Field emission scanning electron microscopy (FESEM)
 - b) Phase analysis & wettability determination – X- Ray diffraction (XRD), Contact angle
 - c) Drug release analysis by UV - spectrophotometer
 - d) Mineralization assay – Von Kossa staining
 - e) Cell cytotoxicity assay - MTT assay
- The purpose of the study is to analyse bone defects and to adapt and increase bone regeneration.

4. MATERIALS AND METHODS:

4.1 MATERIALS:

I. CHEMICALS:

- Albumin
- Andrographolide drug
- Ethanol
- Acetic acid
- Chitosan
- glutaraldehyde
- Distilled water
- NaOH
- DMSO
- Silver nitrate

II. LAB EQUIPMENTS:

- Pipette
- Burette
- Centrifuge tubes
- Magnetic stirrer pellets
- Glass beakers (100 ml)
- Falcon tubes (50 ml)
- Eppendorf tubes (2 ml)
- Glass pipette (10 ml)
- Glass bottle (1000 ml)

III. INSTRUMENTS:

- Sonicator
- Centrifuge
- Magnetic stirrer
- UV-spectrophotometer

4.2 SYNTHESIS OF DRUG LOADED ALBUMIN NANOPARTICLES:

500 mg albumin were dissolved in 10ml of distilled water to make up 5 % albumin solution. Then, 10 mg of andrographolide were dissolved in 1 ml of ethanol and sonicated for fine dispersion. Further, the drug samples were dissolved in the albumin solution of beakers S1, S2, S3 dropwise under constant stirring for 1 hour. Ice cold ethanol was added (-20°C) dropwise till milky white turbid solution was obtained. After this, 200 µl of glutaraldehyde was added which is a cross linker that helps the albumin get encapsulated in the drug. The stirring is continued in dark for 2 hours. The mixture was centrifuged at 20,000 g for 30 mins at 4°C. The supernatant was collected and observed using UV-spectrophotometry, to find the concentration of free drug in supernatant to deduce the entrapment efficiency of drug using the formula

$$E = [(T_{\text{And}} - F_{\text{And}}) / T_{\text{And}}] \times 100\%$$

Where,

T_{And} is the total amount of andrographolide added and F_{And} is the unbound andrographolide in the supernatant.

The pellet was washed twice and lyophilised to get nanoparticle powder.

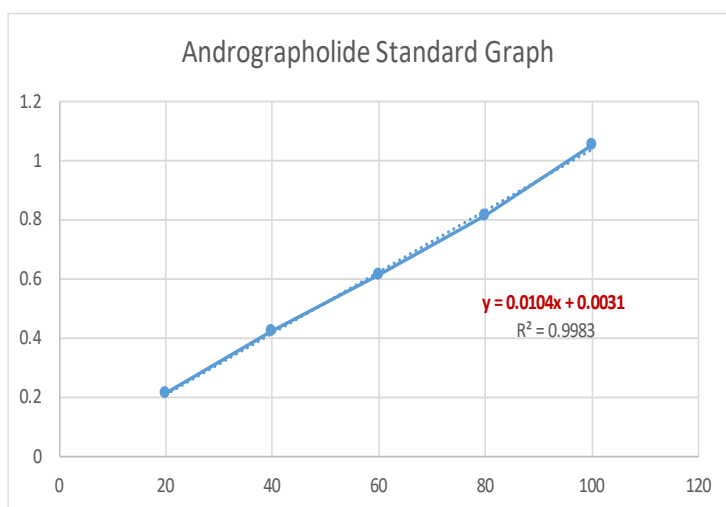


Fig 4.1 Standard graph of andrographolide

$$Y = mX + C$$

Where,

X- Concentration of drug

Y- Absorbance of drug

$Y = 0.0104x + 0.0031$

4.3 FABRICATION OF SCAFFOLD – FREEZE GELATION:

2% acetic acid solution was prepared by adding 600µl of acetic acid to 30ml of distilled water. 0.3 g of chitosan (1% chitosan) was added to the beakers 1% CHIT – A, 1% CHIT – B, 1% CHIT – C, 1% CHIT – D. Then 0.6 g of chitosan (2%chitosan) was added to 2% CHIT – A, 2% CHIT – B, 2% CHIT – C, 2% CHIT – D and was stirred for 3 hrs. In order to prepare NaOH solution, 20 g of NaOH was weighed and dissolved in 500 ml of distilled water and 500 ml of ethanol was added to it and stored in -20°C. 90 mg of AND – NP was added to 1% CHIT – A and 2% CHIT – A, 180 mg of AND – NP was added to 1% CHIT – B and 2% CHIT – B, 270 mg of AND – NP was added to 1% CHIT – C and 2% CHIT – C. 270 mg of ALB – NP to 1% CHIT – D and 2% CHIT – D was added as control. The beakers were kept under stirring for 2 and half hours. Glutaraldehyde was added dropwise to the beaker and sonicated for few minutes. Then the mixture was transferred to small boxes and was refrigerated overnight. Then the casted scaffolds were transferred to a box of bigger size and 80 ml of prepared NaOH was poured and freezed for 24 hrs. The scaffold was thawed for half an hour and NaOH was discarded and distilled water was added to the scaffolds for 10 mins under constant pressing to remove the NaOH. After this, the scaffolds were transferred to the beaker and washed in 50%, 70%, 100% ethanol for 10 mins. Followed by which the scaffolds were dried in hot air oven for drying.

4.3 CHARACTERIZATION STUDIES:

4.4.1 SEM ANALYSIS:

SEM analysis, also known as Scanning Electron Microscopy (SEM) analysis, is a potent analytical technique which we use, to analyse and provide high resolution images of the scaffold we prepared. Scanning Electron Microscope analysis was performed to determine the interlinked pores, pore size, nanoparticle diameter and morphology of the scaffold sample. Small pieces of size 1cm x 1cm of the scaffold samples from all groups were taken, labelled and submitted for SEM analysis.

4.4.2 CONTACT ANGLE:

Contact angle measurement is a qualitative way to evaluate whether the surface of the sample has a hydrophobic or hydrophilic characteristic. To study the wettability of CHIT AND-NP with a contact angle meter. 1cm x 1cm dimensions from each sample was prepared and fixed at a glass strap where, a drop of water from the water contact angle meter's needle was dropped at the different locations, and behaviour was observed in each case. The water contact angle metre included a camera, which allowed it to take pictures automatically.

4.4.3 XRD ANALYSIS:

X-ray diffraction analysis (XRD) can provide precise information on the material's crystallographic structure, its chemical composition, and physical characteristics. By computing or comparing the lattice parameters, crystal structures, and crystallinity values with the accepted values, XRD was used to identify the crystallinity of andrographolide albumin loaded chitosan scaffold.

4.4.4 DRUG RELEASE:

1cm × 1cm scaffolds (N = 3) were incubated under static conditions in 3 mL of phosphate-buffered saline. 1ml was taken out every 24 hours and replaced

with phosphate-buffered saline in an equal amount. At 236 nm, the absorbance of the released andrographolide was measured using a UV spectrophotometer (HITACHI). The standard curve for andrographolide was then used to determine the concentration of the released andrographolide.

4.4.5 INVITRO ANALYSIS OF CYTOTOXICITY & OSTEOGENESIS:

Growth Media – The growth media consist of Dulbecco's Modified Eagle's Medium (DMEM), 10% Fetal bovine serum, 1% Antibiotic

Osteogenic Media – The composition of osteogenic media includes Dulbecco's Modified Eagle's Medium (DMEM), 10% Fetal bovine serum, 1% Antibiotic, Dexamethesone, β - glycerol phosphate and ascorbic acid.

4.4.5.1 MTT ASSAY:

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) assay is done to determine the cell viability and proliferation. The principle behind the MTT assay is that only viable cells have active metabolism and can convert MTT to purple formazan, which has an absorbance maximum at 570nm. The amount of formazan produced can be measured using a spectrophotometer and is proportional to the number of viable cells. To a 12 well plate 1×10^5 murine mesenchymal stem cell were added (N =3) And incubated for 24 hrs. ETO sterilized scaffold was added to the assay plate. On the 3rd day media & scaffolds removed, 100 μ l of 3mg/ml of MTT was added to each well, and kept in CO₂ incubator for 4 hours. The formazan crystals were solubilized in dimethyl sulfoxide and read at 570nm.

4.4.5.2 VONKOSSA STAINING:

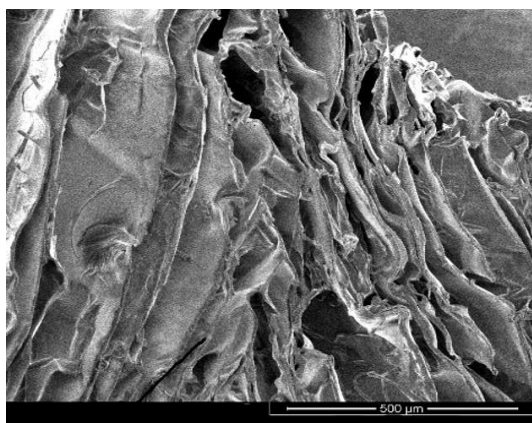
To study the extent of mineralization achieved by the sustained release of andrographolide from a polymeric scaffold, von kossa staining is carried out. The

principle of coloration is based on the transformation of calcium salts present in the extracellular bone matrix secreted by osteoblast into silver salts it is a twostep reaction: primarily silver cations react with components of calcium deposits hence resulting in a transient yellow coloration. However, the second step organic material reduces the bound silver to black metallic silver in the presence of silver. (Schneider, M. R. (2021)

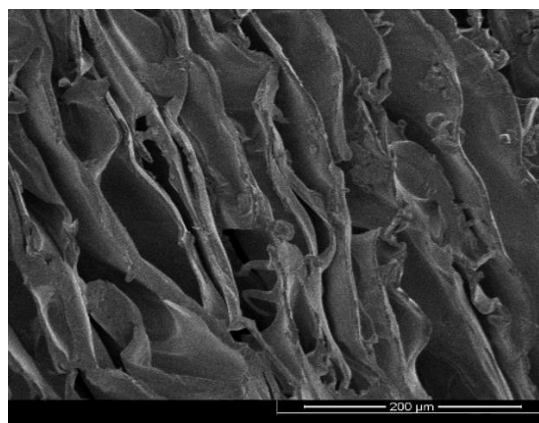
To a 12 well plate 1×10^5 murine mesenchymal stem cell were added and incubated for 24 hrs allowing the cells to attach to the surface. Consequently, third day 1×1 cm of drug loaded scaffold and osteogenic media was added replacing the growth media to induce osteogenesis. On day 14, 200 μ l of silver nitrate is added to each well. The 12 well plate is incubated under UV for 20 mins. 100 μ l of 1% sodium thiosulfate is added. Followed by which the sodium thiosulfate is discarded, to stop the reaction. The cell is then observed using the inverted microscope, 10x IP.

5. RESULTS AND DISCUSSION:

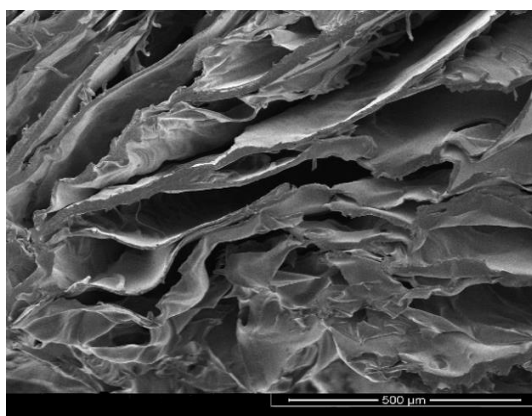
5.1 SEM ANALYSIS OF AND NP – CHITOSAN SCAFFOLDS:



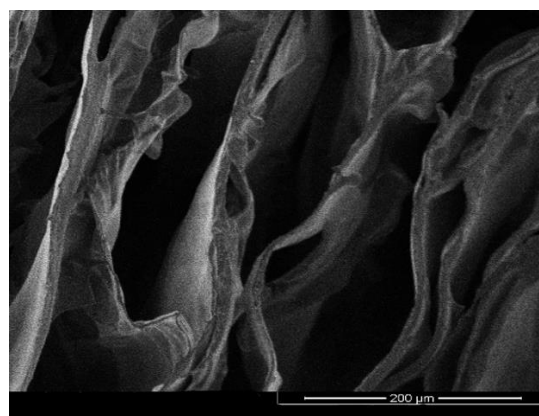
(A)



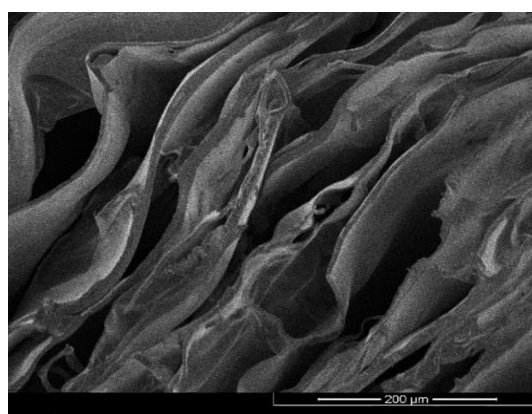
(B)



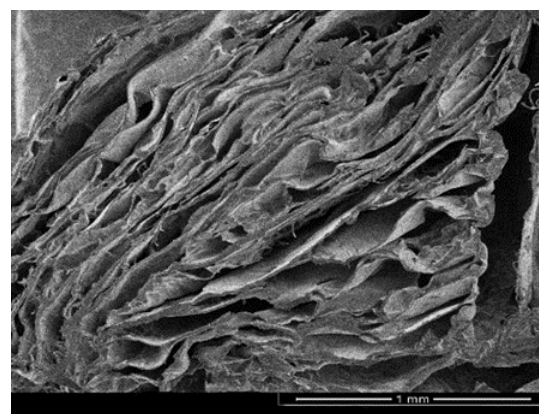
(C)



(D)



(E)



(F)

Fig 5.1 Scanning Electron Microscope images of (A) 1% CHIT- A, (B) 1% CHIT- B, (C) 1% CHIT- C, (D)1% CHIT- D, (E) 2% CHIT- A, (F) 2% CHIT- B, (G) 2% CHIT- C, (H) 2% CHIT- D

Fig 5.1 shows the highly interconnected porous structures with embedded nanoparticles on the surface of the scaffolds were observed under scanning electron microscopy on (chitosan- andrographolide loaded albumin nanoparticle). The pore size ranged from 500 μm to 1 mm, and the nanoparticles were evenly distributed across the surface. Pore size affects a cell's ability to adhere to a scaffold. Various reports highlighted that pores greater than 300 μm promote direct osteogenesis. These observations suggest that these scaffolds promote cell permeability.

5.2 DRUG RELEASE:

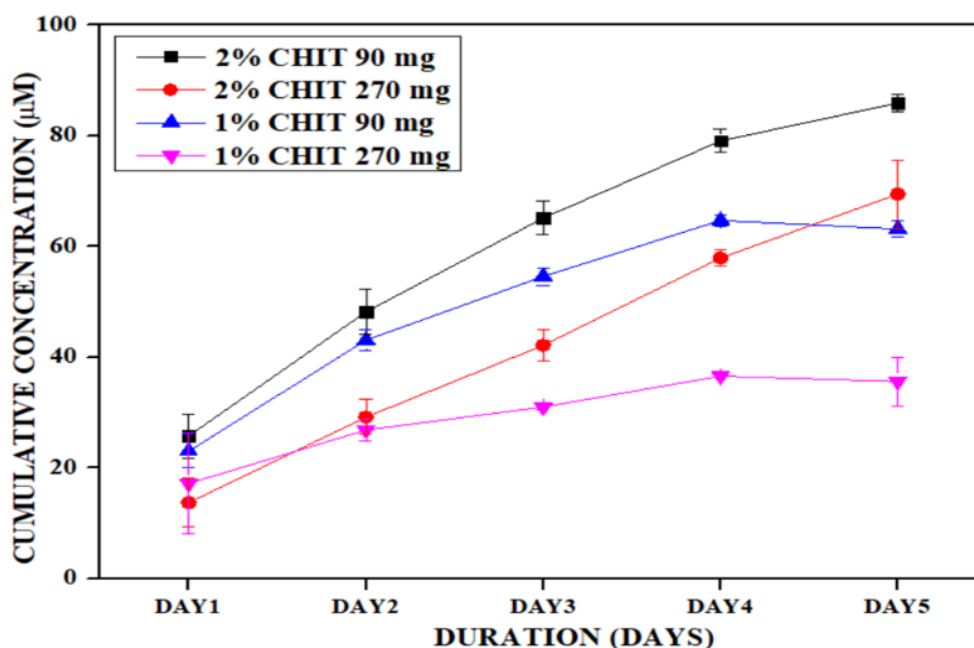


Fig 5.2 Analysis of andrographolide release from CHIT - AND NP scaffolds

With entrapment efficiency of 99.27 ± 0.27 % of drug, the results for cumulative release of andrographolide from chitosan scaffolds are shown in the table below.

Table 5.1: 2% CHIT - A

DAYS	CUMMULATIVE CONC (μM)	STD DEV
DAY1	25.7275641	4.0080766
DAY2	48.15384615	4.0985557
DAY3	65.13141026	3.0165689
DAY4	79.06410256	2.1197507
DAY5	85.91346154	1.5431286

Table 5.3: 1% CHIT - A

DAYS	CUMMULATIVE CONC(μM)	STD DEV
DAY1	23.00320513	3.095231
DAY2	43.05769231	1.923611
DAY3	54.55448718	1.580619
DAY4	64.67307692	0.975328
DAY5	63.125	1.441239

Table 5.2: 2% CHIT - C

DAYS	CUMMULATIVE CONC(μM)	STD DEV
DAY1	13.67628205	4.365803
DAY2	29.1474359	3.113762
DAY3	42.11858974	2.894215
DAY4	57.91025641	1.405148
DAY5	69.43910256	6.197263

Table 5.4: 1% CHIT – C

DAYS	CUMMULATIVE CONC(μM)	STD DEV
DAY1	17.16987179	9.169244
DAY2	26.80769231	2.017449
DAY3	30.96474359	0.4797
DAY4	36.56410256	0.317292
DAY5	35.59294872	4.421683

This data shows the sustained release of drug for 5 days. 1% CHIT- A, 1% CHIT-C released 63.125 ± 1.44 (μM) and 35.592 ± 4.421 (μM) respectively. Similarly, the drug released by 2% CHIT- A was 85.913 ± 1.543 (μM) and 2% CHIT-C was 69.439 ± 6.167 (μM). On comparing 90mg and 270 mg of AND-NP release in both 1% and 2% CHIT scaffolds, 270 mg was released in a more controlled fashion. According to previous studies, the drug leaching from polymeric matrices can be controlled by polymers by regulating the flow of water into the polymeric chains. (Kamath et al., 2013). It can be observed that as the concentration of albumin increases in CHIT- AND NP scaffold, water percolation decreases and thereby controlling the release of drug from the scaffold. This implies that the nano encapsulation of Andrographolide in albumin retarded its release, which is beneficial for therapeutic scaffolds.

5.3 MTT ASSAY:

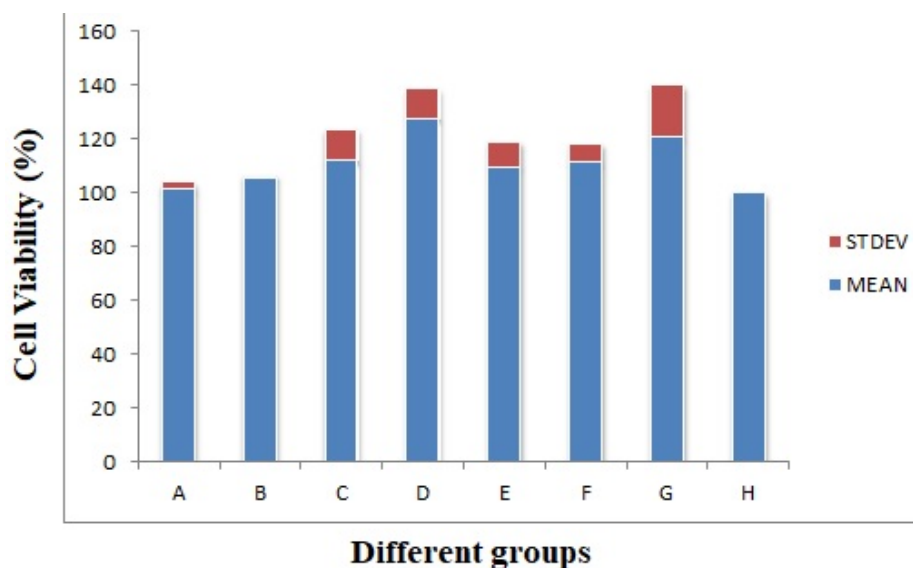


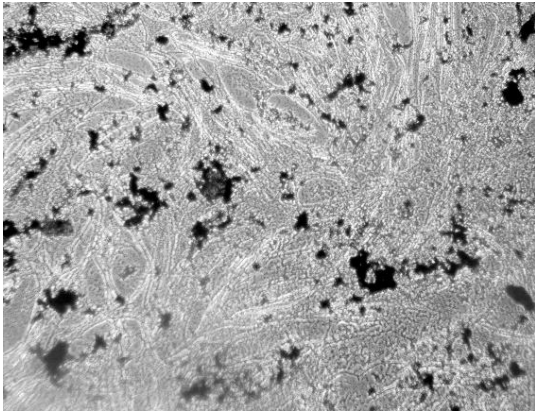
Fig 5.3 The graph represents the % cell viability of (A) 1% CHIT- A, (B) 1% CHIT- B, (C) 1% CHIT- C, (D)1% CHIT- D, (E) 2% CHIT- A, (F) 2% CHIT- B, (G) 2% CHIT- C, (H) 2% CHIT- D

NOTE:

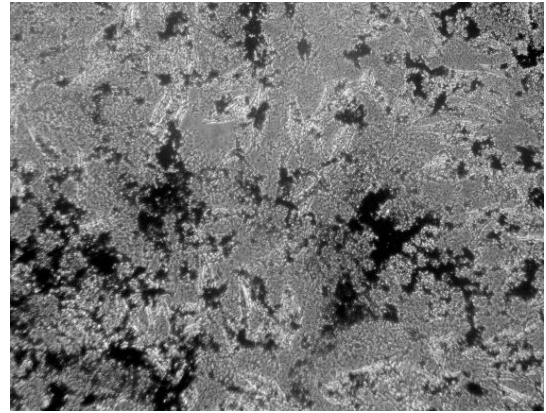
- **Positive Control** – To the adhered murine mesenchymal stem cells 1% CHIT- D and 2% CHIT- D groups were added and treated as positive controls.
- **Negative Control** – The murine mesenchymal stem cells devoid of drug loaded chitosan scaffolds acted as negative control.
- **Test group** – 1% CHIT-A, 1% CHIT-B, 1% CHIT-C and 2% CHIT-A, 2% CHIT-B, 2% CHIT-C was added to the cultured murine mesenchymal stem cells.

The MTT assay Fig 5.3, revealed that the scaffold was not cytotoxic. No significant increase in AND – NP activity was observed among the test groups Fig 5.3 (A – F) compare With CHIT – AND NP (positive control), Fig 5.3 (G). The andrographolide did not increase the proliferation and also did not kill the murine mesenchymal stem cells, it remains standard.

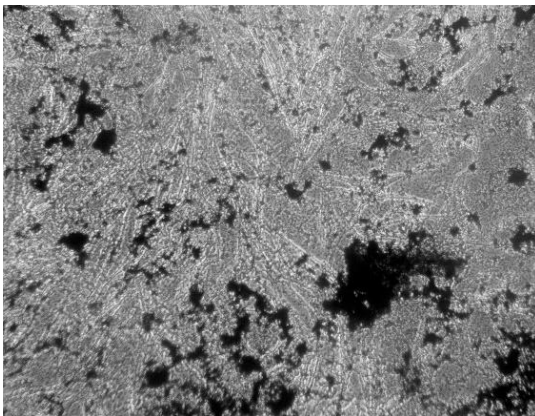
5.4 VON KOSSA STAINING:



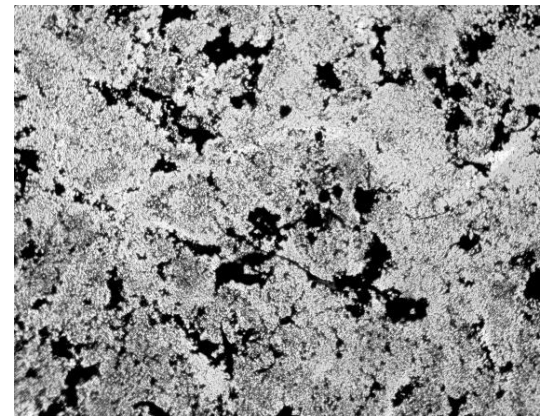
(A)



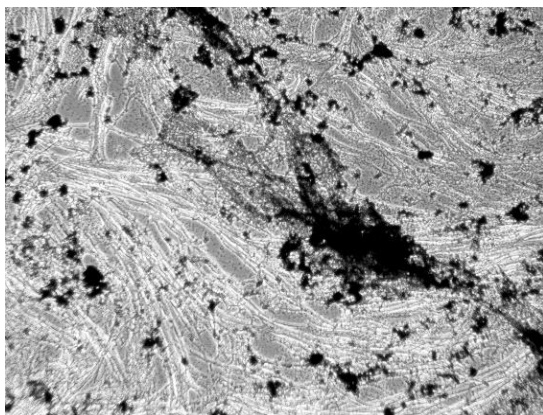
(B)



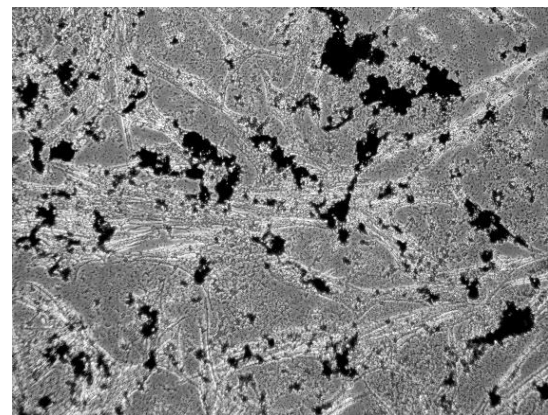
(C)



(D)



(E)



(F)

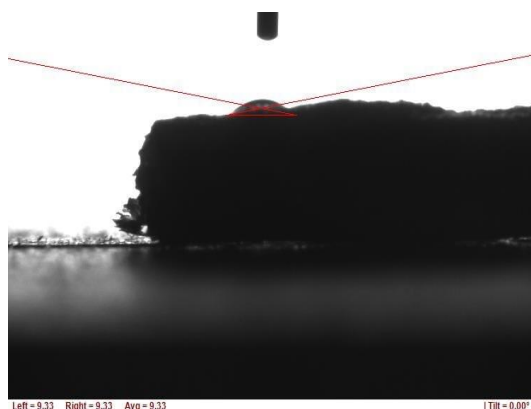
Fig 5.4 Von Kossa staining of fabricated CHIT- AND NP scaffolds, (A) 1% CHIT- A, (B) 1% CHIT- B, (C) 1% CHIT- C, (D) 2% CHIT- A, (E) 2% CHIT- B, (F) 2% CHIT- C, (G) Positive control (H) Negative control

NOTE:

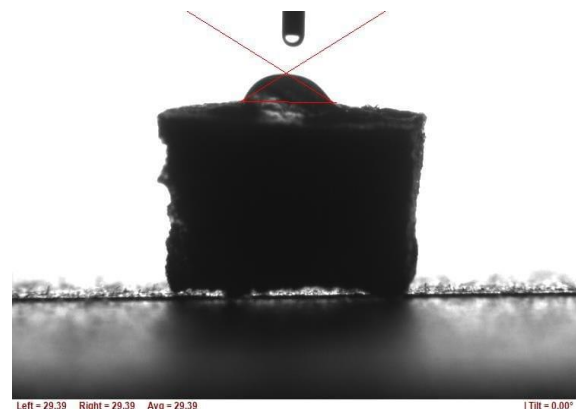
- **Positive Control** – In this group of murine mesenchymal stem cell, growth media was replaced by osteogenic media.
- **Negative Control** – In this group of murine mesenchymal stem cell only the growth media was added.
- **Test group** - To the adhered murine mesenchymal stem cells osteogenic media and 1% CHIT- A, 1% CHIT- B, 1% CHIT- C, 2% CHIT- A, 1% CHIT- D, 2% CHIT- B, 2% CHIT- C, 2% CHIT- D groups was added.

It was observed, from Fig 5.4 that the test groups had more calcium deposits than the positive control. It can be inferred that the AND NP an osteogenic compound had positively influenced the mineralization of the cells.

5.5 CONTACT ANGLE ANALYSIS:



(A)



(B)

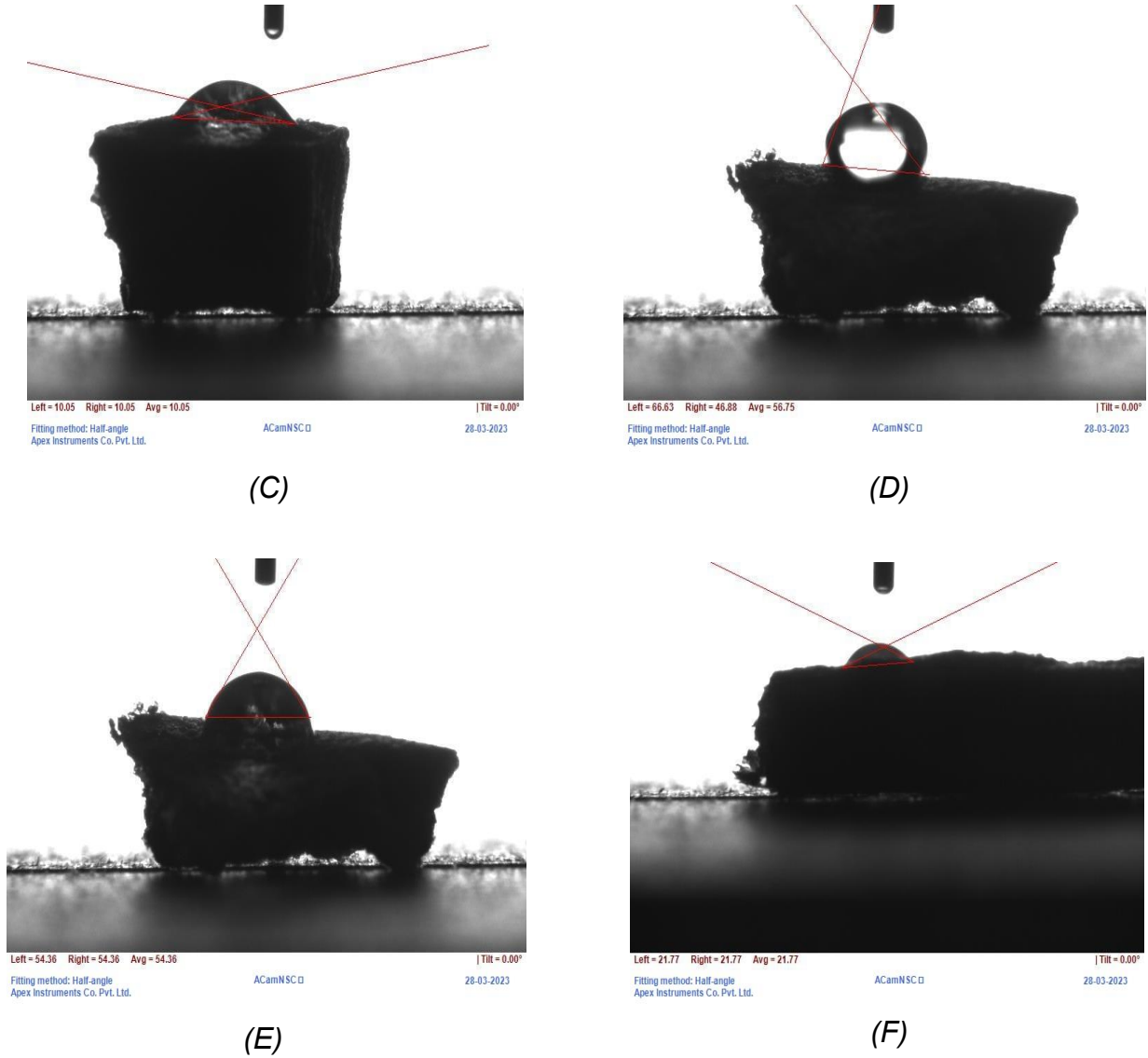
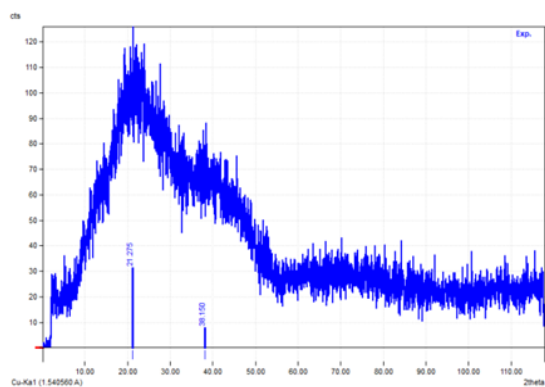


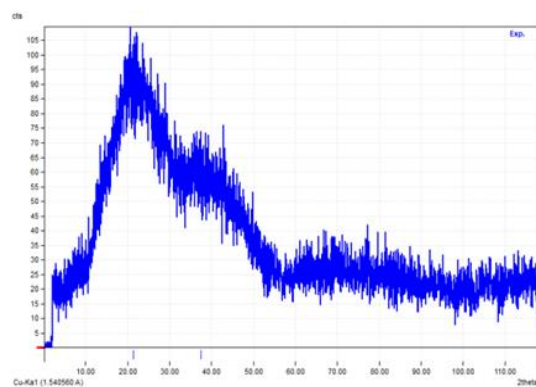
Fig 5.5 Wettability analysis images of (A) 1% CHIT- A, (B) 1% CHIT- B, (C) 1% CHIT- C, (D) 2% CHIT- A, (E) 2% CHIT- B, (F) 2% CHIT- C

The results indicate that the scaffolds with contact angle $10^\circ < \theta_w < 90^\circ$, (B) 1% CHIT- B, (D) 2% CHIT- A, and (E) 2% CHIT- B, are hydrophilic, while CHIT AND NP scaffolds with contact angle, $\theta_w < 10^\circ$, (A) 1% CHIT- A and (C) 1% CHIT- C are super hydrophilic. Several studies report that scaffolds with contact angle less than 90 were found to desirable in drug release (*Salam et al., 2020*). This suggests the potential of CHIT-AND NP scaffold in bone tissue engineering.

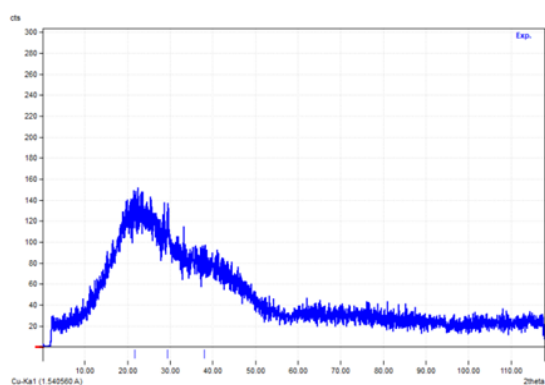
5.6 X-RAY DIFFRACTION ANALYSIS:



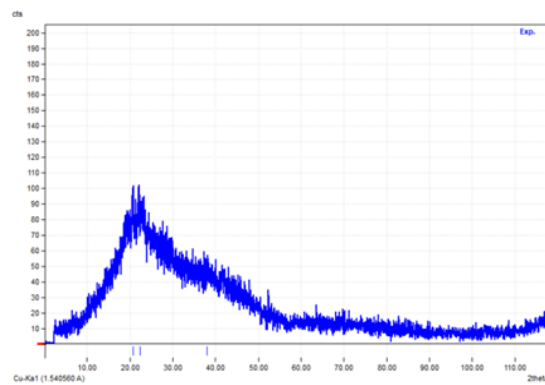
(A)



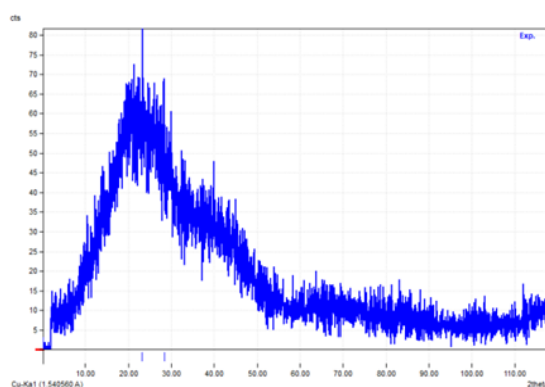
(B)



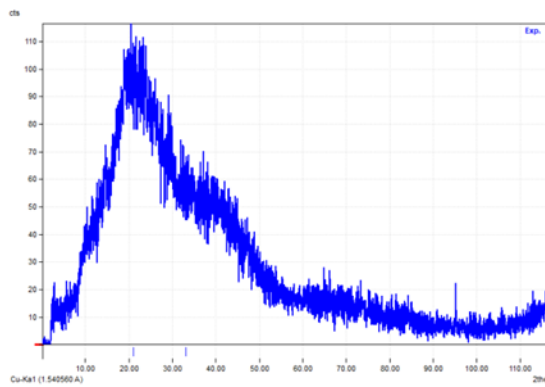
(C)



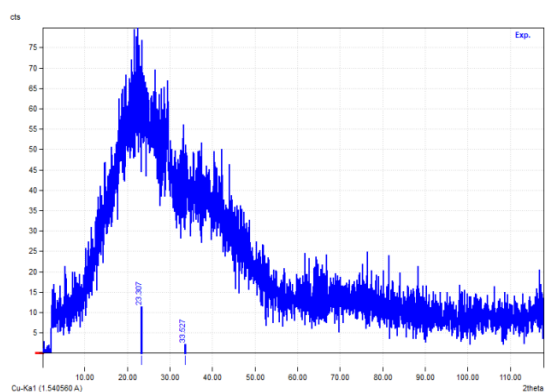
(D)



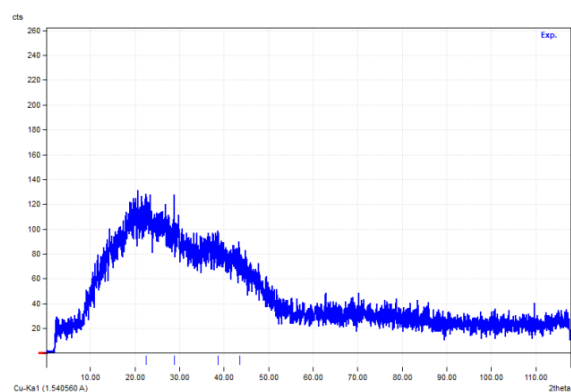
(E)



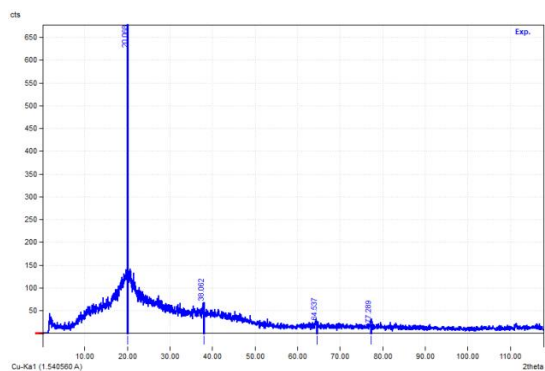
(F)



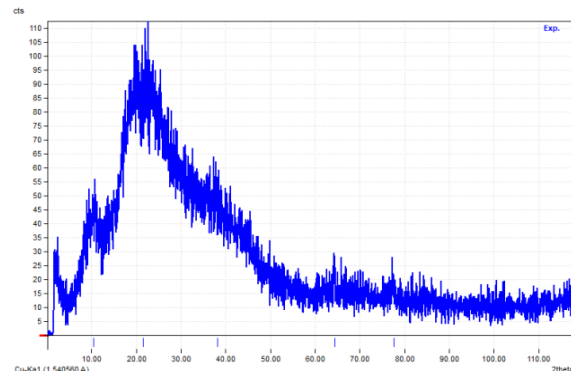
(G)



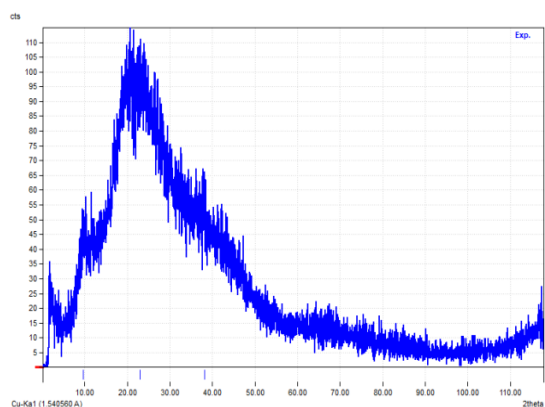
(H)



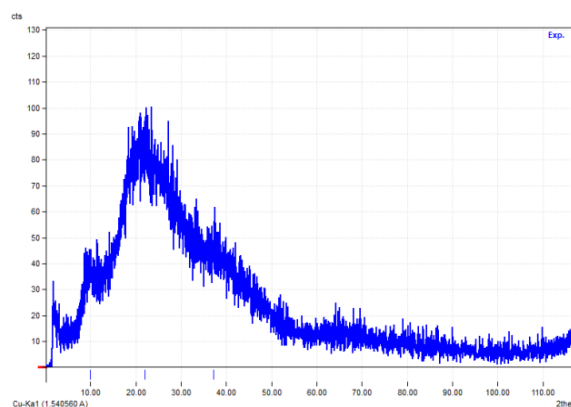
(I)



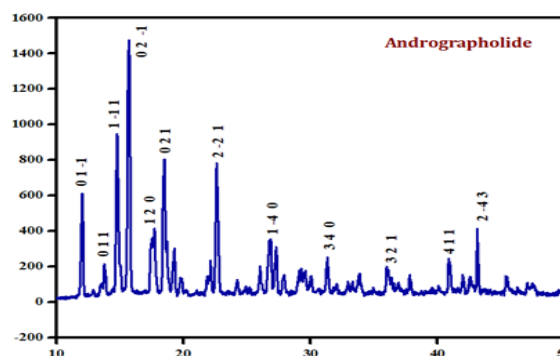
(J)



(K)



(L)



(M)

Fig 5.6 X - Ray diffraction spectra of (A) 1% CHIT- A, (B) 1% CHIT- B, (C) 1% CHIT- C, (D)1% CHIT- D, (E) 2% CHIT- A, (F) 2% CHIT- B, (G) 2% CHIT- C, (H) 2% CHIT- D (I) CHIT particle, (J) ALB particle, (K) ALB - NP, (L) AND NP

X-ray diffraction pattern was used to identify the crystallographic structure of fabricated CHIT - AND NP scaffolds. The XRD pattern of albumin and chitosan compound implies their amorphous nature with 2θ value 21.1 and 20.068 respectively. The AND NP loaded chitosan scaffolds, exhibit amorphous peaks with 2θ value 21.275. In standard andrographolide XRD pattern, the sharp crystalline peaks were seen in Fig 5.6 (M). Whereas no such characteristic peaks of andrographolide were observed for synthesized AND NP Fig 5.6 (L) and CHIT - AND NP Fig 5.6 (A-H), implying that drug was amorphized during the fabrication process. The amorphization of andrographolide is due to the lattice structure distortion during the Nano encapsulation process. However, the presence of andrographolide was established by the drug release studies. In this study the insoluble crystalline drug is converted into amorphous soluble drug. According to several previous reports amorphization of drugs enhances dissolution which is highly advantageous in drug delivery applications (Kamath et al., 2013) (S. et al., 2020), indicating the therapeutic potential of CHIT- AND NP scaffolds.

6. INFERENCE:

In this study 1% CHIT AND-NP and 2% CHIT AND-NP hydrophilic scaffolds were fabricated by freeze gelation for bone tissue engineering application. The morphological and physicochemical studies revealed that chitosan scaffolds fabricated were highly porous and the Nano encapsulated andrographolide was amorphized, which increased the dissolution of drug. The synthesised scaffolds were not cytotoxic with murine mesenchymal stem cells, but also improved calcium phosphate deposition indicating potential for bone regeneration. Further, the drug release analysis showed sustained release of andrographolide for 5 days. To conclude, corresponding to the results above and its low- cost, the chitosan scaffolds with sustained release of andrographolide is advantageous and seems promising for bone tissue regeneration because it promotes osteogenesis and enhances mineralization of bone matrix. Consequently, chitosan scaffolds have been extensively researched for its biopharmaceutical and biomedical applications in bone and dental tissue engineering for over 20 years. Combination of bioactive materials with these chitosan-based scaffolds could also induce angiogenesis and vascularization in various animal models which can eventually be applied on humans in clinical trials.

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