Chapter 2. Literature Review

Although, Bone has inherent regeneration ability, no spontaneous healing occurs in the cases of trauma or tumour. Bone related morbidity and disability affect ~ 80% in elderly and 20 million in total population worldwide. These numbers display the bone disease repair in over $3 billion [25].

2.1 Bone Tissue Engineering

Nowadays, with the rise in incidences of facial trauma had urged the need to optimize materials required for bone-defect healing. The better understanding of the new procedural techniques and osteogenesis process are required. It leads to the advancement in designing and optimizing scaffold for bone tissue engineering (BTE) [26].

Bone in itself is an inorganic-organic composite. The hard connective tissue had been described with a few number of cells embedded in the ECM [27]. It undergoes dynamic biological remodelling in order to maintain homeostasis. Wherein, osteoclasts resorb mature bone tissue followed by osteoblast that regenerate healthy new bone.

This chapter reviews the 3D scaffold materials evaluation. The basis of the selection criteria was defined as per the requirement for the *in vivo* selected applications.

Figure 2.1 summarises the preference to use two types of rabbit osteoblast derivatives. They were the osteoblast from the bone tissue (rT) and osteoblast obtained after Mesenchymal stem cell (MSCs) differentiation (rM). Bone marrow was the source of MSCs. MSCs were the first cell type to infiltrate the bone defect site. These MSCs then differentiate into osteoblast to fasten bone healing. rT osteoblast were the host bone cell which also contribute as scaffold for the infiltrating MSCs.

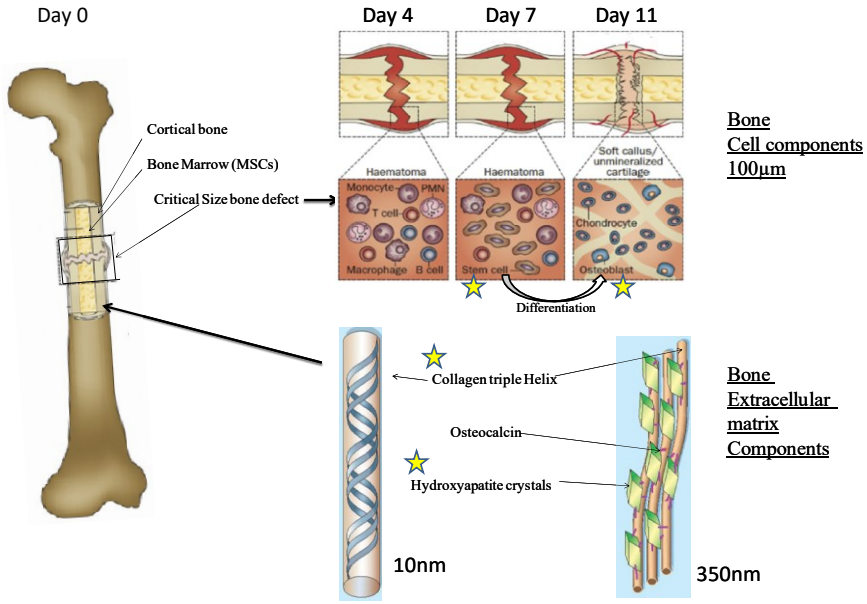

Figure 2.1 Schematic representation of the bone cells and extracellular matrix components.

2.2 Scaffold biomaterial

The challenge is to design and evaluate the cell-scaffold system suitable as the three-dimensional (3D) template. The same is done by mimicking the regenerative process of the human body. The full recovery of the damaged bone tissue is achieved in terms of both its structure and function [28]. Hence, these bone ECM substitutes restore to improve the tissue or organ function.

• During scaffold designing, it is necessary to integrate properties characteristic to bone as it is inspired by the ECM architecture.

• Scaffold must be retained in space and provide adequate space for simultaneous growth and development.

• Scaffold must transport seeded cells to the damaged tissue.

The process of bone formation at the defect site was initiated by the mechanical signals. It was provided by the ECM proteins (both the self-assembled collagen and the presence of the charged proteins). The signal was sensed by the bone-forming cells facilitating the mineral deposition at the nucleation sites [29]. The role of these deposited bone mineral was to regulate a chain of biological reactions. Both the bone-associated proteins and the osteogenic progenitor/stem cells were involved for accelerated bone formation [30,31]. BTE has evolved from the extensively studied class of biomaterials from both natural and synthetic origin [32-36].

On the basis of their origin, materials including bioactive ceramic, bioactive glass and polymers did bond instinctively to the living bone and its substitute. Over a period of time, an array of ceramics have been used as the scaffold component to restore damaged or diseased hard connective tissue.

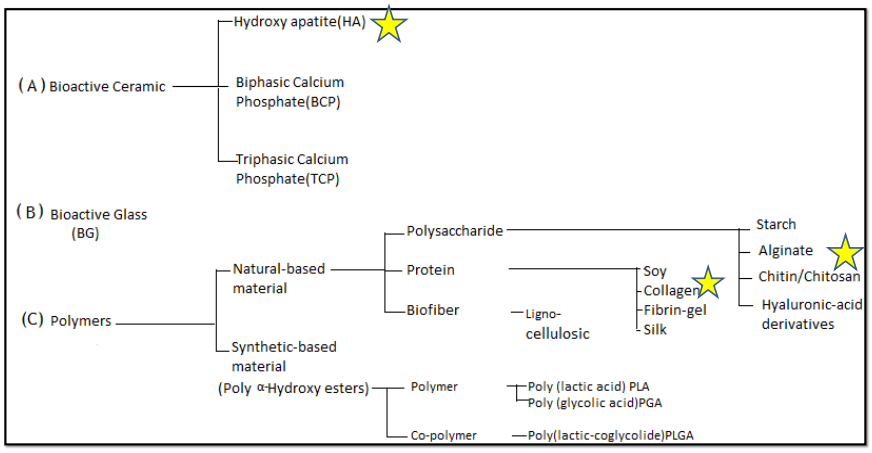

Figure 2.2 Classification of the raw-material on the basis of their chemical origin for scaffold constitution for various research studies in tissue engineering.
*****_Indicates the raw materials utilized for scaffold constitution in this research work_

Table 2.1 Comparative study of the raw materials used in this study w.r.t natural materials using matrix mapping.

Sr. no.	Properties	C	H	G	Collagen	SF	A	HAA
1.	Biomechanical [125,182,189]	2	3	2	1	**3**	1	1
2.	Cost [111,114]	1	2	1	**3**	2	1	**3**
3.	Stability [46,77,115]	2	3	2	2	2	-q	1
4.	**%Composition** [185-188]	**Nil**	**50**	**Nil**	**20**	**Nil**	**Nil**	**0.7**
5.	Biocompatibility	3	2	3	3	2	2	3

* Matrix score: 1-low, 2-intermediate, 3-high or strong.

Chitosan-C, Gelatin-G, Hydroxyapatite-H, Silk Fibroin-SF, Alginate-A, and Hyaluronic acid-HAA

Figure 2.2 presents the class of materials studied for their role as a scaffold component for bone tissue regenerative studies. Hydroxyapatite **(**HA), BCP (mixture of HA and TCP) and TCP are calcium phosphate (Ca-P) based materials. They have been widely studied due to similarity in chemical composition to the inorganic component of the bone ECM [37]. Although as a substitution to the orthopedic implants, HA have been widely researched. As, HA had given excellent cytocompatibility, a priority for being a biomaterial [6].

2.3 Single Component System

The use of natural or synthetic polymer's as three-dimensional (3D) structures for bone regeneration has been quite interesting [38,39]. The natural-origin based scaffolds have been osteoconductive, osteoinductive, cytocompatibile and bioactive in their native physiochemical properties[8]. Several materials like the natural polymers; collagen derived

gelatin [40,41] ceramic material [21,42]; inorganic hydroxylapatite [7,43,44] and chitosan [32] have been observed to be promising for bone tissue regeneration.

2.3.1 Hydroxyaptite: The inorganic component of Bone matrix

The inorganic calcium phosphate (Ca-P) bioceramic deposit, HA, $Ca_{10}(PO_4)_6(OH)_2$ resembles the mineral phase component of the bone [45]. The "calcium to phosphate ratio in terms of their atomic weight" (Ca/P) in the HA component of bone is 1.67 [46]. It has variety of applications in bone fillers, replacement [47]. In addition to its structural topography, the biological property had been critical for the performance of the scaffold material components/ raw materials [48].

Properties of biomimetic Ha that make it suitable as the inorganic component substitute for BTE:

1. As the structural component :

In recent five year period, HA is been investigated extensively due to the similar composition with the inorganic mineral part for bone bioengineering applications.

Osteoconductive nature of HA had promoted the osseointegration of the seeded bone cell by establishing direct chemical crosslink's with the cell synthesized ECM [49]. Innate brittleness and low load-bearing limits this reinforcing material's ability to solely improve the mechanical properties in the scaffolds [50].

The crystal structure supported ionic substitution in chemical-HA enabling it to mimic the inorganic HA of natural bone in being calcium-deficient and carbonate–containing [51]. Chemical-HA was used to promote fabrication of cost-effective scaffold material for future clinical research as observed in the work of D.Yang et.al. [52]. Wherein, an implantable scaffold material was a necessity for the repair of 'critical size defect' in bone related

deformities after trauma and tumors [9]. It was accepted that HA alone had been relatively insoluble *in vivo* [53]. Previous *in vitro* and *in vivo* research reported quick integration of chemical-HA at the implantation site [54,55].

The *in vitro* and *in vivo* studies were done by D.J.N. Harry et.al. and J.Seo et.al., respectively. They have shown that chemical-HA induces surface adherence for both osteoblast and osteoclast, before they start proliferation and begin secreting the ECM [55,56].

Every substitution had influenced the new bone formation during bone remodeling as it alters the innate properties (surface morphology [13], core morphology, thermal stability, antibacterial activity and crystalline behavior) of the scaffold components, both before and after cell seeding [57]. Poor UCS had limited its usage at the load bearing site as ECM in the spatial skeleton of a tissue. The feature univalent to the mineral phase of bone was their time-dependent kinetic surface modification after implantation, which was reported by S.V Dorozhkin et.al. [58]. A chemically and structurally equivalent bioactive carbonate-HA layer initially formed on the 'material-cell' contact surface. It qwprovided an interfacial bonding of the materials with the seeded cell/ implanted tissues [59]. Thus, the bioactivity behavior in these materials could be attributed from the formation of a carbonate containing HA layer [60].

2. As the biological component:

Due to the chemical similarity with the inorganic component of natural bone, property of HA were exploited for using it as the raw material component for biomaterial. These properties include the bioactivity, osteoconductivity, bone-bonding ability and biocompatibility. Modification of this inorganic mineral with fellow bio-molecules displayed prominence in bte.

The HA mineral displays stronger affinity for certain bone-specific proteins. These proteins in turn had contributed in enhancing the bone-bonding behaviour of the scaffold material [61,62].

Poor biodegradability of HA made it sustain longer within the scaffold [63,64]. The degraded byproducts have been non-toxic and non-inflammatory.

HA presented mild percent alkalinity, which can aid in neutralizing the acidity caused by the byproducts from chitosan/gelatin degradation. It ensured a healthy environment for osteoblast and/or mesenchymal stem cells (MSCs). Properties of HA favouring bone cell function were attributed to its particle nature and subsequent increase in the surface area. Incorporation of inorganic HA with organic component favored the biomaterials would be used in BTE.

Also, polymeric nanocomposites have been exhibiting improved mechanical properties in comparison to pure Ha whose brittleness makes it hard to shape [65]. Therefore, the Ha-organic polymers constituted scaffolds compensates for the stiffness of Ha. Previous research studies have incorporated filler material such as polymers matrix. These fillers tend to compensate for the mechanical strength of Ha component in the scaffold material [66–68]. Polymer addition resulted in reduction in porosity with rise in compressive strength. This change in porosity and mechanical strength had less positive impact on in-growth of seeded cell in the bioactive scaffold material, as studied by S.Stratton et.al. [69].

Additionally, Ha had been non-flexible and emigrated from the implanted site to material surface. The migration made it unfit to be used solely as the scaffold material [70,71]. HA cannot disperse well when in slurry rather it agglomerates [72].

Lizette Morejón et.al. [53] reported slight decline in the viable cell population when MSCs were seeded in scaffolds with HA content of 20 wt. % [73,74]. Hence, for scaffold with HA of <20 wt. % was considered suitable for maintaining cell viability of ≥70% for *in vitro* studies for bone bioengineering.

HA with fare compressivity and biocompatibility was used for the structural framework in the scaffold by L.Chen et.al. [75]. *In vitro* studies carried out by Z.A.Uwais et.al. reported ECM growth due to HA bioactivity [76], and promoted bio-mineralization when in simulated Body Fluid (SBF) [77]. The extent of bio-degradability and biomineralization property of natural polymers and proteins was responsible for their selection as scaffold component [78]. Bioactive and osteoinductive HA defines the inorganic part in the scaffold [36,79].

Ceramic HA mimicking the inorganic component of bone releases calcium-phosphate (CaP) stimulating osteogenic cell and accelerating regeneration [7,80]. This HA is responsible for imparting the bone-bonding nature to the scaffold [81]. It is an essential sign of biological response from the osteogenic cells in mineral deposition in the biomaterial. Hence, CaP deposition serves as a parameter in biomaterial selection [53]. The EDX spectrum evaluates the Ca-P dependence of the biomaterial [82]. The intermolecular forces and the particle diameter of CaP directs the morphological and thermal statistics of the biomaterial.

2.3.2 Chitosan

Chitosan was isolated from the crustacean shell with a degree of deacetylation >85%, a natural co-polymer of N-acetylglucosamine. It had been the widely used natural polymer in BTE based research [83].

Properties of biocompatible chitosan that make it suitable for using as the inorganic component in BTE in terms of

1. As the structural component:

Chitosan, unique polysaccharide based polymer shares both structural and chemical similarities with organic collagen of bone ECM matrix and has been used in graft as template for wound healing scaffold [83]. Also, chitosan support the growth and cellular activity of the bone cell.

Alone or in combination with another polymer it can be molded in various forms. It can form interconnected porous architecture after lyophilization.

Selection of biocompatible and osteoconductive chitosan in a dissolving-state confirmation for bone tissue engineered biomaterials was on the basis of previous work [84].

2. As the biological component:

It has displayed biocompatibility, non-toxicity, reabsorbability, and innate antimicrobial properties. For it to be the ideal scaffold constituent for BTE, the bioactivity need to be improved by combining with the other bioactive materials.

The inclusion of a bioactive polymer like chitosan positively contributes to the scaffold-host tissue interaction. The formability, migration and innate brittleness of the Ha particles along the host-scaffold was improved.

Chitosan, a semi-deacetylated chitin derivative is a non-toxic *in vivo* degradative by-product [85]. It supports excellent bone cell adhesion to create microenvironment for ECM deposition [86] as evident from the CLSM images. It holds promising biomaterial choice because of its low immunogenicity, biodegradability and biocompatibility [87]. The physicochemical property of structural scaffold-materials based scaffolds was studied in this research work. The natural polymer [88] based matrix (gelatin) which was infused with inorganic (hydroxylapatite) polymer have been investigated in this work [89].

For scaffolds, the contact surface roughness and core structural modifications have been evaluated using AFM and SEM, respectively [90]. Hence, exhibiting effectiveness of bone tissue regeneration w.r.t the structure of the scaffold materials [91]. These scaffold materials should also be biocompatible for osteoprogenitor growth. Differentiation of osteoblast from MSCs [92] followed by production and maintenance of the ECM produced by the osteoblast should also be promoted by the scaffold[49,50].

2.3.3 Gelatin

Collagen I constitute to the most abundant structural protein in the human body. It is also critical to the strength and toughness of the extracellular matrix of the mineralized tissue, bone [93]. It had been proved that introducing artificial collagen as the mineral component or bone cement could contribute in improving the innate properties. The osteoconductivity, osteoinductivity, cell attachnment, viability and proliferation characteristics and mechanical strength to name a few. The least expensive polypeptide gelatin, denatured collagen biopolymer derivative have been of significant interest. It was due to the non-immunogenicity of its byproducts avoiding non-physiological inflammatory reaction [94].

Gelatin as the ECM constituent was studied by its biodegradation, when the gelatin containing scaffolds were kept in lysozyme at physiological conditions [95]. Gelatin is a linear, amorphous, hydrophilic and mouldable biomaterial in thermal solution directs DSC thermogram. It is biocompatible with relatively slower degradation rate with the by-products not being immunogenic at all [96].

Within the matrix, the arranged collagen responds effectively to the external stimuli. This supports bone healing and ingrowth of surrounding tissue by enabling bone cells embedded in the ECM to sense deformation. Gelatin is a protein derivative and does not exist in nature.

It is prepared after denaturation of fibrous tissue protein of animal origin, collagen. The collagen-derived hydrophilic gelatin contains an array of biological functional group. Hence, displays potential as scaffold material for applications in BTE. Previous work had reported gelatin in displaying characteristics similar to the organic component of the natural bone connective tissue [41,97].

Properties of biocompatible gelatin that make it suitable for using as the inorganic component in BTE in terms of

1. As the structural component/ the structural characteristic/component:

In pharmaceuticals, Gelatin is widely used in adhesives and wound dressings. The property attributed to acquire mold shape, lack of immunogenic byproducts and cell viability supporter. Reasonable at low-cost facilitates its selectivity and mass-production.

2. As the biological component:

The arginine-glycine-aspartate motif (RGD) promotes cell adhesion. It shows cytocompatibility, non-toxicity and reabsorbs upon degradation.

Single component system cannot mimic every property of the natural bone ECM [98]. As the stromal substitute once implanted in the bone defect. It might provide the framework for induction of regeneration from the seeded bone cells. The developed cytocompatible biomaterial to be introduced at the ‘critical size bone defect’ [99]. Partnership between scaffold material and skeletal regeneration was encouraged for bone restoration. Surface and core modification had been done to improve the cell/scaffold material interactions.

Hence, developing a multi-component assembly for bone restoration becomes a necessity [89]. Table 2.1 represents the different scaffold materials for the selection of optimized product. The product is defined as close mimic of the natural bone extracellular matrix (ECM) in terms of variable morphologies, crystallinities, and stoichiometries [100].

The increase in inorganic-HA and organic-polymer interactions were shown. But improved mechanical and biological properties were observed upon chitosan interference [24,101,102]. In favour of cell attachment a positive response has been noted by scaffolds fabricated by 'Emulsion gelate freeze-drying' [103]. These scaffolds have recently shown to be consisted primarily of gelatin in gelatin-Ha scaffold biomaterials [104,105].

Further with next step to mimic the natural biological system of bone was by producing a material constituted by type I collagen variant i.e. gelatin, HA and chitosan. Also, bone cell from tissue and/or marrow obtained from the patient bone tissue and bone marrow respectively were included. A recent study highlighted that highest proliferation rate of the osteoblast seeded on Ha-collagen scaffold was observed when solid content was up to 20 wt.% [106]. The authors also reported clearly that decrease in cell proliferation and increase in cell attachment was observed with the increase in the solid content to 40 wt.% [79,107].

To closely knit the organic gelatin with the inorganic HA components in the organic matrices, the formation of the association was investigated from the previous studies [67]. A close association was noted (between gelatin matrix and Ca-P deposits), when in a homogeneous solution. Among the different scaffold materials, the selected optimized product should be a close mimic of the natural bone ECM in terms of variable morphologies, crystallinities, and stoichiometry.

Table 2.2 Comparative study on the methods of fabrication (in terms of being innovative)

Sr.no	Fabrication Methods	State of scaffold	Contamination	Accessibility	Mechanical property
1.	Lyophilization [22,23,29-30]	Rapid-solidification	Reduce chances	High	Superior
2.	Dry/wet-spinning based extrusion [57-60]	Amorphous consistency	Low	low	Inferior
3.	Gas-foaming [184-187]	Highly viscous	Relatively higher	intermediate	Intermediate
4.	Cryo-tropic gelation [111,125,187]	Interconnections not guaranteed	Intermediate	high	Inferior

	Raw material	Role as the structural component	Role as the biological component	Limitations	References
1.	Gelatin (G)	Induces mineralization, Provides many nucleation site for apatite Improves osteoconductivity, inductivity, Osteogenicity Often contain biofunctional molecules on their surface Biodegradable	Increases attachment and proliferation Retains viability Highly biocompatible and low toxicity Cell spreading, Host Integration biomimic	Limited mechanical properties for load bearing	[108]
2.	Ha	Osteoconductive Osteoinductive, UCS, Load bearing, Bioactive, Crystalline, Bone-bonding Co-precipitation, mouldability,	Supports bone matrix formation for CaP deposition & pore size Mineralization, differentation of MSC to Ob on Ha based scaffold, Host response Enhance the proliferation and the osteogenic-related factors expression.	Easily migrate from the implanted sites	[109] [52] [16]
3.	Chitosan	Osteoconductive, longevity, Improved physi-chemical properties Heavy metal scavenger	Improved Attachment efficiency, Antimicrobial		[110-112]

Table 2.3 Comparative display of the matrix materials studied.

2.4 Recent advances in the Multi-component System

The scaffold parameters w.r.t the overall scaffold structural, physiochemical, thermal and mechanical properties were varied. A comparative analysis was done to determine their possible impact on the bone cell. It enables in development of an appropriate bone substitute material for supporting the ingrowth of new bone [106]. The parameters that directs the cellular organization & present stimuli to direct desired tissue formation were summarised as follows:

(i) The surface characteristic suitable for cell attachment, proliferation and/or differentiation,

(ii) The highly porous, interconnected three-dimensional (3D) network for cell ingrowth and flow of nutrients and metabolic waste,

(ii) Mechanical strength same as the implantation site [113] and

(iv) *in vitro* and *in vivo* cell ingrowth was favoured by biocompatible scaffold with controllable and balanced rate of bio-resorption and degradation [114];

Hence, single component system cannot assist and mimic all the properties of bone. Therefore, developing multicomponent systems for bone repair becomes necessary [115,116]. Fabrication of the multicomponent system by 'gelate freeze-drying' have been evaluated [24,103,117].

On the basis of their characterized properties, the cell-biomaterial combination suitable for clinical bone-bioengineering was optimized. The ECM's supportive potential was analysed by employing hydroxyapaptite as the inorganic phase, gelatin as the organic phase and chitosan as the supportive component. All were in association with the rabbit osteoblast cells (rOb) namely, rabbit-iliac crest derived Osteoblast (rT) and Bone marrow MSCs derived

Osteoblast (rM). The quantification of these cell seeded-biomaterials have determined the proliferative and mineralization potential.

The cross-talk between the scaffold and the seeded-cell had been controllable due to the scaffold characteristics. The cell behaviour had been dependent on the variation in dimensionality experienced by the cell. These characteristic should be adaptable for bone-tissue engineered cell-scaffold to accomplish the regenerative property of bone.

2.4.1 Morphological Studies

2.4.1.1 Surface properties

Surface behaviour of the scaffold material plays a dominant role in the first interaction of the bone cells with the scaffold [111]. With the introduced surface modifications, the cell attachment properties are altered with immediate effect. The ‘average surface roughness’ and ‘surface free energy’ of the scaffolds have been regarded as two of the important factors regulating the cellular functions. Increased surface roughness leads to cell adhesion by favoring interfacial bonding of the material with the cell. Whereas, reduced surface roughness or too smooth surface promotes increased cell proliferation. Previous studies on micro-roughened surface have reported the effect on the osteogenic function of the bone cells [13,118]. For example, B.Langelier et.al. [119] reported enhancement in the attachment property and decrease in the proliferation rate of the osteoblast lineage with increase in roughness. Comparison can be drawn among the scaffold materials surface in terms of roughness parameters. ‘Average Roughness’, a dispersion parameter within the sampling area has been defined as the mean of absolute values of the surface departure, both above and below the mean plane [120].

Increase in differentiation potential of MSCs towards the osteoblast-lineage was promoted on "chitosan" surface (with roughness value 48.990). The *in vitro* studies carried out to find how the variation in surface roughness affected the seeded cell's osteogenic strength, but the results were not consistent [121]. Besides, the reasons behind the influence of surface roughness on Osteogenic-response of seeded cells were not well understood, yet related proposition from previous studies were referred [48]. For example, immediately exposed binding sites for ECM proteins were found. Also, the direct relation between surface free energy with the surface hydrophilicity effects the ECM component production (eg. osteocalcin) and adsorption [122]. Hydrophilic chitosan and gelatin corresponded to the increase in surface energy [123,124]. Molly.M [125] showed that increase in proliferation potential was observed for smooth, hydrophobic surface with lower surface energy.

It was found that obtaining desired surface properties were achievable by effectively controlling the core properties of the scaffold materials designed for BTE. In cumulative, the surface morphology had been the result of surface area, surface charge, surface hydrophilicity and surface energy [126]. The authors had justified the decrement in cell anchorage and proliferation with the increase in surface roughness in the chitosan/gelatin/ Ha scaffold materials [127].

2.4.1.2 Core morphology

The effect of the surface properties on the scaffold microporosity was studied by K.Zhang et.al. [128]. The amount and distribution of void volume had influenced the penetration of cells within the scaffold. Core Morphology of the scaffold material determines the mineral adsorption by the scaffold [57]. Bone like apatite particles growth on scaffold has been evaluated using SEM-EDX.

2.4.2 Elemental study

Even though carbon, nitrogen, oxygen and chloride were detectable and quantifiable, the calcium and phosphorous were of significance interest [129,130]. The inorganic fraction in bone has been primarily constituted from calcium and phosphate in the form of biological-apatite. The incorporation of calcium and phosphate deposits has been considered a key aspect during scaffold synthesis step for bone bioengineering applications. It can be added as a surface coat or to the bulk when preparing the scaffolds.

Calcium and phosphorous ions were introduced on purpose to form the mineral (calcium-phosphate) crystals. Using the semiquantitative method, EDX determines the ratio of calcium to phosphorous (Ca/P) revealing about the nature of the resulting crystal phase [131]. The Ca/P in the calcium rich Tetracalcium phosphate, commercial HA and Tri calcium phosphate (TCP) was 1.44, 2.33 and 2.00, respectively [132]. While, with the in situ calcium phosphate this ratio is 2.33. Limin Sun et. al. [129] reported that materials with a high Ca/P molar ratio are more stable in the biological system. The theoretical value for inorganic HA in Bone ECM is 1.67 [72]. The crystals formed in this study can be defined as calcium rich, to be less soluble in the biological medium [133]. This might be desirable because readily soluble materials could induce more intense response due to the degradative products.

2.4.3 Crystal nucleation and pore size

From both TEM and Selected Area Diffraction pattern for the scaffold materials the self-organization had been noted. Strongly indicative of crystalline HA nucleation, when present in association with either the chitosan or gelatin matrix. It was generally found that the mineralization in the calcified tissue was the result of heterogeneous nucleation.

Homogeneity in the scaffold solution was established following the lyophilisation step. The (002) axis of natural bone's Ha were aligned in parallel with the c-axis of the collageneous fibers in the matrix [134].

2.4.4 Crystallinity and chemical structure

2.4.4.1 Selected Area Electron Diffraction (SAED) based study

The electron diffraction pattern corresponding to the crystallites arrangement of HA was studied from SAED. In these crystallites arrangement, mineral phase arising from fused minerals mimics the natural bone matrice's charge distribution and stereo-chemical arrangement [135]. Similar to collagen, gelatin macromolecules have a regulatory function in crystal formation.

2.4.4.2 X-ray Diffraction (XRD) based study

XRD determined the structural phases present in the scaffolds in terms of their crystalline nature. The mineral phase deposited by the osteoblast once seeded in the scaffolds could be determined quantitatively by XRD pattern studied by S.Dasgupta et.al. [71].

2.4.4.3 Fourier Transform Infrared Spectroscopy (FTIR) based study

The chemical structure of the scaffold materials was determined from the characteristic peaks in fingerprint region and functional group region in FTIR. The spectral changes in the secondary structure of chitosan, gelatin and HA after crosslinking during the scaffold materials formation were recorded [136].

The narrowing of amide I found near 1630 cm^{-1} and decrease in the intensity of amide I component, found around 1660 cm^{-1}. These were indicative of collagen denaturation, as

observed from gelatin peaks [136,137]. For amide I, the peak broadening and peak shifting to lower wave numbers has been found to be associated with the collagen fibre self-assembly.

2.4.4 Diffraction Scanning Calorimetry (DSC) based thermal study

The glass transition temperature (T_g) was obtained from differential scanning calorimetery. T_g was calculated at the midpoint in the heating scan. Shelf life of the scaffold material as the 'product', was determined from its DSC scan. The DSC curve interprets the possible reactions the sample had undergone [114]. The product denaturation and shelf life were also studied from T_{cc}. Thermal stability of the scaffold material has been critical in affecting their: morphology, cytocompatibility, mechanical property and biodegradability [138-140].

2.4.5 Uni-axial Compressive Strength (UCS) study

Primary criterion for the selection of materials to be evaluated for bone bioengineering study, the mechanical property [141]. It serves the measure of strength only along one axis when sample had been cylindrical in shape. The compressive stress is applied along the longitudinal axis of the sample as studied from S.Huang et al. [142]. Variable parameters contribute to the mechanical property of the multicomponent based scaffold materials. These involves: the morphology, Ca/P, porosity, size and size-distribution of the inorganic HA. Also, the innate mechanical property of every component, and the interfacial interactions among the components [143-145].

The bone substitute materials with less elastic moduli in the multicomponent-scaffolds were observed with the chitosan-ceramic interaction [21]. For example, implant material with high elastic modulus results in the drastic tissue loss in cases of joint-replacement [146]. It in turn resulted from large difference in stiffness between the materials and host tissue. Less elastic

modulus with compressive strength can better promote new tissue regeneration in bone. It was to maintain sufficient intergrity in the scaffold material to be implanted, also in accordance with the previous study [147].

2.4.7 Porosity and its influence

The pore size, pore architecture and porosity had been crucial for scaffold osteoconductive properties. The size and spatial distribution of pores in the scaffold influences the infiltration and subsequent proliferation of seeded cells. Both the Cell adhesion and ingrowth had been dependent on porosity characteristically specific for that particular scaffold. Well interconnected porosity, essentially required in metabolic functions for osseointegration [60]. As mentioned above, the scaffold-porosity facilitates the load-bearing mechanical behaviour of the bone-implants, by providing sites for chemical bond formation between the implant and the host bone.

Optimal pore size in the range of 250-350 μm has been reported in balancing cell infiltration and mineralization for bone [148]. The decreases in total porosity with the increase in inorganic chemical-Ha content had been reported [149]**.** This decrease was observed for ≥14 wt. % Ha, when present as the scaffold component. This resulted in reduced access of the seeded-cells to the interior of the scaffold structure. Thereby limiting nutrient transport and hence reduced cell viability. Scaffold synthesising process influences its resultant porosity. In Gelate-freeze drying, the distorted ice-growth increased the microporosity forming cellular microstructures. The micropores enriched scaffold favoured enhanced new-bone volume formation within the scaffolds [150]. Porosity promotes the osteogenic activity but reduces the mechanical behaviour. It is important to strike a balance between the biological behaviour, mechanical behaviour and porosity.

2.4.8 Biodegradibility: Stability in scaffold structure

While developing a scaffold, an effective sterilization-step ensures its utilization for bone TE research studies. As it does not modify it in terms of major change in its porous architecture or degradation. Moreover in bone TE research, as the tissue regeneration proceeds the scaffolds must degrade, without creating cytotoxic by-products [151]. The structural porosity parameter influences the water permeability and so the degradation behaviour. Higher porosity reduces the hydrolysis rate. For bone regenerative scaffolds, decrease in hydrophilicity ensures longer degradation rate. Great care has to be taken in *in vitro* degradative studies as the more severe *in vivo* conditions leads to decrease in the scaffold lifetime [152].

'Creation of artificial degradation conditions' enable the scaffold to perform better in the *in vivo* scenario. Lysozyme, the primary enzyme responsible for the degradation of chitosan through hydrolysis of its N-acetyl glucosamine groups [115], *in vivo*. Its degradation releases amino sugars, which can be either excreted or incorporated into glycosaminoglycans and glycoprotein metabolic pathways [102]. On the scaffold surface, oligomeric units formed after hydrolytic cleavage of chitosan get dissolved when in PBS. Gelatin too hydrolyses quickly in water.

Bone formation had been concurrent with implant resorption. Accelerated degradation rate of the scaffold influences the new bone formation [121].

Although, many *in vitro* studies were done on the biodegradation of polymer/ceramic scaffold [153]. To elucidate the mechanism by which the bioactive mineral phase would

contribute (slow down/ speed up) to the degradation of the scaffolds the mineralization studies were carried out in SBF.

2.4.9 Biomineralization: Bioactivity of the scaffold architecture

When highly porous calcium-phosphate based scaffolds were immersed under physiological conditions in SBF. Their degradation had been accelerated resulting in the release of ions. This release results in increase in Ca^{2+} and PO_4^{3-} ions in the immediate vicinity. It makes them readily available for biological apatite precipitation within the scaffolds [154]. Other scaffolds too can form apatites on their surface when in the biological body fluid, as found from previous studies [155]. Few negatively charged non-collagenous bone ECM proteins tend to form coordinate bond with surface exposed cations. The coprecipitation with these deposited apatite triggers the differentiation of seeded MSCs into osteoblasts.

The chemical formation of these mineralized layer/calcium-phosphate layer (either amorphous, partially crystalline or crystalline) due to ion-exchange (between the surrounding fluid and the scaffold) had been referred to as bioactive. The bioactive behaviour promotes to bone formation by promoting both cell attachment and mineralization [156]. This bioactive behaviour of these scaffold materials was evaluated *in vitro* by checking the mineralization ability using metastable Simulated Body Fluid (SBF). SBF is an apatite supersaturated calcium-phosphate solution, similar to human blood plasma as solution [157]. It had been required due to the presence of obstacles for homogeneous apatite-nucleation and requirement of heterogeneous nucleation sites.

The apatite-coating formation in SBF had been the method to design bioactive scaffold materials as implants for bone-tissue engineering. The property of these apatite layers cause changes in the cell environment by affecting both cell viability and proliferation. Bone-like

apatite formed on the surface has been very similar to the natural bone, in growth behavior, crystallinity (low), mineral composition, structure and size (nanoscale). Through this apatite-layer reproduced in SBF, materials bind to the living bone [158].

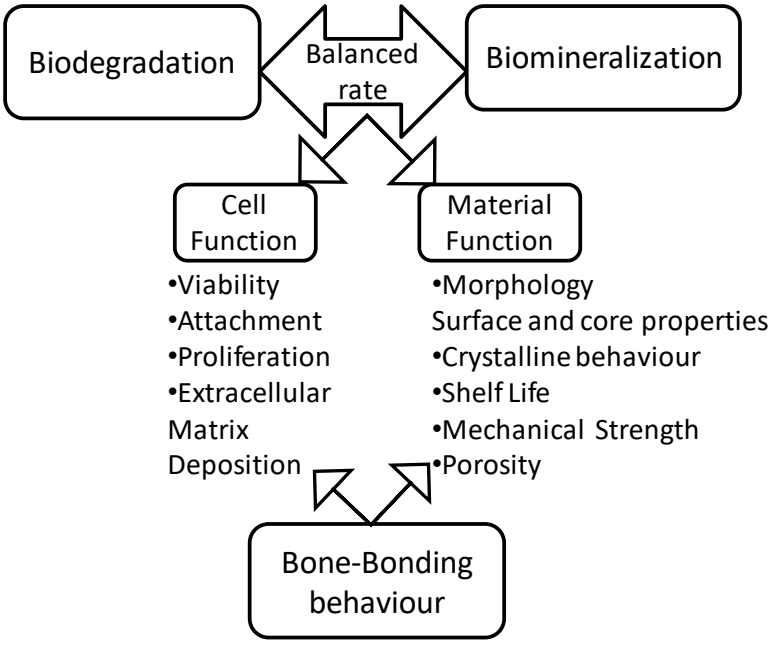

Figure 2.3 Parameters influenced from the balanced rate of biodegradation to biomineralization in the scaffold structure have been summarised: (a) Affected cell function & (b) Affected material function.

2.4.10 Antibacterial Properties

Surgical Site infections poses significant clinical problem during orthopaedic surgery and subsequent healing. Thereby prolonging the recovery period, implant failure and increased cost. Prevention of these infections in the scaffold and at the implant delivery site involves scaffolds with preferably innate antibacterial properties. This makes the healthcare reasonable with the addition of a supportive antibacterial component. Their innate anti-

bacterial activity was evaluated through the 'Agar diffusion' method [159]. These scaffolds with antibacterial properties would present promising solution in healing open fracture.

2.5 Bone cells

2.5.1 Osteoblast

Osteoblasts were found to be located along the surface of bone and show morphology characteristic to any protein synthesizing cell [160]. They constitute 4–6% of the resident bone cell population. Also well known for their functional role, to secrete the osteoid for bone ECM formation, progressive mineralzation and its control [29,161].

2.5.2 Osteoclast

Osteoclasts play crucial part in the remodelling of bone [162,163]. The haematopoietic precursor cells found in the bone marrow and peripheral blood are the precursors of osteoclast. The osteoclast population display bone surface attachment and migration. They were functional to subsequently degrade the bone extracellular matrix components (e.g. Collagen, osteoponin, fibronectin, bone sialoprotein/osteocalcin).

2.5.3 Osteocytes

These are the most abundant bone cell existing throughout the mineralised ECM. They form the interconnected network to respond to local stimuli to regulate osteoblast-osteoclast based bone remodeling [164]. The remodelling process adapts the skeleton to respond to growth factors (GF) involved in replacing the damaged bone.

2.5.4 Mesenchymal stem cells (MSCs)

MSCs can be used in BTE and regenerative studies because of their ability to filtrate to the site of bone injury from the bone marrow in the immediate vicinity [165]. The MSCs then

differentiate into bone specific lineages equipped with immune privileges, and genetic stability [59]. MSCs here commit to bone phenotype with extreme sensitivity to hard tissue-type elasticity. The MSCs matrix once mimics the collagenous bone, prove to be osteogenic from the work of K. Baghaei et.al. and H. Li et.al. [166,167]. During the initial few weeks in MSCs culture, reprogramming to osteoblastic lineages has been possible with the addition of soluble inducible GFs. As after several weeks, the MSCs tend to commit to the lineage specified by the matrix elastic behaviour. The significant implication of the *in vivo* microenvironment on the lineage specification of MSCs decides their therapeutic use.

2.6 Cell selection

In addition to the use of osteogenic differentiating agents and GFs, the selection of the type of cell to be seeded for the creation of scaffold for bone repair is important. The addition of cells into the scaffold can change the interaction between the surrounding tissue and the scaffold, affecting bone healing.

Osteoblasts had been the well-differentiated skeletal cell responsible for the secretion of the bone ECM. They arise from the pluripotent MSCs that are capable of differentiating into a number of committed cell lineages in the presence of a specific set of GFs. Hence, out of several cell types to choose from, two types of the cellular components were used for bone repair. It involves the primary cell line of MSCs and the host tissue specific osteoblast cells [17,168]. The ability of MSCs to differentiate towards the osteogenic lineage have been exploited *in vitro*.

2.7 Cytocompatibility

The Alamar Blue (AB) Assay is based on the fluorometric-based detection of the mitochondrial metabolic activity in viable cells. The chemical reduction of resazurin (blue)

by the mitochondrial NADPH dehydrogenase (with diaphorase activity) to fluorescent resorufin (red) is carried out by non-toxic, water soluble ABA on adhered cells [169].

2.8 ECM Mineralization potential

During differentiation, osteoblasts secreted ECM promotes mineralization. The early differentiation phase of osteoblasts had been marked by the alkaline phosphatase (ALP) activity. ALP activity was measured to determine the matrix maturation during the differentiation of osteoblasts on the scaffolds [170].

Scaffold's mineralization was done to test their osteoinductive nature from the calcium deposits detected by Alizarin Red S (ARS) [171].

2.9 Growth factors gene expression profile

The materials alone cannot offer to support the regeneration ability of bone. The cells in the host bone when infiltrate the scaffold, it is common for the resulting tissue not to be fibro-osseous. It has been found to include specific GFs s to accelerate the growth in a defined direction. The GFs for bone repair occur naturally in the body. To achieve their role for TE, an increased concentration must be used [172–174]. GFs aid the studies done *in vitro*.

Out of the many ways the GFs were introduced for bone repair, chitosan/gelatin/HA scaffold introduction was utilised to facilitate bone regeneration. Isolated rabbit iliac crest cells when cultured in the osteogenic media supplemented with the GF. Bone morphogenic protein-2 (BMP-2) showed the ECM production from culture day 14 onwards [175].

Using these *in vitro* results as the basis for the in vivo study, ECM production was observed later than in vitro i.e. from culture day 30 onwards [176]. Increased time lapse was observed when MSCs were cultured and studied for the ECM production both in vitro and in vivo. But,

additional GFs were required for ECM production from the MSCs differentiated osteoblast. The cells can easily be utilised to include the GFs in the bone tissue engineered scaffold, the scaffold alone cannot be used to introduce one or more GFs [17].

Wang et al. compared the results of a gelatin/Ha scaffold in the osteogenic media when one with and without the GFs BMP-2. BMP-2 regulates ALP expression and Osteoblast mineralization [177]. An array of phenotypic abnormalities found in rabbit or human bone were largely due to an osteoblast defect. Chain of morphogenic proteins (including bone morphogenic proteins (BMPs)) induces the early-osteoblast marker, ALP in MSCs. Differentiation status of the normal rabbit bone cells when cultured till ≥70% confluency in monolayer until passage 4 (P4) was determined *in vitro*. At each passage, real-time quantitative PCR (Q-PCR) analysis of mRNA expression of collagen type I, OCN and BMP-2 was done. The differentiation status of MSCs derived osteoblast during monolayer subculture was analysed.

2.10 Biochemical analysis

2.10.1 Collagen

The imino acid hydroxyproline has been involved in stabilizing the triple helical collagen molecule [178]. The concentration of hydroxyproline was used to calculate the total collagen from cell culture to patho-physiological tissue.

2.10.2 Osteocalcin

Osteocalcin, the primary non-collagenous protein secreted by ost”eoblasts [179], which signals the termination of the osteogenic differentiation [180]. The property was exploited to measure both the new bone formation and the bone cell lineage. The presence of higher amount of osteocalcin (green) deposits were seen in the culture day 14 scaffold. It was indicative of the increase in the osteoprogenitor cells with the gain in the culture duration.

2.11 *In vivo* osteoblast studies: Scaffold ability to promote bone regeneration

Induced bone healing involves a cascade of chemical factors (e.g. GFs, cytokine) induced signaling pathways. Through the activation of genes, these GFs had been involved in the osteoprogenitor proliferation and differentiation. All these pathways occur in an organized process, which eventually results increase in the new bone volume due to bone regeneration [181-183]. The bone-scaffold bonding lead bone regeneration can be evaluated using specific in vivo models. In rabbit critical-defect model, a 22 mm surgical defect that does not heal on its own is created on the external face of the femoral distal head. This defect-model had been used to investigate the role of the scaffold materials in terms of their osteogenic behaviour in induced bone healing.