Chapter 12
Engineering of Bone: Uncovering Strategies of Static and Dynamic Environments

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List of Abbreviation

ECM	Extracellular matrix
3-D	Three dimension
HAp	Hydroxyapatite
Ti	Titanium
PLA	Polylactic acid
PGA	Polyglycolic acid
PLGA	Poly(lactic-*co*-glycolic acid)
PCL	Polycaprolactone
PLCL	Poly(lactide-co-ε-caprolactone
hADSCs	Human adipose-derived stem cells
BMSCs	Bone-marrow-derived MSCs
BSP	Bone sialoprotein
TCP	Tricalcium phosphate
ALP	Alkaline phosphatase
CPC	Calcium phosphate cement
hUCMSCs	Human umblical cord-derived mesenchymal stem cells
PVA	Polyvinyl alcohol
PEO	Polyethylene oxide
PAA	Polyacrylic acid
pDNA-NELL1	Nel-like Type I molecular-1 DNA

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hAFSCs	Human amniotic fluid-derived stem cells
OX2	Osterix
RUNX2	Runt-related transcription factor 2
PEG	Polyethylene glycol
RGD	Arg-Gly-Asp
hESCd-MSC	Human embryonic stem cell-derived mesenchymal stem cells
PEGDA	Polyethylene glycol diacrylate
MSCs	Mesenchymal stem cells
HOB	Human osteoblast cells
ELR	Elastin-like recombinamer
hESCs	Human embryonic stem cells
iPSCs	Induced pluripotent stem cells
BMP-2	Bone morphogenetic protein 2
BMP-7	Bone morphogenetic protein 7
TPS	Tubular perfusion system
IL-1	Interleukin-1
IL-6	Interleukin-6
TNF-α	Tumour necrosis factor alpha
FGF-2	Fibroblast growth factor 2
M-CSF	Macrophage colony-stimulating factor
PDGF	Platelet-derived growth factor
BMPs	Bone morphogenetic proteins
VEGF	Vascular endothelial growth factor
TGF-β	Transforming growth factor *beta*
IGFs	Insulin-like growth factors
bFBF	Basic fibroblast growth factor
LbL	Layer by layer
MMP	Matrix metalloproteinase
PD-MCG	Polydopamine-coated multichannel biphasic calcium phosphate granule system
BCP	Biphasic calcium phosphate scaffolds
CFD	Computational fluid dynamics
RPM	Rotations per minute
RWV	Rotating wall vessel
EMF	Electromagnetic field
PEMF	Pulsed electromagnetic field
GMP	Good manufacturing practice
Micro-CT	Microcomputed tomography
CAD	Computer-aided design

1 Bone Tissue Engineering: An Introduction

As a highly specialized and dynamic tissue, bone is characterized by its mineralized matrix, rigidity and hardness with certain degree of elasticity. Bone provides support and protection to internal organs and also aids in locomotion. It is also involved in haematopoiesis and is an important reserve of calcium and phosphorus [1]. Further, the extracellular matrix (ECM) of bone comprises (a) non-mineralized organic phase, predominated by collagen type I, and (b) a mineralized inorganic phase, constituted of carbonated apatite [2]. This remarkable nanocomposite architecture confers properties such as high compressive strength and fracture toughness to bone. Additionally, there are other non-collagenous protein components that contribute to bone-specific events such as mineralization, osteoblast differentiation and bone remodelling [3].

During embryogenesis, bone formation either happens through intramembranous ossification or through endochondral ossification [4]. Once formed, at the morphological level, bone exists as compact (cortical bone) or spongy bone (cancellous bone). Cortical bone comprises tightly packed collagen fibrils that provide mechanical strength to bone. Cancellous bone, on the other hand, is a loosely arranged matrix that contributes to the metabolic functions of the bone. Being a highly dynamic tissue, bone undergoes constant modelling and remodelling [5]. During bone modelling, bone formation or resorption occurs on a bone surface. On the contrary, bone remodelling refers to bone formation or resorption that occurs sequentially in an organized manner with the aim to maintain structural integrity of the skeleton. Two major players involved in these processes are osteoblasts (bone-forming cells) and osteoclasts (bone-resorbing cells).

When subjected to trauma such as traumatic injury, soft tissue damage, tumour resection, age-related diseases such as osteoporosis or complications such as diabetes, bone responds by its ability to regenerate. However, if the defect size is greater than critical size, bone falls short of its regenerative capacity [6–10]. As a result, there is intervention in form of conventional treatment regimes such as autologous and allogenic grafts. Although autologous grafts remain the gold standard due to non-immunogenicity and recapitulating all properties of the requisite bone graft, it is limited by availability, donor site injury and morbidity [11]. Allogenic grafts from cadavers might be an alternative; however, it is associated with infections and graft rejection [12]. These issues led the researchers to harp on alternate bone repair strategies such as bone tissue engineering.

Bone tissue engineering aims to develop functional tissues and substitute the lost bone. In this context, tissue engineering encompasses three crucial elements, namely three-dimensional (3-D) transient structures called scaffolds, cells and growth factors, which drive the generation of a successful graft. Apart from these three elements, bioreactors have been shown to recapitulate certain aspects of in vivo bone microenvironment such as shear stress and mechanical stimulation [13–15]. This chapter will discuss the recent advances in the role of scaffolds, cells, growth factors and bioreactor-based strategies that have been studied towards the development of a successful graft.

2 Biomaterial-Based Scaffolds in Bone Tissue Engineering

Typically, scaffolds are porous, biodegradable and biocompatible materials with appropriate mechanical properties that facilitate adhesion, proliferation, differentiation and regeneration of damaged tissue [16]. For bone tissue engineering specifically, an ideal scaffold should be osteoinductive (able to induce osteogenesis by recruiting pre-osteoblasts/progenitor cells), osteoconductive (should support adhesion, proliferation and migration of osteoblasts throughout the construct) and osseointegrative (should be able to integrate into the surrounding bone). While a graft is osteoinductive, osteoconductive and osseointegrative, it should also recapitulate the mechanical properties of the surrounding anatomical site of implantation and maintain its integrity against the wear and tear caused during remodelling inside the host [17, 18]. Therefore, a biomaterial for the engineering of cortical or cancellous bone should be chosen based on their compressive strength that varies between 100–200 MPa and 2–20 MPa, respectively [19]. As an example, inspired by the excellent structural and mechanical properties of honey comb, Zhao and Liang [20] developed a 3-D-printed biomimetic comby scaffold using chitosan/hydroxyapatite (HAp) powder. Compressive strength (1.62 ± 0.22 MPa) and Young's modulus (110 ± 22 MPa) were found close to cancellous bone. Similarly, Chen et al. [21] fabricated biocompatible highly porous titanium (Ti) scaffold by powder metallurgy method with magnesium powder used as space holder; mechanical properties of the resulting scaffolds were close to human cortical bone.

In addition to the mechanical properties of the scaffold, bone regeneration is also influenced by the type of biomaterials, scaffold architecture and scaffold functionalization. Each of these will be discussed in the following section.

2.1 Choice of Biomaterials for Bone Tissue Engineering

According to the European Society of Biomaterials, a biomaterial is defined as "A material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body". Scaffolds used for bone tissue engineering are majorly categorized as **polymeric scaffolds, ceramic scaffolds, metallic scaffolds and their composites**. Figure 1 depicts examples of each of the aforementioned category [22–25].

Polymeric scaffolds are fabricated using either natural polymers (such as collagen, hyaluronic acid, agarose, chitosan and silk), synthetic polymers (such as polylactic acid (PLA), polyglycolic acid (PGA), poly (lactic-*co*-glycolic acid) (PLGA), polycaprolactone (PCL)) or their combination. Natural polymers hold superiority over synthetic polymer in terms of biocompatibility, biodegradability as well as biological information that supports cell attachment. However, they exhibit batch-to-batch variability, immunogenicity and risk of disease transfer as well as lack requisite mechanical strength. On the other hand, synthetic polymers are known for excellent

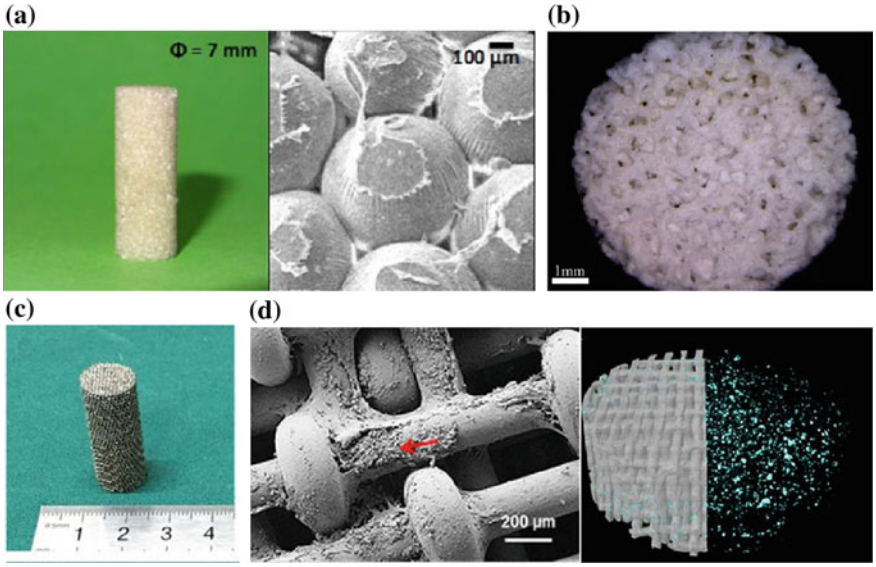

Fig. 1 Biomaterials in bone tissue engineering; **a** digital photograph of silk fibroin scaffold (left) and SEM of silk fibroin scaffold showing silk fibroin microparticle arrangement in hexagonal fashion (right); **b** digital microscopic photograph of mesoporous bioactive glass; **c** digital photograph of porous Ti6Al4V scaffolds for bone tissue engineering. **d** SEM of human mesenchymal stem cell (hMSC)-seeded polylactic acid (PLA)/hydroxyapatite (HAp) composite scaffolds. Arrow depicts cell mineralization (left) and segmented microcomputed tomography image of PLA/HAp composite scaffolds depicting mineralized nodules (right)

mechanical properties and tailorability into various forms [26, 27] and however, they lack bioactive sites and hence are improvised by incorporating various bioactive molecules such as HAp or by blending with natural polymers [28]. As an example, in a study, silk fibroin (natural polymer) was blended with poly (lactide-co-ε-caprolactone) (PLCL) (synthetic polymer) in order to make the latter more conducive to cell–scaffold interaction [29]. It was found that blending of a natural polymer with a synthetic polymer supported differentiation of human adipose-derived stem cells (hADSCs) to osteogenic lineage. Furthermore, in vivo implantation of blended scaffold demonstrated enhanced bone volume, bone mineral density and new bone areas as compared to pure PLCL scaffold. In another study, human foetal osteoblasts were cultured on electrospun composite nanofibrous scaffolds based on HAp, chitosan and collagen in order to assess biomineralization. It was found that in comparison to HAp-chitosan scaffolds, collagen-doped composite scaffolds were highly biomimetic and osteoinductive [30]. To add to this, introduction of multiwalled carbon nanotubes to collagen–HAp composite scaffolds promoted the proliferation of bone-marrow-derived MSCs (BMSCs) as well as expression of osteogenic markers such as bone-sialoprotein (BSP) and osteocalcin as compared to collagen–HAp scaffolds [31].

Ceramic Scaffolds, specifically calcium phosphate scaffolds, such as tricalcium phosphate (TCP), Hap or their combination as biphasic systems are extensively studied in bone tissue engineering because they perfectly mimic the mineral composition of the bone and their biophysical properties make the scaffolds osteoconductive and osteoinductive [32–35]. Apart from the calcium phosphate ceramics, there are other bioactive ceramics such as calcium silicon-based ceramics termed akermanite (combination of calcium silicon and magnesium) and bioglass (combination of sodium oxide, calcium oxide, silicon dioxide and phosphorus pentoxide) that have also been explored for bone tissue engineering [36–38]. While calcium silicon-based ceramics are well known for their mechanical properties and controlled degradation rate, bioglass is known for its biodegradability and osteogenic potential [39]. However, brittleness of bioglass limits its use as a stand-alone scaffold and is therefore used as composites for bone regeneration [40].

Furthermore, scaffolds containing metals find application in bone regeneration due to properties such as high mechanical strength and fracture toughness [41, 42]. Commonly used metals are stainless steel 316L(ASTM F138), cobalt-based alloys (ASTM F75 and ASTM F799) and titanium-based alloys (Ti–6A1–4V, ASTM F67 and F136) [43]. However, metallic scaffolds exhibit poor biological recognition and release toxic metal ions due to corrosion or rusting, thereby resulting in allergic reactions and inflammation [44]. To overcome this, the surface of metallic scaffolds is usually modified or coated to improve biocompatibility; cell-recognizing ligands and growth factors have also been integrated within the proximity of the construct to enhance cell growth [45]. Surprisingly, a study demonstrated that Ti scaffolds have an intrinsic potential to promote osteogenesis. Briefly, BMSCs seeded on uncoated highly porous Ti scaffold and HAp-coated Ti scaffold were evaluated for their osteogenic potential. Results demonstrated that uncoated Ti scaffolds induced better bone formation and ingrowth when implanted in sheep stifle joints as compared to HAp-coated scaffolds, suggesting that Ti-based scaffolds are self-sustained to promote osteogenesis and have the potential to be used in healing large bone defects [46]. There are also reports of few other metals such as magnesium alloy W4 and copper-containing scaffolds in promoting osteogenesis [47, 48].

2.2 Role of Scaffold Architecture

Bone tissue engineering is influenced not only by the type of biomaterial but also by the design and geometry of scaffolds. Several studies have shown the influence of scaffold architecture in modulating cell behaviour and their differentiation [49]. This section will be discussing the influence of scaffold architectures, such as hydrogels, macroporous scaffolds and fibrous scaffolds, in bone tissue engineering (Fig. 2) [50–53].

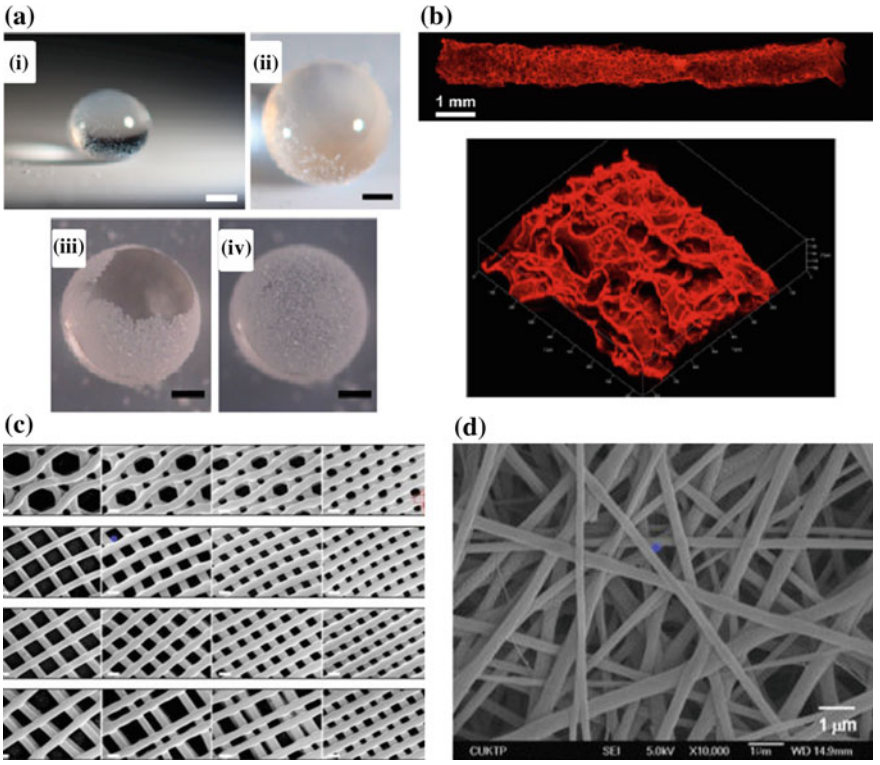

Fig. 2 Types of scaffolds in bone tissue engineering; **a** gelatin methacrylate drop on a hydrophobic surface coated with hydrophobic bioactive glass nanoparticles (i) and (ii). Droplet encapsulated with nanostructured film (iii), resulting in semitransparent liquid marble (iv). The polymer liquid core was then crosslinked with UV light resulting in bioactive hydrogel marble; **b** confocal micrographs of complete and surface of rhodamine-labelled scaffold based on pullulan, modified with a cholesterol moeity as obtained by freeze drying; **c** SEM micrographs of 3-D-printed bioactive glass ceramic (Sr-HT gahnite) scaffolds of different geometries; **d** SEM micrograph of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) scaffolds generated by electrospinning

2.2.1 Hydrogels

Hydrogels are products of physical or chemical cross-linking between hydrophilic polymers (natural or synthetic) with the tendency to swell in biological fluids. Highly hydrated state of hydrogels makes them an ideal choice for cell encapsulation as well as differentiation [54, 55]. They also possess excellent capacity to entrap and release bioactive agents, thereby serving as promising candidates for bone regeneration. Various natural polymers such as collagen, gelatin, alginate, hyaluronic acid, agarose, chitosan and silk and synthetic polymers such as PLA, polyvinyl alcohol (PVA), polyethylene oxide (PEO), polyacrylic acid (PAA) and poly(propylene fumarate-co-ethylene glycol) have been used for hydrogel fabrication [56, 57]. Further, many hydrogels have been reported as injectable systems that can be introduced to the

site of action by minimally invasive procedures and support in situ bone formation [58]. Amongst various injectable hydrogels reported, alginate is one of the well-studied biomaterials for bone tissue engineering [59]. In a study, Han et al. [60] prepared an injectable calcium silicate/sodium alginate composite hydrogel; in situ gelation was induced by calcium ions released from calcium silicate following the addition of D-gluconic acid δ-lactone. The composite successfully induced HAp formation and promoted osteogenesis of rat BMSCs and angiogenesis of human umbilical vein endothelial cells. Other polymers such as chitosan, collagen and *N*-isopropylacrylamide have also been used as injectable systems for bone tissue engineering [61–63]. Additionally, composite hydrogels using HAp, nanosilica, bioglass and zinc have been utilized in bone tissue engineering to provide mechanical stability and promote in vivo calcification [64–70]. Furthermore, on the basis of type of cross-linking, injectable hydrogels can be thermoresponsive, photocrosslinked, chemically crosslinked or enzymatically crosslinked [71–73].

2.2.2 Macroporous Scaffolds

Macroporous scaffolds are highly interconnected, porous, 3-D structures that are based on natural or synthetic polymers. These can be generated using techniques such as particulate leaching, freeze drying, solvent casting, gas foaming, thermally induced phase separation and 3-D printing [74–76]. Various composite macroporous scaffolds have been reported for bone tissue engineering. In one such example, highly porous chitosan–silica composite scaffolds were fabricated by freeze drying; the scaffolds favoured osteoblast proliferation, with enhanced alkaline phosphatase (ALP) activity as well as mineral deposition in comparison to chitosan-only scaffolds [77]. Further, 3-D printing has also been used in bone tissue engineering [78–81] and allows for precise control over the architecture and geometry of the scaffold [82]. Commonly used biomaterials for 3-D printing of bone that can recapitulate the mechanical properties of bone and can promote vascularization include calcium phosphate composites, bioactive glass mixture, zirconium oxide, silica, graphene and strontium [83]. One such study utilized 3-D-printed porous scaffold based on bioactive glass and chitosan nanoparticles, loaded with an osteoinductive protein, Nel-like Type I molecular-1 DNA (pDNA-NELL1) and tested for efficacy of BMSCs to repair bone defects in rhesus monkey [81]. It was observed that enhanced alveolar bone regeneration was observed in 3-D-printed bioactive glass–chitosan nanoparticles loaded with pDNA-NELL1 and BMSCs as compared to other control groups.

Within macroporous scaffolds, pore size and interconnectivity are known to influence cell infiltration and bone regeneration [84]. In a study, bilayer HAp-based scaffold corresponding to cortical-cancellous organization in bone with pore size of 200 μm (as outer layer) and 450 μm (as inner layer) was compared to trabecular-like organization with a uniform pore size of 340 μm for their ability to support bone regeneration in a 10-mm segmental rabbit radius defect model. Result showed that uniform pore-sized scaffolds supported better functional bone, greater flexure strength as well as toughness when compared to scaffolds with bimodal pores [85].

In another interesting study, Gupte et al. [86] fabricated nanofibrous PLA scaffolds of controlled pore architecture using thermally induced phase separation and sugar porogen template method and evaluated BMSCs differentiation as a function of pore architecture. They found that smaller pore size (125–250 μm) favoured chondrogenesis while larger pore size (425–600 μm) supported mineralized bone tissue via the ingrowth of blood vessels within the porous architecture. This study suggested an important contribution of pore architecture in bone tissue engineering application.

Apart from pore architecture, few researchers have also explored the role of pore shape in modulating osteogenesis. As an example, Xu et al. [87] compared varying shapes (square, triangular and parallelogram) of porous ceramic scaffolds and found out that highest ALP activity was observed in scaffolds with parallelogram shape. While it is not very clear why parallelogram shape demonstrated highest ALP activity, nevertheless, it would be interesting to understand this at the mechanistic level.

2.2.3 Fibrous Scaffolds

Fibrous scaffolds have been widely explored for bone tissue engineering [88] since they mimic the fibrillar extracellular collagen networks within the bone. They can be fabricated via electrospinning, self-assembly or phase separation method. Electrospinning is one of the most versatile techniques since it can be used to generate scaffolds with controlled morphology (nanofibre diameter and orientation) as well as porosity [89]. A study reported the effect of electrospun fibre diameter and orientation on differentiation of BMSCs for bone repair. Results showed that BMSCs demonstrated more elongated and spindle-shaped morphology on aligned fibres compared to random fibres. Further, aligned, submicron/micron-sized fibres (906 ± 178 μm) showed higher expression of osteogenic markers such as Osterix (OSX) and Runt-related transcription factor 2 (RUNX2) as compared to other test groups (random fibres of diameter 1,183 ± 174 μm, aligned fibres of diameter 404 ± 107 μm and random fibres of diameter 449 ± 96 μm) [90]. Few others have also reported the effect of fibre orientation on enhanced osteogenesis [91–93].

2.3 Role of Scaffold Functionalization

Scaffold surface plays a crucial role in cell–scaffold interaction. In order to enhance a scaffold's bioactivity, its surface is modified to incorporate specific functions such as small functional groups, growth factors, small peptide sequences or complex bioactive molecules [94–96]. In a seminal work by Benoit et al. [97], phosphate-functionalized polyethylene glycol (PEG) surfaces led to osteogenic differentiation of hMSCs, thereby demonstrating that simple functional groups can be used to control complex cellular events. To add to this, Arora and Katti [98] also showed that phosphorylation and polysialylation of gelatin led to enhanced mineralized and osteogenic differentiation, respectively, on murine MSC line, C3H101/2. In another

study, polyethyleneimine and citric acid-grafted 3-D-printed PLA scaffolds were subjected to simulated body fluid to generate calcium-deficient PLA-HAp. These scaffolds led to enhanced ALP activity and expression of various osteogenic markers as compared to PLA scaffolds demonstrating the role of surface modification on biological activity of PLA scaffolds [99]. Furthermore, conjugation of cell adhesion peptide Arg-Gly-Asp (RGD) to polymeric surfaces also modulates cell behaviour [100]. This was corroborated by a study performed by Chen et al. [101], wherein they investigated the osteogenic differentiation of human ESC-derived MSC (hESCd-MSC) on calcium phosphate cement–chitosan–RGD scaffolds. In vitro results showed significant attachment, proliferation and mineralization of hESCd-MSC when seeded on CPC–chitosan–RGD as compared to CPC–chitosan scaffolds.

Other moieties such as heparin have also been used to functionalize polymeric surfaces [102]. In one study, the effect of heparin functionalized chitosan scaffold on the activity of MC3T3-E1 pre-osteoblast cells was investigated. Result showed that scaffolds with covalently bounded heparin led to enhanced ALP and osteocalcin secretion in comparison to electrostatically bound heparin containing chitosan scaffold and heparin-free chitosan scaffold [103]. In another study, methacrylated polyethylene glycol diacrylate (PEGDA)/chondroitin sulphate-based hydrogels were subjected to chondroitin sulphate-mediated recruitment of ions like calcium and phosphate. Under in vitro conditions, human tonsil-derived MSCs seeded on biomineralized surfaces promoted expression of various osteogenic markers, and the hydrogel with 10% chondroitin sulphate demonstrated highest bone mineral density in critical-sized cranial defect model as compared to other conditions [104].

2.4 Cell Sources for Bone Tissue Engineering

Bone is a highly dynamic organ and comprises four active bone cell types—osteoblasts, osteoclasts, osteocytes and bone-lining cells [105]. Each of these cell types has a specific function contributing to the generation of a healthy bone, thereby suggesting that an ideal bone graft may require seeding of these cells on a scaffold. However, limited cell source is always a drawback for generation of such a graft [106]. Type of cells, their source and the protocol of cell seeding greatly influence the formation of any engineered tissue. Use of pre-differentiated osteoblasts from autologous source is the most obvious choice for bone tissue engineering, but their slow proliferation has led to use of alternate sources [107] which includes mesenchymal stem cells (MSCs) from bone marrow, adipose tissue and dental pulp, embryonic stem cells as well as genetically engineered osteogenic cells. A comparison between various cell sources has been depicted in Table 1. Clinical performance of these cells in bone tissue engineering would depend upon the ease of harvesting, in vitro expansion, in vivo osteogenesis, low/no immunogenicity as well as no transmission of pathogens. The following section will discuss the latest updates on various cell sources used in bone tissue engineering.

Table 1 Advantages and disadvantages associated with cell sources for bone tissue engineering

Cell type	Advantage	Disadvantage
Bone marrow-derived mesenchymal stem cells (BMSCs)	• High osteogenic potential • Highly stable in culture media, even a small number of cells can yield high quantity • Extensively studied	• Extracting procedure is highly invasive and painful • Low yield at isolation and high risk of contamination • Loss/decreased multipotency after extensive passages or when isolated from older age group
Adipose tissue-derived mesenchymal stem cells (ADSCs)	• Isolation is easy and less invasive • Cell yield is higher than bone marrow aspirate and better genetic stability • Low donor tissue morbidity	• High tendency of spontaneous differentiation into adipocytes
Dental pulp-derived mesenchymal stem cells (DPSCs)	• Highly proliferative with enhanced ability to differentiate into osteoblasts	• Low yield on isolation
Embryonic stem cells (ESCs)	• Highly pluripotent cells • Unlimited self-renewal capacity	• Formation of teratomas • Sufficient studies not available to enumerate stable and reproducible differentiation • Immune incompatibility • Ethical concerns
Induced pluripotent stem cells (iPSCs)	• Highly pluripotent cells with no ethical or immunological issues • Can be generated through any cell source • Highly patient-specific.	• Associated with tumorigenicity and spontaneous teratoma formation
Genetically engineered osteogenic cells	• Result in differentiation of engineered cells towards osteogenic lineage only and thus in enhanced bone regeneration	• Associated with immunogenicity, acute immunomodulatory effect and malignant transformation (due to uncontrolled insertional mutagenesis)

2.4.1 Mesenchymal Stem Cells (MSCs)

MSCs show various properties such as self-renewal, multi-lineage differentiation and immunomodulation. As a result, they demonstrate potential as a promising tool in cell therapy and tissue engineering applications [108–110]. Stability of MSCs in the culture medium, ease of preparation and their potential to differentiate into osteoblasts present them as one of the most suitable candidates in the field of bone regeneration. Different sources for MSCs have been used in the past; this section will discuss major sources such as bone marrow-derived, adipose-derived and dental pulp-derived MSCs.

a. Bone-Marrow-Derived Mesenchymal Stem Cells (BMSCs)

BMSCs have gained enormous success in the recent past and have been extensively explored from the pre-clinical perspective. As an example, Nassif et al. [111] seeded BMSCs on chitosan scaffolds that were pre-treated with dexamethasone, an osteogenic inducer, and showed that BMSCs had osteoinductive properties when implanted on chitosan scaffolds under both in vitro and in vivo conditions as compared to empty scaffolds. Another study involved the encapsulation of BMSCs in matrigels which were then loaded on 3-D-printed porous titanium scaffold (Ti6Al4V), in order to provide appropriate mechanical strength. In vivo results showed that the scaffolds with BMSC-loaded matrigels showed better new bone formation in rats with full thickness critical mandibular defects in comparison to the rats treated with locally injected BMSCs scaffolds and pure matrigel-loaded scaffolds [112].

Though BMSCs have multilineage potential, their ability to differentiate into osteoblasts is the highest [113]. However, harvesting protocols for these cells is quite invasive and is also associated with a high risk of contamination, especially if the isolation is done from a patient having any other disease. Further, the number of cells isolated varies from patient to patient and declines with the age of the donor [114]. Additionally, strength of the population obtained is also governed by the isolation technique. Therefore, other cell types have also been explored for bone regeneration.

b. Adipose Tissue-Derived Mesenchymal Stem Cells (ADSCs)

ADSCs have appeared to be a successful alternative to BMSCs primarily due to the ease of extraction, abundance, rapid in vitro expansion and better genetic stability under in vitro conditions [115]. Many studies have explored ADSCs in bone tissue engineering applications. As an example, human ADSC-seeded collagen/HAp scaffolds were evaluated for ectopic bone formation following subcutaneous implantation in mice in comparison to cell-free scaffold. The results indicated augmented calcium deposition and vascularization in ADSC-seeded scaffolds as compared to cell-free scaffolds and demonstrated potential in cases of elderly or those

with reduced regeneration capacity [116]. Another study compared the osteogenic capacity of human ADSCs and human osteoblast (HOB) cells on microchannel-patterned collagen–fibroin–elastin-like recombinamer (ELR) blend films. Although both ADSC and HOB-seeded constructs closely mimicked the ultrastructure of bone, ADSCs showed better osteogenic properties as compared to HOB when seeded on collagen–fibroin–ELR constructs, thereby demonstrating potential in bone tissue engineering [117].

ADSCs have appeared to be a popular source for bone tissue engineering applications. However, they demonstrate high tendency of spontaneous differentiation into adipocytes [118].

c. Dental Pulp-Derived Mesenchymal Stem Cells (DPSCs)

DPSCs have also been explored as an alternative to BMSCs due to enhanced proliferation rates. Moreover, DPSCs are a very homogenous population and have demonstrated enhanced ability to differentiate into osteoblasts, thereby demonstrating potential in bone tissue engineering [119]. In a study by El-Gendy et al. [120], potential of hDPSCs on 45S5 bioglass scaffolds to promote bone-like tissue formation under in vitro and in vivo conditions was tested. The authors found out hDPSCs promoted greater osteogenesis under basal as well as osteogenic conditions on 45S5 bioglass scaffolds as compared to cells grown on 2-D surfaces. Furthermore, peritoneal implantation of DPSC-seeded scaffolds demonstrated sporadic woven bone-like spicules as well as calcified tissue, showing potential in bone repair. Similar results have been reported in other systems; Petridis et al. [121] reported the healing response of cranial defects in rats when implanted with DPSC-seeded hyaluronic acid-based scaffolds. Results demonstrated superior bone regeneration in DPSC-seeded scaffold as compared to cell-free scaffolds. Even though DPSCs show potential in bone tissue engineering applications, full realization of clinical potential pertaining to DPSCs in bone tissue engineering requires the establishment of new strategies in this direction [122].

2.4.2 Pluripotent Stem Cells: Human Embryonic Stem Cells (hESCs) and Induced Pluripotent Stem Cells (iPSCs)

Pluripotent stem cells such as hESCs and iPSCs have also been explored as alternative cell sources for bone tissue engineering [123, 124]. In a study, Marolt et al. [125] showed that cultivation of hESCs on 3-D osteoconductive scaffolds in bioreactors (bioreactors are discussed later in this chapter) with interstitial flow of culture medium led to formation of compact, homogenous, stable bone-like tissue without differentiating into other lineages. In vivo implantation of engineered bone further resulted in maintenance and maturation of bone ECM without teratoma formation, a phenomenon constantly observed following implantation of undifferentiated hESCs. However, these cells exhibit ethical constraints since isolation of cells destroys the

embryo. Further, on implantation, the cells invoke infiltration of inflammatory cells and subsequent rejection [126, 127].

Recently, iPSCs have been widely explored as a prospective cell source for bone tissue engineering [128]. In a study, human iPSCs cultured in macrochannelled PCL scaffolds demonstrated enhanced osteogenesis under in vivo conditions as compared to cell-free scaffolds [129]. Similar results were obtained when iPSC-derived osteoprogenitors were encapsulated in self-assembling peptide nanofibre hydrogels, followed by their implantation in a calvarial bone defect rat model [130]. While the iPSCs appear to be a promising source for bone regeneration, there are certain concerns especially tumorigenicity and spontaneous teratoma formation [131]. To address this concern, Xie et al. [132] used a new class of iPSCs called the iPSC-MSCs that act as a source of MSCs and at the same time are less tumourigenic compared to iPSCs [133]. The cells outgrowing from embryoid bodies that were in turn generated from iPSCs were designated as iPSC-derived MSCs. The derived cells were then seeded on HAp/collagen/chitosan-based biomimetic nanofibres and investigated for their bone regeneration in cranial bone defects in rats. It was revealed that iPSC-MSC/HAp/collagen/chitosan demonstrated nearly two-fold higher bone mineral density compared to other groups, and the system could be used as the new-stem cell–scaffold system in the field of bone tissue engineering.

2.4.3 Genetically Engineered Osteogenic Cells

Although MSCs have been profusely used in bone tissue engineering, their entry into distinct lineages comes at the cost of high concentration of exogenous growth factors [134]. In order to overcome this, researchers are genetically engineering MSCs with certain bone-specific genes in order to induce differentiation into osteogenic linage only, thereby demonstrating potential towards enhanced bone regeneration [135]. In a study by Huynh et al. [136], MSCs were genetically engineered for over-expression of RUNX2 with concomitant SMAD3 knockdown, and cell-seeded PCL scaffolds were tested for differential matrix deposition potential. Interestingly, genetically engineered MSCs demonstrated enhanced mineral deposition while the unmodified MSCs demonstrated enhanced glycosaminoglycan deposition, thereby showing application towards the regeneration of complex tissues. In another study, Kuttappan et al. [137] investigated the bone regeneration capabilities, more specifically in repair of segmental defects by using bone morphogenetic protein 2 (BMP-2)-engineered MSC-seeded composite scaffolds. The authors demonstrated that BMP-2-engineered MSCs showed better new bone formation in critical-sized rat femoral segmental defects as compared to non-transfected MSCs. In yet another study, Kargozar et al. [138] tried to accelerate the bone regeneration capacity of BMSCs by transfecting them with a plasmid containing bone morphogenetic protein-7 (BMP-7)-encoding gene. Both the modified and unmodified cells were seeded on bioactive glass/gelatin nanocomposite scaffolds, which were then evaluated for osteogenic potential in calvarial critical-sized defect in rats. In vivo results showed that higher rate of osteogenesis was observed in the group of animals

implanted with modified BMSCs in comparison to cell-free scaffolds and group with unmodified BMSCs. Although genetically engineered osteogenic cells demonstrate enhanced osteogenic differentiation, they are associated with immunogenicity, acute immunomodulatory effects and malignant transformation (due to uncontrolled insertional mutagenesis).

Worldwide, series of clinical trials and studies are going on based on pluripotent stem cell-based therapies for the treatment of diseases such as mascular degeneration, and neurological disorders, haematological disorders and cardiovascular disorders; however, clinical application for bone regeneration is still underway [139, 140].

2.4.4 Co-culture Strategies for Bone Tissue Engineering

It is evident that bone is a complex construct having four active bone cell types, namely, osteoblasts, osteoclasts, osteocytes and bone-lining cells with well-defined functions, working in synchronization to achieve bone homoeostasis [105, 141]. Therefore, single-cell-type culture usually fails to mimic the bone microenvironment, thereby eliciting the need of co-culture strategies in bone tissue engineering. Moreover, an implant that is unable to generate vascularization inside the construct can lead to a necrotic graft further delaying the possibilities of clinical application.

In this regard, various co-culture models have been developed to understand the effect of the aforementioned cell types on bone regeneration [142]. As an example, Beskardes et al. [143] investigated the effect of perfusion co-culture based on osteoblast derived from MSCs and osteoclasts derived from THP-1, human acute monocytic leukaemia cell line on chitosan-HAp superporous scaffolds. On similar lines, Jeon et al. [144] recapitulated bone tissue remodelling by co-culturing osteoblast and osteoclasts derived from human iPSCs-MSCs and human iPSCs macrophages, respectively, on HAp-coated PLGA/PLA scaffolds. Subcutaneous implantation of the HAp-based 3-D co-culture model into the dorsal region of 6-week-old athymic female nude mice showed better bone-like tissue formation as compared to monoculture of iPSCs-MSCs.

Furthermore, once a tissue-engineered construct is implanted at the defect site, delivery of nutrients and oxygen poses a major challenge in success of a graft. Under in vivo conditions, it is the responsibility of the blood vessels to take care of exchange of nutrients and waste materials. Any tissue that is within 100–200 μm vasculatures would receive nutrients [145]. Therefore, establishment of pre-vascularized construct is the need of the hour and indeed is an important challenge [146]. In an interesting study, a 3-D co-culture system was established using DPSCs cultured within microcarriers and endothelial cells embedded in type I collagen. Co-cultured constructs demonstrated higher expression of osteogenic markers as compared to monocultures, thereby showing the potential of this system in vascularized bone tissue engineering [147]. In another study, Nguyen et al. designed an in vitro co-culture system to simultaneously culture hMSCs with endothelial cells by encapsulating them in collagen and/or alginate hydrogels and concluded that simultaneous co-culture on collagen hydrogel led to superior outcomes which were further augmented in tubular perfusion system (TPS) bioreactor [148]. Thus, development of such dynamic

platforms for pre-angiogenesis is beneficial and can be induced in the construct before in vivo transplantation towards better clinical outcomes. More details on dynamic bone tissue engineering are explained in the later sections.

2.5 Role of Growth Factors in Bone Tissue Engineering

Growth factors are endogenously produced large polypeptides and induce various cellular functions such as cell recruitment, their proliferation, migration and differentiation [149]. They also help in formation and maintenance of the newly formed bone tissue [150–152]. During bone fracture healing, associated signalling cascade can be broadly classified into inflammatory factors, angiogenic factors and osteogenic factors [153].

Inflammatory factors: Inflammation is the initial stage of bone fracture repair. This phase of healing comprises recruitment of inflammatory cells at the bone fracture site by pro-inflammatory signals, released by platelets [154]. Inflammatory cytokines trigger invasion by lymphocytes, plasma cells, macrophages and osteoclasts. Factors that play a key role during inflammation include interleukin-1 (IL-1), interleukin-6 (IL-6), tumour necrosis factor alpha (TNF-α), fibroblast growth factor 2 (FGF-2) and macrophage colony-stimulating factor (M-CSF) [155, 156].

Angiogenic factors: Vascularization plays a prominent role in providing oxygen, nutrients and regulatory factors apart from recruiting additional osteoblasts and promotes cell differentiation and endochondral ossification. Impaired or lack of blood supply during bone growth or repair results in tissue hypoxia, bone loss and ultimately in necrosis [157–159]. Hence, impaired blood supply is a major factor in reduced bone healing. Some key angiogenic factors involved in bone regeneration include platelet-derived growth factor (PDGF), bone morphogenetic proteins (BMPs), FGF and vascular endothelial growth factor (VEGF).

Osteogenic factors: Bone regeneration calls for recruitment of osteogenic progenitor cells which could differentiate into bone-forming osteoblasts. During ossification, osteoblasts secrete ECM molecules like glycosaminoglycans which interact with growth factors for modulating downstream signalling cascade [160]. Growth factors responsible for triggering the differentiation of progenitor cells into osteogenic lineages are PDGF, TGF-β, insulin-like growth factors (IGFs), FGF and BMPs. Of these, BMPs are the most widely studied for bone regeneration; BMP-2 and BMP-7 are FDA approved and used clinically for bone regeneration [161, 162].

In summary, in order to achieve effective bone formation, tissue engineering utilizes combination of progenitor cells on bioengineered constructs along with these bioactive molecules.

2.5.1 Delivery of Growth Factors

Considering the importance of growth factors in tissue regeneration, their delivery to the site of genesis is of utmost importance. Typically, growth factors do not act in an endocrine fashion; instead, they exhibit short-range diffusion through the ECM and possess short half-lives. Earlier studies involved introduction of growth factors either by direct injection or by systemic local administration. However, these led to suboptimal functioning of the growth factors since these biomolecules have a short half-life due to their rapid degradation in vivo. As a result, in order to maintain supraphysiological concentrations of certain growth factors such as BMP-2, high dose of recombinant BMP-2 is administered. This, however, is associated with cancer development in case of lumbar spinal arthrodesis [163]. Therefore, it becomes imperative to design delivery systems for sustained and controlled release of growth factors in order to reduce multiple administration cycles and associated clinical risks [164]. Depending on the site of delivery and biological requirement, an efficient growth factor carrier must have control over growth factor release kinetics which may be extended, multifactorial or sequential release [165]. As an example, delivery of BMP-2 requires initial burst release followed by slow and gradual release in order to improve bone regeneration [166]. Additionally, the delivery system must be capable of delivering physiologically relevant doses in absolutely targeted fashion while preserving the bioactivity of growth factors for prolonged time periods.

For successful delivery of the right dose and right type of growth factors, various strategies have been employed. These include physical entrapment of growth factors with the scaffold, covalent binding of growth factors to scaffold, affinity-based entrapment and growth factors incorporated within nanocarriers. Each of these has been discussed in the following subsections and depicted as a schematic in Fig. 3.

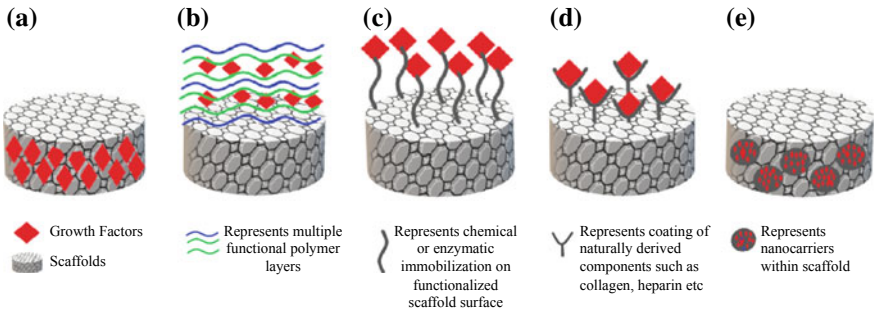

Fig. 3 Schematic depicting growth factor immobilization strategies in bone tissue engineering; **a** physical entrapment; **b** layer-by-layer approach; **c** covalent immobilization; **d** affinity-based binding; **e** growth factor-loaded nanocarriers entrapped within scaffolds

Physical Entrapment

In past few decades, physical immobilization of growth factors was limited to their adsorption over the scaffold surface. However, this was associated with poor delivery as described in a study by Ziegler et al. [167], wherein the authors immobilized BMP-2 and basic fibroblast growth factor (bFBF) directly on synthetic bone implants and found that the growth factors lost their biological activity after initial burst period. This drawback strengthened the requirement of immobilizing or entrapping growth factors within the polymeric carriers in order to obtain sustained release with improved biological activity. Thereafter, researchers encapsulated the growth factors within the 3-D constructs by blending them with the carrier polymers prior to fabrication. Physical entrapment of growth factors within the carriers usually does not affect the bioactivity of encapsulated growth factors or scaffold properties.

In a study, Murphy et al. [168] reported the release of VEGF from mineralized, porous PLGA-based scaffolds; VEGF was incorporated during gas foaming/particulate leaching process, and the study showed that VEGF activity was over 70% up to a period of 12 days and had no effect on porosity of the scaffolds. Growth factors can also be entrapped within microspheres to further delay their release. In one such report, Reyes et al. developed a brushite–PLGA system to study the release rates of integrated PDGF, TGF-β and VEGF with respect to bone regeneration. The system effectively controlled the release kinetics and maintained the concentration of growth factors at the site of defect, thereby promoting enhanced osteogenesis [169].

Direct adsorption also demonstrated difficulties in controlling the release rates of multiple growth factors. Therefore, researchers came up with a layer-by-layer (LbL) approach, wherein sequential deposition of multiple templates of various synthetic and natural polymers along with bioactive molecules is performed and can be employed for spatial and temporal release of growth factors [170, 171]. The strategy exploits electrostatic interaction between charged substrates and growth factors for the deposition of multiple functional polymer layers over a template [172]. The first LbL film capable of microgram-scale release of BMP-2 was developed by Macdonald et al. [173]. Briefly, they developed a tetralayer architecture using poly (β-aminoester), chondroitin sulfate and BMP-2. In vitro release kinetic studies demonstrated around 10 μg BMP-2 release over a period of two weeks, with less than 1% release in first 3 h when compared to the plain collagen matrices which delivered about a phantom of BMP-2 far quickly to stimulate osteoinduction. The system also retained its biological activity and induced bone differentiation in MC3T3 E1S4 pre-osteoblasts. This method can also be employed to deliver and tune the release of multiple growth factors within the LbL architecture [174]. The aforementioned studies demonstrate potential of LbL technique in precise control over release of multiple growth factors by using polymers with different degradation rates.

Furthermore, rate of release of entrapped growth factors is majorly governed by the polymer property, cross-linking and geometry of the carrier device and is both a diffusion- and degradation-dependent process [175, 176]. Although physical entrapment does not hinder the bioactivity of encapsulated growth factors, only those constructs that do not utilize harsh conditions during scaffold fabrication can be

utilized. Further, the loading efficiency is small; only a fraction of growth factors can be bound and have previously demonstrated unpredictable release kinetics. Release of growth factor is also dependent upon the degradation of encapsulating polymer. Thus, in order to control the release kinetics, polymers are functionalized so as to maintain sustained delivery [177].

Covalent Immobilization

This type of approach is generally adapted to promote systemic and prolonged release of growth factors to the cells [178]. Typically, the growth factors are bound to functionalized surfaces by chemical or enzymatic reactions, and their release is mediated by hydrolysis or enzymatic cleavage. Recently, Luca et al. [179] studied the effect of covalently linked BMP-2 and TGF-β3 on additively manufactured 3-D scaffolds which were modified with poly(oligo (ethylene glycol) methacrylate) brushes for application in osteochondral tissue regeneration. There was significant upregulation of osteochondral differentiation when the growth factors were homogenously linked to the substrate as compared to simple addition of growth factors in soluble form. However, they did not observe any effects when the growth factors were added in a gradient fashion suggesting further optimization of the system.

One of the most popular methods for covalent coupling is through carbodiimide coupling [180]. This has been utilized by Karageorgiou et al. [181] for covalent immobilization of BMP-2 on silk fibroin films. The authors showed that human BMSCs differentiated into osteoblasts when cultured on BMP-2 coupled silk fibroin films in comparison to unmodified silk films in presence of osteogenic stimulants, thereby concluding that the entrapped BMP-2 was more efficient than its delivery in soluble form.

It has also been demonstrated that dopamine can be easily introduced over any organic or inorganic material forming a polydopamine layer which is structurally very similar to 3, 4-dihydroxy-L-phenylalanine [182]. Using this idea, in a study, BMP-2 was loaded onto polydopamine-coated multichannel biphasic calcium phosphate granule system (PD-MCG), and the system showed sustained release of BMP-2 for 30 days. Pre-osteoblast MC3T3-E1 cells seeded on the dopamine-coated biphasic calcium phosphate system displayed enhanced differentiation, and in vivo implantation showed superior bone formation when compared with MCG system without dopamine coating and dopamine-coated MCG void of growth factor. The results demonstrated that PD-MCG could be used as an effective injectable bone substitute to promote new bone formation than those without dopamine coating [183].

While covalent conjugation has been widely explored for growth factor delivery, it can be quite labour intensive and may interfere with the active site of the protein leading to reduced bioactivity. Conjugated growth factors also demonstrate limited diffusion and hence are available only to proximally close cells.

Affinity-Based Binding

Inspired by ECM–growth factor interactions, another pattern for encapsulating growth factors within scaffolds is through the introduction of naturally derived components such as collagen, fibronectin, gelatin and hyaluronic acid as they provide specific biological site for immobilization of growth factors [184–188]. As an example, heparinized scaffolds explore the natural affinity of heparin sulphate and various growth factors such as FGF1, FGF2, VEGF and BMPs [189–191]. In one such example, Kim et al. functionalized PCL/PLGA scaffolds with heparin–dopamine conjugate followed by sequential coating with BMP-2 towards the investigation of osteoblast activity in vitro and bone formation in vivo [192]. It was found that BMP-2-loaded heparin–dopamine-functionalized scaffolds (BMP-2/Hep-DOPA/PCL/PLGA) showed enhanced ALP activity and calcium deposition with osteoblast-like cells in vitro as compared to BMP-2/Hep/PCL/PLGA and PCL/PLGA scaffolds. These results corroborated with better bone formation in vivo as well. In another interesting study by Martino et al. [193], delivery of PDGF-BB and BMP-2 from adhesive fibronectin fragment-functionalized fibrin matrix in a critical-sized calvarial model led to enhanced bone formation at low doses as compared to fibrin-only, fibronectin-functionalized fibrin matrix and fibrin matrix with growth factors.

This type of growth factor encapsulation mechanism protects growth factors from proteolytic degradation and helps them to maintain prolonged biological activity [194, 195]. Such systems are advantageous since there are no chemical treatments done during incorporation of growth factors as the modification of the scaffold is done prior to encapsulation.

Nanocarriers

Nanocarriers have become popular for growth factor delivery due to their high drug loading and retention capacity, large surface area and ability to protect encapsulated protein from in vivo enzymatic degradation [196]. Nanocarriers may be based on synthetic polymers, proteins, polysaccharides, lipids, silica or even nanocapsules. These can be synthesized using various methods like emulsion–solvent evaporation, phase separation, solvent displacement, self-assembly and electrospraying [197]. There are several studies which have reported the effectiveness of nanoparticles in growth factor delivery in context to bone regeneration [198, 199]. In a recent study, osteoinductive and compressive strength of 3-D HAp-based scaffolds was improvised by incorporating BMP-2-loaded nanoparticles. BMP-2-encapsulated PLGA nanoparticles were prepared by double emulsion–solvent evaporation method and were uniformly distributed on the scaffolds using PCL coating. The modified scaffold demonstrated improved bone regeneration capacity in rabbit calvarial bone defect model as compared to uncoated scaffolds.

2.6 Dynamic Environment for Bone Tissue Engineering

Cells in our body reside in a constant flux including the exchange of nutrients and gases (oxygen and carbon dioxide) between cells and the surrounding interstitial fluid. In bone tissue, the shear stress generated by the flow of interstitial fluid provides a mechanical stimulus. This interstitial fluid flow is generated through mechanical loading during movement and locomotion. It has also been demonstrated that longer periods of rest or inactiveness negatively affect bone formation and remodelling [200]. In vitro studies have determined that bone cells respond to the stimulus caused by fluid shear stress by releasing osteogenic factors such as prostaglandins and nitric oxide indicating the relevance of shear stimulus on bone formation [201].

Bone tissue engineering has been previously been reported by many researchers, wherein both static and dynamic conditions have been explored [202]. Static culture is usually performed in a suitable growth medium without any external stimulus or change in dynamics of the system. Under such culture conditions, transport of nutrients and oxygen happens through diffusion, and a diffusion gradient is generated since the cells at surface consume nutrients and oxygen at a faster rate as compared to their supply. Therefore, owing to mass transport limitations at the core of the scaffold, the cells present at the inner core regions do not receive sufficient amount of nutrients and oxygen, thereby leading to cell death and formation of a necrotic core [203]. As a result, a dynamic environment of mass transport and waste removal is not formed, and a physical stimulus similar to the flow of interstitial fluid during in vivo conditions is not present in static atmosphere. These limitations associated with static conditions do not provide a comprehensive and reliable analysis of cell behaviour on the scaffolds through in vitro analysis; therefore, these results cannot be entrusted for advanced analyses such as pre-clinical studies. Such limitations can be overcome by the use of dynamic environments for the cell culture on 3-D scaffolds and can assist in proving realistic answers to the drawbacks associated with the seamless upscaling of in vitro studies to in vivo animal models and thereafter to clinical applications. A comparison of static and dynamic culture is depicted in Table 2. A dynamic environment can either be created in vitro through the use of bioreactors system or by harnessing the in vivo environment to act as a bioreactor for the development of tissue-engineered grafts. Upcoming sections of this chapter will discuss the recent advances in the area of in vitro and in vivo bioreactors.

2.6.1 In Vitro Bioreactors

Bioreactors have been used in vitro for expansion of cells on a biomaterial scaffold before in vivo implantation. They allow in vitro culture of cells in a dynamic environment which can be monitored to ensure maximum cell growth under the influence of shear stimulus as well as uniform supply of nutrients and oxygen throughout the scaffold along with the removal of waste products. It has been experimentally demonstrated that shear stress affects the differentiation of MSCs into osteoblasts

Table 2 Comparison between static and dynamic culture

Property	Static culture	Dynamic culture
Growth	Non-uniform cell growth	Uniform cell growth
Mass transport	Mass transport purely through diffusion	Mass transport through flow currents generated via shear stress
Monitoring of culture conditions	Monitoring cannot be performed	Such system can be monitored for flow velocity, inflow of growth medium and outflow of waste
Closeness to in vivo environment	Higher disparity from in vivo environment	Closely mimic in vivo environment
Scalability	Does not provide scalable results for follow-up in vivo studies	Provide scalable results for follow-up in vivo studies

[204, 205]. Shear stress is mainly caused by the flow of liquid medium and can be manipulated by the change in flow velocity, wherein the flow velocity is directly proportional to the shear stress on the cells. Three kinds of flow patterns have been analysed for shear stress including pulsatile, oscillatory and continuous flow. Of these, continuous flow pattern is most utilized for bioreactor studies. The fluid flow velocity and shear stress can be determined by optical measuring techniques such as particle image velocimetry or through computational fluid dynamic (CFD) modelling like finite element analysis and Lattice–Boltzmann method [206, 207]. There are multiple kinds of bioreactors based on the technique applied to create a fluid flow across and around the 3-D scaffolds. Few of these bioreactor types are discussed below and represented in Fig. 4d [208].

Spinner Flask Bioreactors

In order to overcome the limitations associated with static culture, spinner flask bioreactors were introduced to cause a convective flow of media through hydrodynamic forces generated by the spinning of a magnetic stirrer rod placed at the bottom of a cylindrical flask having side arms with filter cap for removal/addition of media or cells. The scaffold is attached at a fixed position through threaded needles which in turn in connected to the top of the container [209, 210] as shown in Fig. 4d. The shear stress caused by convective flow depends on the stirring speed; a study used 30 rotations per minute (rpm) as the stirring speed of the medium in a 120-ml flask, and it was observed that spinner flask culture showed 60% enhanced proliferation at first week and 2.4 times higher ALP activity at 2 weeks in comparison to static culture [211].

In another study, speed of 50 rpm depicted a positive response on osteogenic activity [212]. These are the simplest form of bioreactors, and several studies have

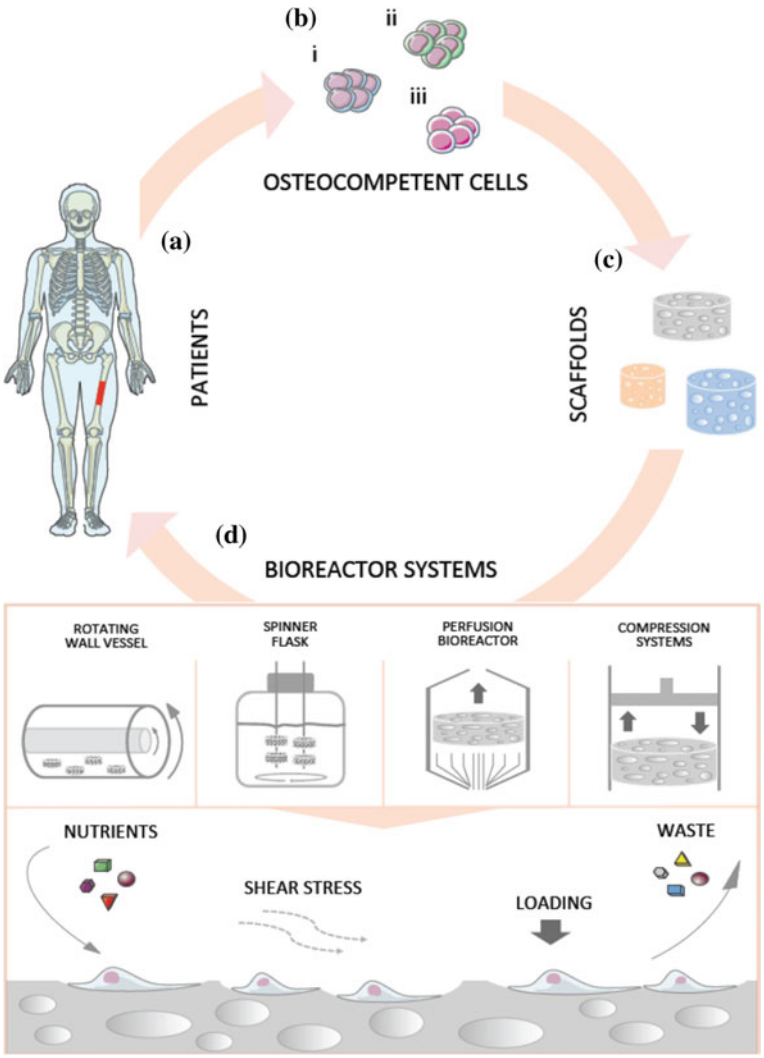

Fig. 4 Schematic representation of bone tissue engineering paradigm using in vitro bioreactors: **a** patient having a bone defect shown in red colour; **b** osteogenic cells having patient specificity and derived from sources like (i) adult tissue, (ii) induced pluripotent stem cells or (iii) blastocysts generated via somatic cell nuclear transfer; **c** cell-seeded biomaterials scaffolds of different dimensions and porous architecture; **d** dynamic culture of cells on the porous biomaterial scaffolds in different types of bioreactor set-up for the development of bone tissue-engineered grafts. These include **rotating wall vessels**, **spinner flask bioreactors,** p**erfusion bioreactors and compression stimulation-based bioreactors.** The function of these bioreactors is to ensure efficient mass transport of nutrients as well as oxygen and to ensure functional regeneration under the effect of mechanically stimulated environment

confirmed the utility of such systems in comparison to static culture for bone formation. Another interesting study by Kim et al. [213] performed on porous silk scaffolds demonstrated elevated levels of ALP and mineralized matrix along with increased cell proliferation during dynamic culture in spinner flask as compared to static culture. Despite being useful for dynamic culture of cells, spinner flask culture may be limited by non-homogeneity of hydrodynamic forces due to high to low gradient of the flow effect from bottom to top of the flask. Another limitation includes formation of a dense cell layer on the outer surface of the scaffold [214].

Rotating Wall Vessel (RWV) Bioreactors

RWV bioreactors are primarily composed of a cylindrical vessel that rotates along a central axis, and this rotational movement produces low shear stress that is sufficient enough to positively affect cellular activities including proliferation and differentiation. As shown in Fig. 4d, the scaffold inside such rotating chambers can be either present freely, attached to the vessel wall or present as circular discs along the horizontal axis.

Numerous studies have shown that RWV bioreactors show favourable response towards bone formation. A study by Song et al. [215] reported that RWV bioreactors with fixed scaffold set-up (scaffolds attached on vessel wall) showed five times higher cell expansion in comparison to stirrer flask bioreactor and static culture. The cells also showed significantly higher mineralized nodule formation, collagen fibres and neo-osteoid tissue formation with respect to stirrer flask bioreactor and static culture. However, in certain studies, lower values for osteogenic markers were found as compared to spinner flask bioreactor or static culture [216]. This may be attributed to either the collision of free floating scaffolds or due to lower values of shear stress causing insignificant stimulus to the cells.

Perfusion Bioreactors

An improved method of dynamic cell culture using hydrodynamic shear stress was developed in the form of perfusion bioreactor [217]. Instead of indirectly applying shear forces towards the scaffold through spinning motion (spinner flask bioreactor) or through rotation (RWV bioreactor), perfusion system directly applies a laminar fluid flow through the scaffold which enables efficient mass transport of nutrients and oxygen throughout the scaffold. As shown in Fig. 4d, a perfusion bioreactor set-up comprises a closed loop of media flow through the scaffold via the assistance of a peristaltic pump. Inlet and outlet ports are also connected to this system for media replenishment and waste removal, respectively. There can be two kinds of flow-perfusion bioreactor systems, indirect and direct perfusion systems. In indirect perfusion, the scaffold inside the cassette is not sealed tightly, allowing most of the media fluid to pass through the path of least resistance, i.e. around the scaffold, although some fluid also passes through the scaffold. Thus, shear stress caused by the

fluid flow is not able to reach the core regions of the scaffold although studies have shown that such systems have been able to provide a favourable osteogenic response as depicted by increase in ALP and osteocalcin protein expression levels [218]. In direct perfusion, scaffold is placed in another cassette that is tightly sealed inside the chamber in a press-fit manner, thus forcing the fluid inflow through the scaffold [219]. The mass transport can easily happen inside the direct perfusion bioreactors, therefore providing better results in comparison to indirect perfusion. Such perfusion bioreactor systems can assist in the development of large tissue-engineered scaffolds for bone applications without formation of a necrotic core [220].

Stimulation-Based Bioreactor Systems

A dynamic set-up wherein stimulation to osteogenic cells is applied through forces such as mechanical, electromagnetic or ultrasonic stimulus can be considered as stimulation-based bioreactor systems. These are discussed as follows.

Compression-Based Bioreactors

Mechanical loading and unloading can be sensed by the osteoblasts and osteogenic cells through a phenomenon called mechanosensing [221]. Therefore, scaffold deformation and relaxation through mechanical loading and unloading are sensed by the cells, indirectly via extracellular matrix or directly by change in cell–cell distance or cell shape. This may further modify the local cell environment via change in extracellular gradients or concentration of secreted ligands/growth factors. The compression bioreactor set-up mainly comprises of a cell-seeded scaffold present below a loading piston in which unidirectional load can be applied as static or dynamic compression [222] as shown in Fig. 4d. Several studies have demonstrated the importance of compression loading for bone formation/regeneration. As an example, Matziolis et al. [223] demonstrated that even short-term mechanical stimulation, involving a 24-h cyclic load of 4 kPa and 25% strain at 0.05 Hz, was sufficient to enhance bone formation during culture of human BMSCs on human cancellous bone–fibrin composites inside a compression bioreactor. Another study was performed on biodegradable cryogels composed of L-lactide and dextran with 2-hydroxyethyl methacrylate end groups. These cryogels were seeded with human osteoblast-like cell line, and studies were performed in compression bioreactor with mechanical stimulation at 1.5% strain and at a frequency of 1 Hz for 1 h/day. This study also suggested a positive role of mechanical stimulation in osteogenesis [224].

Electromagnetic Field (EMF)-Based Bioreactors

Electric and magnetic stimuli have been shown to assist in bone formation and fracture healing since decades [225]. Pulsed electromagnetic fields (PEMF) have been utilized in multiple cases of reduction in bone loss following a fracture [226]. In our body, EMFs and PEMFs are generated during muscle movement. EMFs affect

different cellular pathways involved in proliferation and differentiation of cells [227]. The EMF bioreactor set-up for bone tissue engineering applications is essentially composed of Helmholtz coils and PEMF power generator. The cell-adhered scaffolds are placed between the two coils inside a sealed chamber, and electromagnetic field of required intensity is applied. It has been demonstrated through various studies that EMFs induce and promote osteogenesis in MSCs [228] and osteoblasts [229], respectively. In a representative study, Fassina et al. [230] used an electromagnetic bioreactor with a stimulation regime of 2 ± 0.2 mT intensity of magnetic field and an electric tension of amplitude $= 5 \pm 1$ mV, frequency $= 75 \pm 2$ Hz and pulse duration $=$ 1.3 ms. The Saos-2 human osteoblasts were cultured in porous polyurethane scaffold under the EMF stimulation. Encouraging results were obtained in form of higher cell proliferation and increased coating with decorin and type I collagen along with increased calcium deposition. However, the major disadvantage associated with such system is high cost for set-up, although their advantage lies in the ease of handling due to non-invasiveness and higher possibilities of good manufacturing practice (GMP) approval.

Combined Stimulation-Based Bioreactors

In certain bioreactors, two or more types of stimulation methods are applied to enhance the osteogenic response of cells. Some examples of such combined stimulation-based bioreactors include perfusion–compression bioreactor system [231, 232] and EMF-compression-ultrasonic bioreactor system [233]. The cumulative effect of different kind of stimulation increases the osteogenic response as compared to individual stimulation effects due to closer mimicking of native environment of the body, wherein several factors work together to stimulate the cells in order to enhance osteogenesis.

2.6.2 In Vivo Bioreactors

In comparison to static methods of culture, in vitro bioreactors are one step closer to the final goal, viz. regeneration of human bone. These bioreactors provide important cues that assist in mimicking the native bone environment; although they can still not match the native environment containing a plethora of pathways and mechanisms working towards cell fate processes. Therefore, in vivo bioreactors have been developed as a recent technology in an effort to provide native environment to the cell–scaffold system, wherein the body of the organism acts as a bioreactor for efficient bone formation using body's own reparative capability [234, 235]. The beauty of such bioreactor system lies in minimal dependency of the cell/scaffold construct on exogenous growth factors, stimulation factors or media supplements since all these requirements can easily be obtained locally from the body of the organism. The term in vivo bioreactors was first introduced in the year 2005 independently by two different research groups. Depending on the model organism being used,

studies performed using in vivo bioreactors can be categorized to three types: (a) small animal models, (b) large animal models and (c) clinical studies. These are further discussed as follows:

Small Animal Models

The pioneering studies in the area of in vivo bioreactors were performed in small animal models such as mice, rat and rabbit [236]. In a study by Stevens et al. [237], they demonstrated that an engineered bone having biomechanical properties identical to that of the native bone could be developed by using a "bioreactor" space created in vivo between the tibia and periosteum of New Zealand White rabbits. The engineered bone was harvested after 6 weeks, and thereafter, it was implanted as autologous bone transplantation in contralateral tibial defects leading to complete integration. Another successful study was performed by Holt et al. [238] in a rat model, wherein neovascular in growth and bone formation was achieved by ligating the superficial inferior epigastric vessels through a cylindrical coralline scaffold. In another study, pedicled periosteal flap was utilized as an in vivo bioreactor for development of a vascularized bone graft in a rabbit model [239]. In another in vivo bioreactor study in a rabbit model, tibial periosteum capsule was loaded with 3-D-printed PLA-HAp composite scaffolds. Bone marrow stromal cells were seeded on these scaffolds, and cell-seeded scaffolds were further connected through a vascular supply as illustrated in Fig. 5 [240]. Although these models paved a way for in vivo bioreactors as a new strategy, the scaffold size was too small to understand the complications of large bone implants as applicable under clinical set-up. As a result, large animal models were sought after, as a strategy for advanced studies on in vivo bioreactors.

Large Animal Models

As mentioned previously, large animals like minipigs, sheep, and non-human primate models were used for in vivo bioreactor studies in order to avoid the limitations associated with small animal models. Though non-human primates have many advantages that make them the best models for in vivo bioreactor studies, the associated costs and regulatory concerns limit their usage for broader applications. Akar et al. [241] have listed the prerequisites involved with the use of large animals as the model for in vivo bioreactor studies. As per this list "an in vivo bioreactor should: (1) mimic the clinical surgery techniques; (2) allow evaluation of vascularized bone formation of large volume and complex shape; (3) have an implantation site with high regenerative capacity and low infection risk; (4) be adaptable for different tissue engineering components; (5) allow quantitative evaluation of results; and (6) be available/adaptable in a wide range of clinical research centres". It has been evaluated that ovine (sheep) and porcine (pig) periosteum-guided models fulfil these criterion and therefore have been studied as large animal models for in vivo bioreactor

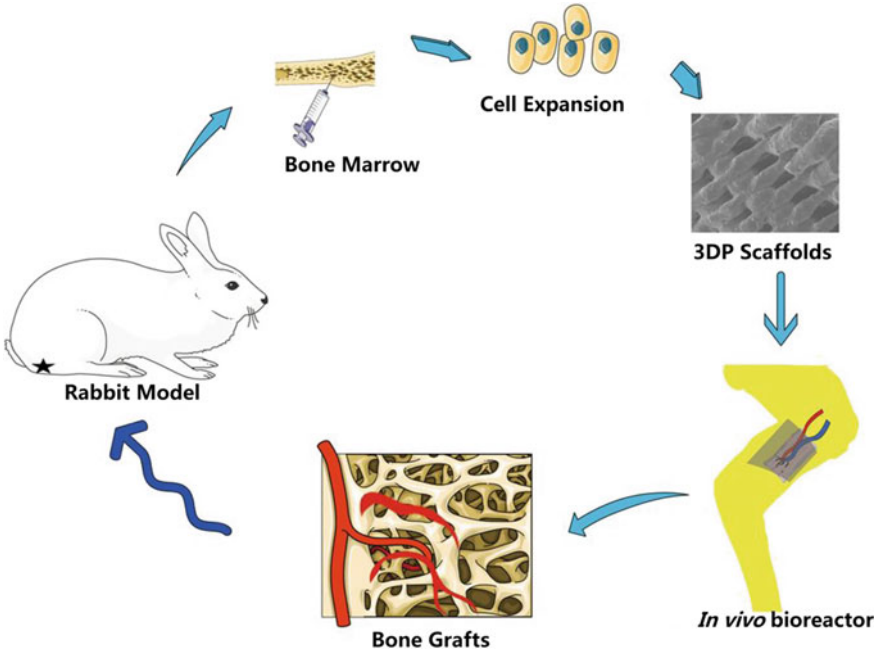

Fig. 5 Schematic representation of in vivo bioreactor culture of three-dimensionally printed (3-DP) polylactic acid-hydroxyapatite composite scaffolds in a rabbit model. Bone marrow stromal cells were first expanded and then seeded on the 3-DP scaffolds followed by in vivo bioreactor culture in tibial periosteum while being connected to a vascular supply. Such an in vivo bioreactor set-up leads to the formation of vascularized bone tissue

applications. As an example, Cheng et al. [242] observed active bone formation leading to efficient vascularized graft development in an ovine model. In this study, poly (methyl methacrylate) chambers were implanted around rib periosteum. Both muscle fascia and periosteum have also been used as an in vivo bioreactor site, though it has been observed that periosteum serves as a better site in comparison to muscle fascia [243]. In another study, a sheep model implanted with particulate autologous bone graft was studied for development of bone segments via in vivo implantation at the site of rib periosteum [244]. Thus, large animal models help in the understanding the procedure for bone graft development using in vivo bioreactors with the final aim for clinical translation of the strategy.

Clinical Studies

Human body has been used as an in vivo bioreactor in certain studies although such applications are limited in numbers. Warnke et al. [245] used human body as a bioreactor to grow bone on a titanium mesh cage containing bone mineral blocks and BMP-7 along with bone marrow from the patient. These constructs were first

implanted at the in vivo bioreactor site for 7 weeks, i.e. latissimus dorsi muscle followed by transplantation for repair of mandibular defects in the same patient. The outcome was favourable as the patient's mastication was improved along with the aesthetics. In another study, a similar scaffold construct was implanted at gastrocolic omentum as the in vivo bioreactor site, before transplantation in mandibular defects. Encouraging outcome was observed in form of better mastication, speech and aesthetics [246]. Some of the points to be taken into consideration for future clinical studies are: (1) evaluating the timing for removal of bone graft from the in vivo bioreactor site or the timing of transplantation at the defect site and (2) involving techniques like microcomputed tomography (micro-CT) and computer-aided design (CAD) to develop custom-designed patient-specific scaffold grafts [247].

2.7 Conclusion and Future Perspective

Bone tissue engineering has experienced significant advancements in the recent past, especially in the area of scaffold fabrication, growth factor delivery strategies, usage of cell type as well as bioreactors. More specifically, scaffold fabrication for bone tissue engineering has experienced efforts in the kinds of polymers/metals/ceramics, scaffold architecture as well as surface functionalization. Efforts have also been taken in the direction of growth factor incorporation and cell types utilized. Furthermore, there have been some very interesting reports on the application of dynamic environments (in vitro and in vivo bioreactors). However, very few of these products have reached the clinic. This could be attributed to the uncertainties associated with recapitulation of complex bone environments (physical, mechanical as well as biological properties) within the 3-D matrix. Another critical challenge is the development of a fully vascularized bone graft. This can be achieved by either fabricating scaffolds with large, interconnected pores and also by incorporating angiogenic growth factors. An ideal scaffold that can make a mark in the clinic will be a patient-specific bone graft. More specifically, techniques like 3-D printing may be utilized to print a patient-defect site-specific biomaterial embedded with progenitor cells. The biomaterial could then be subjected to bioreactor-based culture followed by implantation of the patient-specific graft at the defect site.

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