In-Vitro and In-Vivo Tracking of Cell-Biomaterial Interaction to Monitor the Process of Bone Regeneration

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15.1 Introduction

Skeletal structure, major part of which is composed of bone, provides the mechanical support, aids in haematopoiesis, acts as the primary storage of mineral as well as enhances mobility coordinating with muscles in vertebrate animals including human (Terranova et al. 2016). This multifunctional tissue is highly sensitive towards wide variety of bio-chemical and bio-mechanical stimuli due to which preservation of structural integrity often needs well distribution of stress/strain caused by mechanical loading and weight-bearing physical activities (Bailey and McCulloch 1990). Having the natural capability for regeneration and repair, small wounds in the skeletal structure often do not need any clinical management. Large segmentation non-union bone fractures, in contrast, can only be healed either by grafting artificial bone substitutes or placing titanium, zirconia or other bio-inert metallic implants as the 'gold standard' of such therapy (Yang et al. 2013; Montazerolghaem et al. 2016). In spite of increasing demand of biomaterials worldwide, many patients suffer from unavailability of such technologies which eventually force them to choose the cost-effective option such as limb amputation requiring long-term hospitalization indirectly causing a negative impact on the socio-economic status (Wang et al. 2014).

Bone, having a multi-scale structure, is primarily divided in two parts among which cortical bone creates the dense, non-porous external surface of the bone and comprises of about 99 and 90% of total available calcium and phosphate of the human body. In contrast to that cancellous bone is distributed throughout the core of the bone with interspersed haematopoietic stem cells, adipose tissues, blood vessels and spanning lamellar trabeculae. Although the trabecular or cancellous bone

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comprises about 20% of the total weight of the bone, effective surface area is much higher compared to cortical bone possibly owing to its superior porosity (McKittrick et al. 2010). Difference in age, genetic diversity, inherited unique biological features, defect geometry all together influence in variation of structure and composition of bone thereby requiring different requirements to be fulfilled for effective bone regeneration. In lieu of this, biomaterials guide the progenitor cells to fill the bone defects by restoring the structure, function of bone and type of cells present in the native bone. Ideal biomaterial should not only mimic the biological and mechanical performance of the bone but also should meet the requirements of being non-toxic, in-vivo biodegradable, bioactive. Moreover, excessive immunogenic reaction against implantation of such foreign bodies should not cause hindrance to apposition of new bone, matrix mineralization. Tunable properties in implants/scaffolds such as nano- or micro-structuring help to alter cellular morphology as required for cellular attachment and migration on the non-porous implants. The multi-scale porous scaffolds whereas provide sufficient space and microenvironment requisite for tissue in-growth through pores while maintaining regular supply of oxygen, nutrient through blood vessels, nerve conduction and deposition of minerals (Fedorovich et al. 2011). Due to patient-specific difference in site and structure of native bone and bone defects, interior and exterior structure of the implants should be customized to fit in to the defect site to render early primary stability (Saiz et al. 2013). This approach has been found to encourage cellular interaction resulting in successful artificial-to-biological translation (Hench 2015; Knight and Hankenson 2013). This necessitates the tracking of the in-vitro and in-vivo biological response at host-implant interface to ensure complete integration of tissue in and surrounding the biomaterial without causing incompatibility as summarized in the following sections of this article.

15.2 Significance of Tracking Bone-Biomaterial Interaction

The bone regeneration and reconstruction were initially started with implantation of autologous bonegrafts collected from ribs, calvarium, iliac crest from patient's own physiological system (Frohlich et al. 2008; Zomorodian and Baghaban 2012; Romagnoli and Brandi 2014). The use of autografts was still limited by restricted access as well as chronic pain at the site of tissue collection and potential risk of infection at the donor site despite some positive aspects including low immunogenicity (Romagnoli and Brandi 2014). The autografts and allografts moreover may only be considered as osteogenic to initiate osteogenesis and neo-vascularization provided that the host-graft interface is sufficiently stable and macroporous with optimal presence of pre-osteogenic cells at host bed (Delloye et al. 2007). Often the terminology 'osteogenic' creates confusion with other two related terms 'osteoinductive' and 'osteoconductive' both of which bears separate meaning. Cells can migrate from the host to osteoconductive scaffolds to be proliferated and differentiated further in to matured osteoblasts leading to ingrowth of bone whereas the scaffolds only can induce in-vivo osteogenecity when preserved with

osteoinductive factors such as transforming growth factor-beta (TGF-β) type 1 or 2 capable to differentiate mesenchymal stem cells, i.e., osteoprogenitor cells to mature osteoblasts (Urist 1965). With expanding clinical requirement, the research paradigm on bone reconstructing scaffolds has immensely shifted towards more 'bioactive' materials with capability to integrate with surrounding bones which will be eventually resorbed and replaced by body's own tissue with minimal dissimilarity at a time-dependant manner (Stevens 2008). Despite several proofs of similarities of inorganic or organic biomaterials in providing structural support, concerns exist predominantly over the insufficient toughness and mechanical stability provided by both the polymeric and metallic scaffolds especially for load-bearing applications (Kokubo 1995). Inorganic biomaterials such as tricalcium phosphate, bioactive glass have the ability to precipitate naturally mimicking hydroxyapatite (HA) in-vitro and in-vivo in presence of biological fluid. These are found to stimulate complex osteogenic gene transduction network, although they are unable to provide adequate mechanical strength due to the brittle nature especially of the bioactive silica glass even after enforcing crystalline HA in to their molecular structure (Kokubo 1995; Tsigkou et al. 2007; Jell and Stevens 2006).The rate of resorption can, however, be manipulated by simultaneously tailoring the surface chemistry, topographical features and controlling the in-vitro and in-vivo release of ions from the scaffolds in to bone microenvironment. Unlike those materials, the natural (collagen etc.) and synthetic polymers (polycaprolactone, copolymers of polylactic acid, and polyglycolic acid) can be utilized in fabrication of three-dimensional (3D) scaffolds with controllable porosity, topology and cross-linking density employing several conventional and advanced layer-by-layer manufacturing methods including computed tomography (CT) scan-guided computer-aided 3D printing (Hollister 2005). Majority of the biomaterials are being developed to mimic the natural architecture of bone which is composed of compact central cortical region with recapping osteon surrounded by spongy shock-absorbable trabecular bone with interspersed hydroxyapatite crystals and collagen fibres thus making it strong, stiff and mechanically stable (Shao et al. 2022). Poor mechanical properties thereby elicited by the polymers somehow can be improved by incorporating inorganic materials to mimic Young's modulus similar to native bone without compromising interwoven network formation by the pores while maintaining optimum degradation rate, bioactivity and viscoelastic properties (Filippi et al. 2020). Literature review also revealed improved osteogenic potential of surface modified biomaterials compared to that of unmodified materials as mentioned by Kazimierczak and Przekora (Kazimierczak and Przekora 2020). Apart from inorganic coatings, plasma modifications, hydrothermal and laser sintering methods, alteration of surface chemistry by incorporation of combinations of few among fibroblast growth factor (FGF), TGF-β, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), bone morphogenetic protein (BMP) type 2 or 4 in to scaffold are reported to induce superior osteogenic gene expression simultaneously accelerating angiogenesis and osseointegration at the implantation site (Przekora 2019). This approach probably provides cues directly to stimulate

innate biological response and chemotactic cell behaviour aiding cell adhesion, proliferation and differentiation (Alam et al. 2007).

All of these above-mentioned materials and their composites have tried to create a niche cellular microenvironment to meet the necessary features applicable for bone repair since long but all the approaches have not been able to achieve that huge diversity. Moreover, lack of clarity in elucidating detailed molecular mechanism involving cell-ECM interaction, cell-cell interaction and cell-scaffold interaction make it difficult to explore the novel theoretical and practical directions to achieve successful biological translation (Gao et al. 2017). A few experimental demonstrations can be obtained from literature in this regard to identify some of the associated pathways involved in osteogenic differentiation of osteoprogenitor stem cells. Among all, TGF-β/BMP pathway gets activated through binding of TGF β-1 to the respective receptor followed by TGF β-2 binding, autophosphorylation and recruitment of SMAD proteins to the receptors. This leads to differentiation of undifferentiated stem cells to mature osteoblasts by inducing chemotaxis of the stem cells to the extracellular matrix (ECM) and the ending of the process is marked with deposition of mineral as well as collagen synthesis in the matrix (Gao et al. 2017). Similarly the role of Wnt/β-catenin signalling pathway, Hedgehog signalling pathway, Notch signalling pathway, MAPK signalling pathway in activating early and late stages of osteogenic differentiation in response to several stimuli also has been explained in various in-vitro and in-vivo studies (Engin et al. 2008; Delpiano and Acker 1985; Shi et al. 2015; Wang et al. 2016; Yuan et al. 2016; Hu et al. 2013). Variation in the physical parameters such as porosity irrespective of being micro or nanosized has been found to significantly promote protein adsorption, focal adhesion, cell viability and upregulate osteogenic gene expression accentuated by the large effective surface area and porous volume. This necessitates the detailed study of cell-biomaterial interaction apart from conventional tracking of biomarkers (Gao et al. 2017). However, the major concern lies in understanding the complex bone regeneration modulated by a combination of growth factors, inflammatory modulators, that is, cytokines released in the diversely populated bone microenvironment. This further necessitates understanding the effect of administered active biomolecules or drugs in the bone remodelling process either positively or negatively. Since the introduction of several molecules sometimes may destroy the natural bone healing phenomenon by disrupting homeostasis between growth factors/cytokines.

15.3 In-Vitro Evaluation Methods

Mediated by ligand-integrin receptor binding, the bone tissue engineering scaffolds strongly influence the adhesion and migration of surrounding cell niche consisting of adult stem cells, pluripotent stem cells, umbilical cord blood mesenchymal stem cells, embryonic stem cells leading to in-growth of tissues through the porous 3D architecture of the scaffolds. The adipocytes, in addition to those cells, result in the formation of microvascular networks albeit it takes days even weeks to transform in

to fully functionalized vascularized bone tissues (Kang et al. 2015). Vascular smooth muscle cells or pericytes whereas are required for structural stabilization of newly developed endothelialized blood vessels in the bone (Neff et al. 2011; Bergers and Song 2005). Cell-biomaterial interaction directly modulates these cytoskeletal activity, matrix modelling and cellular contraction requisite for fracture healing (Friedl et al. 1998; Yannas et al. 1989). Modification of scaffolds by alteration in the concentration of adhesive ligands or polymers (natural or synthetic) has been found to significantly influence the strength of cell-scaffold interaction while causing differential integrin binding according to the availability of receptors (Murphy et al. 2013). This phenomenon was explained by a previously published literature where comparatively less compact and mechanically stable gelatin methacrylate scaffold (fabricated with 5% gelatin) with low compressive modulus exhibited superior osteogenecity compared to those fabricated with high concentration of gelatin (Celikkin et al. 2018). Therefore, the optimization of cellular interaction at cell-scaffold interface is necessary utilizing the standard procedure to evaluate stimulation of cellular adhesion, migration, proliferation and differentiation of osteoprogenitor cells towards maturity. All the established in-vitro assays are summarized in Fig. 15.1.

15.3.1 Cytotoxicity and Cell Metabolic Activity

According to the guidelines framed by International Standard ISO 10993-5:2009, direct as well as indirect contact approaches are employed to evaluate 'biocompatibility' of biomaterials with an aim to protect human from potential biological risk arising from medical devices. In-vitro cytotoxicity therefore inspects the impact of the developed material towards targeted cells cultured and maintained in appropriate conditions. Direct contact approach involves culturing of cells seeded on scaffolds or implants followed by fluorescence microscopy-based observation of live cells tagged with Calcein (excitation/emission at the wavelength of 495/515 nm), a specific fluorescent dye for living cells and dead cells tagged with propidium iodide (excitation/emission at the wavelength of 560/720 nm), another specific dye for dead cells, although the evaluation is seemingly qualitative (Lagonegro et al. 2017). Quantitative measurement from the same experiment can be done by measuring the total area covered by the cells normalized to that covered by the cells cultured on negative control, that is, without any biomaterial. The pattern of cell growth although can depend on the type of substrate as it has been commonly found that cells are grown in clusters when cultured on flat surfaces while giving a confusing estimation about the cellular coverage measured by the fluorescence assay (Lagonegro et al. 2017). Literature also showed that an optimum gap of 50–70 nm between integrin binding motifs can aid in formation of focal adhesion and cellular attachment which can be inhibited once the spacing exceeds 100 nm usually observed with nano-structured biomaterials eventually leading to cell death (Cavalcanti-Adam et al. 2006; Bershadsky et al. 2006). Indirect contact test on the other side analyses the effect of release of cytotoxic agents from the material of interest. Briefly, the extracts

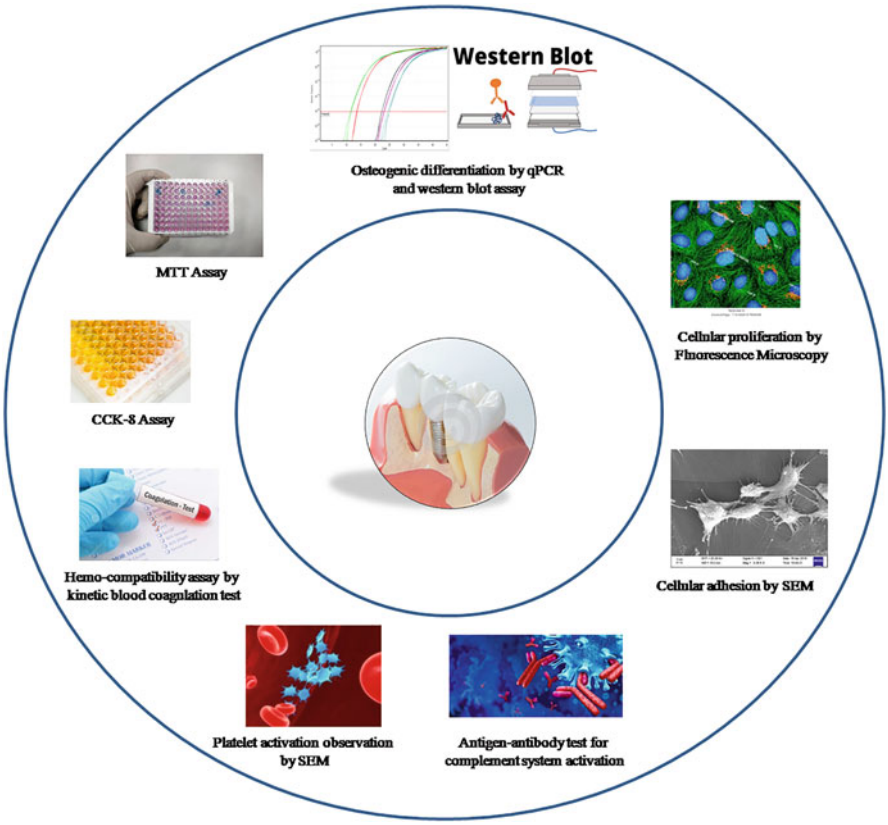

Fig. 15.1 Summarized representation of in-vitro assays to evaluate cell-biomaterial interaction

are prepared by immersing the materials in pristine culture medium to develop the conditioned medium. That conditioned medium is added then to the fresh culture medium at increasing concentration followed by culturing of targeted cells for a short duration, that is, 24 h. Cell viability is further determined following the standard protocol of MTT colorimetric assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye). The outcome may be further validated by several independent methods including chemiluminescence while quantifying the intracellular ATP present in metabolically active cells. Cell counting kit-8 also may be used in this regard where the incorporating reagent WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) gets bioreduced in exposure to cellular dehydrogenase secreted from the metabolically active cells. This in turn produces formazan product, on solubilization of which in aqueous cell culture media gives orange colour and therefore can be detected by spectrophotometry (Tong et al. 2016).

One of the major drawbacks associated with the indirect test is lack of clarity in revealing the reason of cytotoxic effects caused especially by calcium phosphate-based biomaterials. These apparently lead to reduced cell viability owing not only to the release of ions due to spontaneous dissolution of calcium phosphate but also introduction of ionic reactivity resulting in drastic changes in the ionic composition of culture medium turning it to be an unfavourable environment (Klimek et al. 2016; Przekora et al. 2014). The commercially defined cytotoxicity evaluation of ceramics especially can show misleading results as evident from the long- and short-term ion interaction assay while altering the concentration of divalent cations (Ca++ and Mg2++) and phosphate ions in pristine cell culture medium (Przekora et al. 2014). This assay depicted that abnormally high concentration of calcium in the culture media released from the ceramics might lead to in-vitro cytotoxicity on human foetal osteoblast cells (hFOB) although the ion reactivity of calcium-lacking hydroxyapatite scaffold was strongly affected by the composition of medium and duration of exposure. Alongside, the increased ion-adsorption especially with scaffolds fabricated from unsintered hydroxyapatite lead to critically low concentration of divalent cations resulting in inhibition of cellular metabolism, activation of integrin receptors, cell adhesion as well as viability (Malafaya and Reis 2009). The increased concentration of phosphate ions, on the contrary, showed less toxicity of hFOB cell line as evident from the results of MTT assay (Przekora et al. 2014). Other limitations include incompatibility of silicon oxycarbide nanowires with the chemical reagents of MTT assay which has been found to adversely affect the outcome as the assay is based on the principle of redox reactions (Lagonegro et al. 2017). In lieu of this, covering of cell monolayer by agar mixed culture medium following incubation with scaffolds/implants has been found to be more reliable in this aspect. Cells cultured in this manner are usually inspected to track changes in cell morphology, cell lysis and instances of probable detachment, loss of membrane integrity and vacuolization by means of microscope. This approach also has been executed by the same group (Lagonegro et al. 2017).

15.3.2 Hemocompatibility and Immunogenic Response

Biomaterials including implants or scaffolds meant for use in bone tissue engineering must possess hemocompatibility as a series of complex physiological reactivity happens while differing the degree of adjustment at blood-biomaterial interface. The risk of potential deleterious effect ensuing out of incompatibility may extend up to implant failure along with other severe consequences. Adsorption of circulating platelets and plasma proteins on the surface of substrates leads to activation of blood coagulation cascade mediated by leukocyte adhesion, in-vivo adhesion and activation of platelets resulting in blood coagulation (Liu et al. 2014a). Cellular matrix is formed provisionally on colonization of cells leading to the conversion of prothrombin to thrombin. It leads to formation of dense fibrin network of clots surrounding the implants, although the phenomenon is more prominent in cardiovascular implants, stents. After coagulation persists, reverse mechanisms are

initiated to dissolve the thrombus where zymogen, plasminogen is converted to plasmin and results in breaking of fibrin network to form fibrin degradation products (FDP) (Zorio et al. 2008; Liu et al. 2014b).

Primary evaluation of hemocompatibility of scaffolds involves detection of FDPs on incubation of targeted cell seeded scaffolds in freshly collected whole human blood compared to the FDPs formed by incubation of whole human blood without scaffolds (Weber et al. 2021). Briefly, after incubation of about 90 min, blood is transferred into a fresh container coated with anticoagulant and subjected for the measurement of blood cell numbers to find out significant deviation in platelets, erythrocytes and leukocytes. The analysis of complement activation markers also can be performed to evaluate the potential of the biomaterial in triggering inflammatory response leading to activation of polymorphonuclear (PMN) leukocytes. The level of PMN elastase and proteolytic enzyme released from the leukocytes in the exudates blood needs to be measured therefore. Activation of coagulation cascade can be tracked by measuring the level of thrombin-antithrombin III (TAT) complex which is formed by counter activity of fibrin by coagulation inhibitor antithrombin III. The same can also be evaluated indirectly by performing kinetic blood coagulation test. The formation of blood clot can be replicated in-vitro by adding drops of whole human blood on surface of implants and is incubated at 37°C for about 5 min. Recalcification then may be done by uniformly mixing calcium chloride in warm blood followed by dilution with distilled water to separate the supernatant comprising of lysate-free red-blood corpuscles (RBCs). The absorbance of the supernatant is measured by means of UV-Vis spectroscopy at about 540 nm (Lagonegro et al. 2017). The outcome may be corroborated by quantification of p-selectin by specific immunosorbent assay. Release of p-selectin has been usually found to be significantly high with response to oxide containing biomaterials (silicon oxide, titanium oxide) compared to the control polypropylene tissue culture substrate (Lagonegro et al. 2017). P-selectin is considered to be an important biomarker expressed on platelet membranes while indicating platelet aggregation mediated by fibrinogen binding on being released from alpha-granules (Body 1996; Shattil et al. 1987). Increased surface area available due to micro-roughening often results in increased plasma protein deposition resulting in high platelet activity, although it should also be noted that the micro-structured metallic implants with features of <3 μm may not provide enough area for adhering platelets having size of 3 μm at resting condition (Park et al. 2001). Accelerated protein adsorption seems to be the primary reason behind high index of blood coagulation on those structures compared to machined implants.

Complement system activation as a result of interaction of blood with foreign material takes place and gets terminated by formation of SC5b-9 complex thus indicating its suitability to be the important marker, whereas platelet activation can be detected by the presence of β-TG in blood which also gets released from alpha granules in the interacting platelets.

In addition to these above-mentioned biochemical assays, blood-biomaterial interaction can be examined by Scanning Electron Microscopy (SEM) imaging where the biomaterials are subjected to interact with platelet rich plasma for a brief

duration. The samples then are to be fixed with 4% w/v paraformaldehyde solution following dehydration with serially increasing concentration of ethanol. The shapes of the platelets indicative of the activation might be determined from the three-dimensional images acquired from SEM of the gold-sputtered plasma-soaked implants or scaffolds and the adhering platelets might be quantified by lactate dehydrogenase assay as well (Lagonegro et al. 2017; Park et al. 2001). Apparently, the platelets appear to be star-shaped at early activation phase, whereas the fully activated platelets are found to be octopus-shaped. Additionally, the platelets also can be observed for the pseudopod formation, aggregation, and spreading on the implant surface. The appearance of the dense fibrin matrix surrounding implants can also be observed by SEM imaging. The aliquot of platelet rich plasma also can be subjected to flow-cytometry.

15.3.3 Cellular Adhesion and Proliferation

Accomplishment of cellular adhesion which is divided in three consecutive major steps, that is, cellular attachment, cell spreading and focal adhesion, is observed to be crucial to produce cellular mass sufficient for cellular proliferation and further differentiation thus necessitating the determination of cellular adhesion. The adherence of the anchorage-dependant cells, that is, osteoblasts, pre-osteoblasts, mesenchymal stem cells on biomaterial substrates, is therefore necessary. In-vitro immersing or in-vivo implantation of the bone scaffolds instantaneously induces adsorption of several tissue and plasma proteins at the surface of the substrates. The plasma proteins (albumin etc.) and adhesive glycoproteins (fibronectin, vitronectin, vinculin, laminin etc.) undergo conformational change thereby significantly affecting the strength of adsorption and further cell adhesion (Salakhutdinov et al. 2008; Burmeister et al. 1996). Experimental evidence demonstrated that the hydrophobic implants induce greater deformation of adsorbed proteins resulting in unfolding compared to that observed on the surface of hydroxyl group-functionalized hydrophilic implants (Keselowsky et al. 2003). The more hydrophilic group the implant surface contains, the more protein gets adsorbed on the surface (Chang and Wang 2011). Implants and scaffolds pre-coated with those adhesive proteins and immunoglobulins even sometimes with adhesion-specific peptide sequence RGD (arginine-glycine-aspartic acid) tend to be recognized more by the recruiting target cells present in the vicinity of the implant (LeBaron and Athanasiou 2000; Yamada 1991). These biological cues specifically attach to the integrin binding domain in the cell membrane of osteoblasts or osteoprogenitor cells thus mediating the cell adhesion to a great extent. Cellular attachment followed by interaction of actin cytoskeleton to the proteins present in ECM results in formation of subcellular structures including focal adhesion plaques (FAP). This phenomenon gets further accentuated when the surface of the implant is nano-structured while fabricating ridges, pores, channels, fibres as the integrin binding sequences are also nano-sized (Ercan and Webster 2014; Le et al. 2013).

Biological activity of the implants and scaffolds is usually evaluated visually by microscopical observation. After incubation for desired duration, the cell-scaffold constructs are fixed with 2.5% of glutaraldehyde solution followed by dehydration with increasing concentration of ethanol solutions. The dried samples afterwards are observed under SEM to characterize cell distribution, cell-scaffold interaction, intracellular connection and morphology (Lagonegro et al. 2017; Lee et al. 2006). SEM observation, although being the extensively used evaluation method, qualitative fluorescence analysis also can be performed on the cell-scaffold constructs to observe cytoskeleton and focal adhesion of the cells growing on the surface. On washing with phosphate-buffered saline (PBS), the non-specific binding sites of the samples are blocked by bovine serum albumin and then staining was done using primary antibody of any of the before-mentioned cytoskeletal proteins labelled with fluorescent dye tagged secondary antibody. Further staining of actin cytoskeleton and nucleus also is performed using other dyes phalloidin and DAPI (4',6-diamidino-2-phenylindole) respectively prior to observing under fluorescence microscopy (Lagonegro et al. 2017). Similar to the cytotoxicity evaluation, samples can be evaluated quantitatively by MTT assay or CCK-8 method as well (Zhang et al. 2018).

15.3.4 Osteogenic Differentiation

On protein adsorption on the surface of substrates and subsequent cellular adhesion, osteoconductive implants aid in cell proliferation of partially differentiated osteogenic lineage and ECM formation. Osteoinductive implants or scaffolds including stem cell-incorporated or osteogenic growth factor-loaded systems have been found to recruit osteoprogenitor cells leading towards differentiation (Albrektsson and Johansson 2001; El-Ghannam 2005; Xu et al. 2017). This phenomenon is a complex dynamic metabolism-controlled process comprising of three consequent phases. While proliferating, the osteoblasts start to synthesize primarily collagen I protein and other ECM proteins including osteopontin, osteocalcin, bone sialoproteins. At initial stage, bone alkaline phosphatase (bALP) activity seems to be low accompanied with low ability to produce osteopontin which increases exponentially afterwards leading to synthesis and maturation of ECM. The bALP activity reaches the maximum point at this stage when cells stop proliferating as well as matrix mineralization is also observed to be low. Increased level of osteopontin, osteocalcin and collagen type I at this second phase seems to crosslink with osteoid to form the template for deposition of calcium phosphate crystals moving towards the third and last stage of matrix mineralization. The end of osteogenic differentiation is marked with sharp decline in bALP activity, high serum level of osteocalcin and osteopontin, both of which are calcium binding proteins along with formation of mineralized stable bone matrix (Polo-Corrales et al. 2014). This process, however, summarizes the characteristic markers at the different phases. Starting from the initial stages of osteogenic differentiation, that is, last stage of cellular proliferation to ECM formation, can be characterized by increasing activity of bALP by means of q-PCR

(polymerase chain reaction), enzyme-linked immunosorbent assay (ELISA), Western-blot assay, immunofluorescence-based staining. The late stage of osteogenic differentiation whereas can be evaluated by studying relative gene expression of relevant osteogenic markers (osteocalcin, osteopontin, collagen I, bone sialoprotein) and levels of corresponding proteins in bone. Alizarin Red S assay is also a reliable method for qualitative and quantitative evaluation of matrix mineralization.

15.4 In-Vivo Evaluation Methods

Biological response to the metallic, polymeric and composite materials used for fabrication of bone tissue engineering implants and scaffolds largely relies on the size, shape, surface modification, surgical site and method of implantation as well as the duration of implantation therapy. Similar to the in-vitro cellular activity, in-vivo biocompatibility and osteogenecity have been found to be correlated with the acute and chronic inflammatory responses happened right after the surgical implantation of scaffolds. In lieu of this, the interaction of a plethora of immunogenic and non-immunogenic cells including macrophages, fibroblasts, osteoblasts, osteoclasts, multinucleated giant cells surrounding implants is needed to be evaluated. Knowledge about in-vivo activation and communication of cells with materials otherwise would be incomplete if gross morphological, immunohistochemical and histomorphometry analyses were not performed at all.

15.4.1 Inflammatory Response

Naturally obtained polymers such as collagen-based biomaterials display physiological reactivity determined by mononuclear cells and sometimes pathological reaction while inducing multinucleated giant cells after subcutaneous implantation in small animal model (Ghanaati 2012; Ghanaati et al. 2011a). These materials are usually observed to be well-integrated with the surrounding subcutaneous host connective tissues with an evident mild peripheral vascularization pattern (Ghanaati 2012). Ghanaati et al. showed that the presence of stable collagen membrane allowed the penetration of anti-inflammatory mononuclear cells inside the core resulting in tissue in-growth over time while the polytetrafluoroethylene (PTFE) membrane as negative control prevented penetration of mononuclear cells rather inducing the formation of pro-inflammatory multinucleated giant cells at both of the surfaces. Thus, the negative control membranes posed as an effective functional barrier inhibiting tissue in-growth. Even the silk-fibroin membrane, although having superior biocompatibility, has been found to delay the penetration of both the pro-inflammatory multinucleated giant cells and anti-inflammatory mononucleated cells resulting in lagging events including membrane breakdown followed by neotissue formation and transmembrane vascularization. The outcome of this above-mentioned study, however, contradicts other findings where significant decrease in thickness was observed after

in-vivo implantation of non-cross-linked collagen membrane over time with instance of transmembrane vascularization on breaking down of the membrane (Rothamel et al. 2005; Schwarz et al. 2006). Among the bone substitutes, subcutaneous placement of deproteinized bovine bone grafts sintered at low temperature in mice model experimentally showed a temporary immune response in terms of localization of multinucleated giant cells at early phase (Barbeck et al. 2014). The response was remarkably decreased with in-vivo time-based degradation of small granules present in the graft. Synthetic biomaterials including bioactive glass also showed similar pathological immune response, although at a comparatively milder level (Ravarian et al. 2013). This emphasizes the advantage of pre-processing of bone engineering scaffolds or grafts especially fabricated with hydroxyapatite, silk fibroin, beta-tricalcium phosphate either with human blood or sometimes culturing of human osteoblasts with or without endothelial cells before implantation (Barbeck et al. 2015a, 2016; Ghanaati et al. 2011b). All of these have been found to attribute to significant induction of multinucleated giant cells with markedly increased vascularization. Pre-seeding scaffolds only with osteoblasts even have been found to release several soluble factors including pro-angiogenic stimuli enough to stimulate host endothelial cells to adhere, proliferate and form neovessels (Ghanaati et al. 2011b).

As it has been previously mentioned, the inflammatory and in-vivo biodegradation patterns are usually determined on subcutaneous implantation of the synthetic as well as natural bone graft substitutes. The resorption rates of these materials are directly related with induction of multinucleated giant cells and transmembrane vascularization even up to the central region which is integral to the process of guided bone regeneration, therefore highlighting the importance of such evaluation. Briefly the experimental animals are treated with the samples implanted in a pre-formed subcutaneous pocket at the subscapular region (Barbeck et al. 2015b; Ghanaati et al. 2010). After desired duration, the animals are subjected to euthanasia and explanted samples are fixed with 4% paraformaldehyde solution followed by decalcification and dehydration with increasing concentration of ethanol. Histomorphometrical analysis is performed thereafter with histological sections prepared from paraffin-embedded scaffold implantation bed. The presence of multinucleated giant cells with surface expression of tartrate-resistant acid phosphatase (TRAP) as 'look-alike' osteoclasts typical to the induced phagocytotic cells is detected. The cells, however, lack some of the other quintessential characteristics of osteoclasts such as ruffled border (Barbeck et al. 2015b). In-vivo implantation of similar grafts in human also showed the presence of those multinucleated giant cells; however, they lack the osteoclast activity seeming to influence bone regeneration (Al-Maawi et al. 2017). The phenomenon happens possibly owing to the fact that resident multinucleated giant cells in host tissue, being induced through integrin β 3, behave similarly to the osteoclasts, while biomaterial-adherent ones, being positive for integrin β 2, act as interleukin-4 mediated immunogenic foreign body giant cells only (Barbeck et al. 2017; McNally and Anderson 2002).

Trindade et al. evaluated host-biomaterial relation in lieu of immune system activation on implantation of titanium implants in osteotomy defect created at distal femur in rabbit model compared with sham-operated rats (Trindade et al. 2018).

After euthanasia, implants from half of the animals were retrieved to collect the bone for evaluating relative expression of genes responsible for induction of neutrophils, T and B lymphocytes, activation of complement system, macrophage activity. Bone samples with embedded implants were collected from rest of the animal for performing histological analysis for the expression of relevant components in innate immune system. Those components included bone resorption markers: receptor activator of nuclear factor kappa-B ligand (RANKL), osteoprotegerin (OPG), tartrate resistant acid phosphatase (TRAP) and cathepsin K (CathK). Both the studies resulted in evident innate immunity building through up-regulation of neutrophil activity as a marker of acute inflammation, increased macrophage fusion, thereby inhibiting neutrophil apoptosis as a marker of chronic inflammation surrounding titanium implants. The uprising balance towards reparative M2 phenotype of macrophages apparently shifted the paradigm towards anti-inflammatory activity and wound healing while decreasing the level of inflammatory M1 phenotype over time. This outcome corroborated with the previous hypothesis (Gensel and Zhang 2015).

15.4.2 Biomechanics

In-vivo osseointegration surrounding implants largely depends on achievement of the early implant stability. The stability further relies on several factors including surgical technique adapted, quality and quantity of the residual host bone before implantation, compatibility of implant-bone interface as well as microscopic and macroscopic surface features of the implant (Büchter et al. 2006). All these contributing factors effectively delay the total time needed for the completion of prosthetic reconstruction. Surgical trauma and incapability of attachment of adjacent tissue directly on the implant surface often result in fibrous tissue formation rather than direct apposition of newly formed bone in close agreement with the host tissue. Early achievement of mechanical stability thereby biological and functional restoration, that is, osseointegration, may be ensured by reducing the possibility of micromotion by decreasing the gap at bone-implant interface (Szmukler-Moncler et al. 2000). Micro-movement at a minimum keeping the range of 500–3000 μ strain moreover can be achieved by improved geometry of the implants especially for the metallic ones (Buser et al. 1991; Joos et al. 2000). Comparative study among implants with different geometries such as cylindrical, threaded cylindrical, cylindrical with steps, threaded cylindrical with steps and double-disk experimentally depicted that the threaded implants distributed the strain homogeneously on the surrounding tissue leading to physiological and non-physiological transmission of mechanical strength (Joos et al. 2000). This appears to be necessary for long-term implant stability.

Biomechanical evaluation is usually performed via measuring the removal torque and resonance frequency measurement (RFM). After in-vivo implantation of implants in targeted bone (tibia, femur, maxilla and mandible) of the small animal, vibrational frequency of the implant with tissue is measured from intact euthanized

animals to evaluate implant stability right after the implantation as well as after euthanasia. High frequency thereby is associated with increased stiffness at bone-implant interface and higher peri-implant bone density (Meredith et al. 1996; Pagliani et al. 2013). Less lateral displacement also has been found with more mechanically stable implants which can be further reflected as improved osseointegration. In lieu of this, multiple probes need to be placed perpendicular to the transducer (/sensor) at various regions surrounding the implant.

Loosening of the implantable screws is often a matter of concern as frictional force acting at contact region of both the biological and implant surfaces significantly affect the preload values of the implants (Lang et al. 2003). The more force or torque is applied during tightening of the screws, the less frictional coefficient it will have in future. This can be directly correlated to the increased resistance to micro-motion and friction resulting in inhibition of loosening of implants. This feature largely relies on the structure, threading, texture and degree of lubrication of the implants (Burguete et al. 1994; Wang et al. 2009). Nano-structuring of titanium implants has been found to accelerate bone growth and osteoblast response establishing high shear strength after 4 week of implant placement. The micro-roughened implants although ensured tissue in-growth through the micropores resulting similarly high interfacial bonding but after around 12 weeks of implantation (Xia et al. 2012). Counter-clockwise rotation is usually applied at a fixed degree of rotation per second on the retrieved samples and the torque rotation curve is recorded to measure maximum torque on the curve at which the implant eventually gets removed from the newly grown tissue. The interfacial stiffness also may be calculated from the same curve according to standard procedure (Büchter et al. 2006).

15.4.3 Histomorphometry and Immunohistochemistry to Evaluate Osteoblast and Osteoclast Response

In-vitro immunohistochemical evaluation of the growth of osteoblasts, formation of blood capillaries along with the expression of relevant osteogenic proteins such as osteocalcin, osteoprotegerin, vascular endothelial growth factor should be ideally validated from the similar study performed on the explanted implants after in-vivo implantation. After decalcification and paraffin-embedding, the samples are cut in thin histological sections followed by observation under optical microscope. Suitable dyes (such as haematoxylin, eosin, toluidine blue) are used for staining of the cells or tissues along with counterstaining. The samples are usually observed for formation of osteoblasts, osteocytes and its morphology, presence of macrophage and multinucleated giant cells, reconstructed vasculature, newly formed bone with lamellar structure, ratio of cortical bone to trabecular bone in the peri-implant zone (Bahraminasab et al. 2021). At different time points, the morphology of different cells gets altered depending on the texture of the implant. Osteocytes, the terminally differentiated form of osteoblasts, may be considered as a key player both in new bone formation as well as in bone remodelling mediated by receptor activator of

nuclear factor-κB ligand (RANKL), osteoprotegerin, receptor activator of nuclear factor-κB (RANK). This necessitates visualization of lacuna-canalicular structure formed by osteocytes residing inside lacunae for alteration of size, morphology, alignment, connectivity with other proximal and distant osteocytes, accessibility to blood vessels (Shah et al. 2016; Bohner et al. 2017). Direct bone-to-implant contact may also be observed by staining the interfacial thin tissue layer with Alizarin S and Brilliant-Cresyl-blue (Büchter et al. 2006). These lamellar structures also may be observed under fluorescence microscope without labelling (Büchter et al. 2006). Moreover, the significant difference (if any) in the bone-to-implant contact ratio at different time points (e.g. 7 days, 14 days, 28 days, 56 days) can be calculated via quantitative histomorphometric analysis. The calculated parameters are bone-to-implant contact (%BIC) and bone area fraction occupancy (BAFO) where %BIC defines percentage of implant surface directly in contact with anchored bone over the length of implant and BAFO indicates total area of mineralized matrix deposited by bone between the pitches, valleys of threads over the unit microscopic field (Folkman et al. 2020). Several studies although did not find any influence of different surgical techniques and drilling dimensions on those parameters. Preparation of bone before surgery, insertion torque rather apparently influenced the parameters as high insertion torque and no preparation of bone (i.e. cortical perforation) caused micro-fractures, generation of high temperature, necrosis of host tissues due to compression leading to failure in osseointegration (Cha et al. 2015; Stocchero et al. 2019).

15.4.4 In-Vivo Imaging for Bone Tissue Engineering

Several non-destructive real-time imaging techniques are available to track the progress of peri-implant bone healing and even the accuracy of the surgical placement of the implants. These techniques include X-ray computed tomography (CT), magnetic resonance imaging (MRI) and ultrasound (US), optical imaging (OI), single photon emission computed tomography (SPECT) and positron emission tomography (PET) which provide anatomical and sometimes metabolic information during and post-implantation (Fragogeorgi et al. 2019).

Nuclear imaging such as SPECT and PET detects the emitted photons from the isotope combined chemically and biologically active substances using ionizing radiation. Region of interest can be identified and measured with high sensitivity through SPECT, although the uptake of radiotracer agents in metallic hardware (screws, plates, rods) can overlap the region of interest in the reconstructed 3D images of the pre-clinical/clinical models (Lima et al. 2020). This overlapping is usually represented using the term 'shining metal artifact' which apparently indicates significantly increased projected region statistics (Duncan and Ingold 2018). If ignored, this phenomenon might be misinterpreted as having loosening or infection around implants due to which high radiotracer activity is observed in reality (Römer et al. 2005; Murer et al. 2020). These evaluation techniques, however, are limited by the brief biological half-lives of the radiotracer elements or radiopharmaceuticals as

the suitability of using such compounds for long-term tracking the progress of slow physiological process (i.e. bone regeneration, osseointegration of implants) cannot be justified (Fragogeorgi et al. 2019). The poor resolution in these methods sometimes renders it inconclusive in terms of identification of the source of new bone formation; confusion occurs whether the bone formation is happening at the peri-implant region or in the host tissue (Wong and Piert 2013). In contrast to that, PET imaging provides quantitative measurement of radiotracer uptake in bone and relative osteoblast activity through compartmental analysis integrated with PET platform (Cheng et al. 2013).

Micro-CT imaging utilizes X-ray to perform scans of the experimental animal or object from multiple angles followed by identification and measurement of region of interest subjected to 3D reconstruction of composite images. This imaging can be performed non-invasively for the small animal models under anaesthesia to track quality and quantity of newly formed bone tissues surrounding implants and also to the tissue embedded implants retrieved from the euthanized animals at different time points of post-implantation. The morphological information may be obtained macroscopically from the objects treated with contrast agents as well as microscopic features including structure of osteoblasts, vasculature, lamellar structure, trabecular connectivity all can be identified via advanced synchrotron-radiation enabled micro-CT (Fragogeorgi et al. 2019). With an objective of whole-body imaging, majority of the pre-clinical study prefers to fuse the information obtained from nuclear imaging to the anatomical information obtained from micro-CT based 3D reconstructed images. This owes to the fact that micro-CT provides improved spatial resolution especially in dense tissues (Beckmann and Maier 2011). A few drawbacks are noted for long-term micro-CT imaging such as detrimental effect of long-exposed radiation, risk of having inconclusive data as the poor integration at bone-implant interface or unclear delineation of host bone tissue to scaffold/implant engineered bone can be as complicated as an artifact (Ventura et al. 2014). Figure 15.2 describes a protocol which can be adapted for micro-CT imaging of implants retrieved from euthanized small animal models to track the progress of bone healing.

MRI selectively measures the movement of protons present exclusively in water containing tissues. On application of magnetic field, the protons spin and get displaced from their original position thereby creating a magnetization vector (Ventura et al. 2014). This is the reason behind the fact that MRI exclusively produces high-contrast images for water-abundant soft tissues compared to the low water containing hard tissues such as bone. Around 20% volume of the cortical bone is composed of water specifically present in haversian canals and canalicular network, whereas some of the rest volume is strongly bound to the collagenous matrix and crystals of hydroxyapatite (Timmins and Wall 1977; Elliott and Robinson 1957). This necessitates employing indirect measurement of peri-implant bone apposition based on gradual disappearance of MRI signals (Washburn et al. 2004). In contrast to that interaction of high frequency sound wave with hard tissues can be measured via US imaging systems depending on the attenuation, absorption and reflection of the sound. Irrespective of the hardness, tissues bear a natural feature to act as an impedance barrier to acoustic waves depending on speed of the sound wave

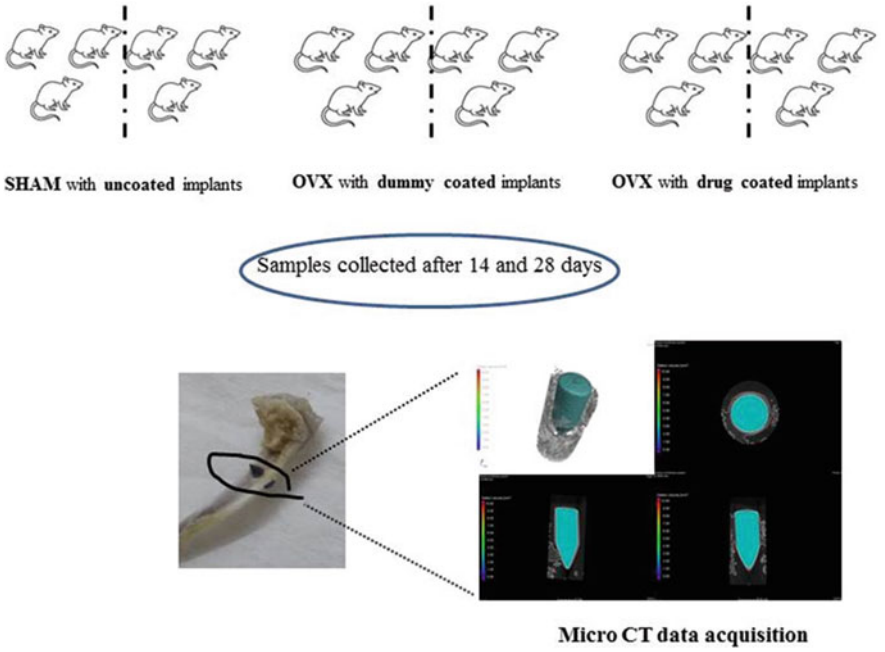

Fig. 15.2 Representation of an experimental protocol for micro-CT imaging for evaluation of in-vivo peri-implant bone growth

and density of the tissue. Several pre-clinical and clinical scenarios may hereby be noted with diverse application of US imaging ranging from monitoring drug treatment, gene-therapy to bone healing engineered by scaffolds/implants (Bez et al. 2017; Hériveaux et al. 2019). Some other highly sensitive imaging modalities include fluorescence imaging (FLI), bioluminescence imaging (BLI) and fluorescence molecular tomography (FMT) which enable photon detection but with poor penetrability (Ventura et al. 2014).

15.5 In-Silico Evaluation Methods

Early achievement of primary stability leading to faster osseointegration can be made possible by fabricating customized patient specific implants. It was conceptualized from the lack of flexibilities in customizing implant length, diameter, surface geometry, threading parameters for a particular fracture (Chen et al. 2012). Customized root-analogue dental implants, since its inception, have been an attractive approach to provide the scope of minimally invasive implantation without requiring traumatic surgical procedures such as sinus lift, drilling, bone augmentation (Pirker and Kocher 2008). It is therefore necessary to perform the in-silico finite

element analysis (FEA) to predict the relationship of biomechanical behaviour of the implants namely magnitude of stress/strain surrounding implants with peri-implant bone growth (Dos Santos et al. 2017). Several studies compared the overall stress distribution pattern and resulting stress on neighbouring bone using general threaded implants with press-fit design accompanied with same standard abutment with that caused by customized route-analogue implant. Summarized results indicated towards more uniform stress distribution, comparatively low alveolar bone stress, improved primary stability and reduced micro-movement with targeted press-fit dental implants (Anssari Moin et al. 2016; Chen et al. 2017). The process initiates the virtual design and development of implant models with requisite geometrical features which are to be virtually placed into simplified bone model composed of equal proportion of cortical and trabecular bone (Dantas et al. 2020). The models are then imported in to the working interface of COMSOLMultiphysics software to develop finite element models. Previously fed with the information regarding mechanical properties of several metals and non-metals acquired from the published literature, external loads are virtually applied from opposite directions (axial and oblique) on the models provided all the bone-bone and bone-metal interface are perfectly bonded. The fixed constraint is applied then in all the possible degrees of freedom at the interface of cutting section and also at the bottom of trabecular and cortical bone. Quantitative analysis finally reveals the effect of applied loading pressure on shear strain on cortical bone. Displacement of the placed implant along with tooth from the socket also can be calculated by means of standard mathematical formula. Therefore, it is absolutely necessary to identify the zone of bone resorption as the major loss of implant stability happens due to improper loading and poor distribution of stress (Dantas et al. 2020). Lorkowski et al. also performed FEA to virtually assess the severity of femoral strain caused by multi-hole plate stabilator accompanied with cerclage wire loops especially in osteoporotic bone suffering from peri-prosthetic fractures compared to that caused in non-osteoporotic healthy bone (Lorkowski et al. 2021). The more screws or wire loops it needs to stabilize the fixture, the more strain it is found to cause leading to reduced bone elasticity, implant destabilization, repeated micro-fractures especially evident in the osteoporotic bone (Lorkowski et al. 2014). X-ray based imaging, being one of the routine examinations for the treatment of peri-prosthetic fractures, is apparently the best source to approximate minute details about altered morphology of bones especially for the elderly patients required for the 3D modelling of the patient-specific bone structure. This seems to be convenient to understand the severity of osteoporosis or illustrate ongoing changes while reducing inconvenient sufferings caused by traumatic revision surgeries, implant exchanges or surgical interventions of repeated fractures (Lorkowski et al. 2021; Lee et al. 2018).

15.6 Future Directions

The advanced technology of biomaterials and cellular biology together has a significant role in driving stem cell fate by mimicking the sequence of physiological events involved in cellular adhesion, proliferation, differentiation, even the production, clearance and interaction of osteoprogenitors at the surface or through pores of the novel biomaterials. Cell-biomaterial interaction, however, can be controlled either by co-culturing cell-sheets on scaffolds prior to implantation and some other approaches include intelligent cell seeding on surface, functional coating on implant surfaces as well. Cell morphology seeded to be an important parameter significantly effecting the cell migration and proliferation on the biomaterial surface. Frost et al. explored the ideal morphology while culturing the porcine dermal fibroblasts on different prototypes of implant surface (Frost et al. 2021). Elongated cellular morphology, being quantified with cell aspect ratio (CAR), was found to be accelerating the cellular proliferation where high CAR value was indicative of rounded cell morphology favouring cell migration. They also outlined the future potential of this work in performing high throughput screening of the prospective surface prototypes before going for in-vitro cell-based assays. Biomaterial, screened by this methodology and designed accordingly might produce better response by generating dynamic cell morphology depending on the sequence of bio-molecular events necessary for tissue regeneration. A novel bio-engineering approach involving microfluidics was applied in a recent research where the clustering pattern and migratory response of retinal progenitor cells were evaluated in presence of some of the widely used extracellular substrates such as concanavalin A, laminin, poly-L-lysine and it was found that the migration of the cluster of the cells was subjected to exogenous chemotactic signalling of fibroblast growth factor (Pena et al. 2019). Although these above-mentioned novel approaches were not applied in optimizing implant/scaffold-based bone regenerative therapies, they may have a pivotal role in investigating potential of the bio-substrates to obtain optimum cell-biomaterial interaction, if utilized. Prior screening of such implants may help to control peri-implant microenvironment experimentally to avoid delayed osseointegration, risk of fibrous encapsulation and loosening of implants. All the evaluation strategies, discussed in this article, however, are not limited only in conventional in-vitro (stem cells etc.) and in-vivo models (small animals specifically rodents) but Vimalraj et al. also highlighted the preference towards zebrafish models as an excellent platform to study host bone-implant interaction (Vimalraj et al. 2021). Apart from several known advantages such as cost-effective, ability for whole-body imaging for optical transparency, genetic adaptability and short life cycle, several inductive signals generated during bone morphogenesis in zebrafish have also the potential to influence bone formation in mammals similar to conventional in-vivo models. The flexibility to study any kind of fabricated scaffold such as hydrogel, film, porous freeze-dried scaffold, electrospun network in such versatile in-vivo platform certainly will provide the edge over others to give insight into biocompatibility, bioactivity of novel materials under investigation.

Conflict of Interest Statement The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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