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Therapeutically relevant aspects in bone repair and regeneration

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Over the past few years, attention has been focused on the therapeutic roles in designing bone scaffolds for successful repair and regeneration. Indeed, biologically dynamic events in the bone healing process involve many of the molecules and cells adherent to the scaffold. Recent bone scaffolds have been designed considering intrinsic chemical and physical factors and exogenous/extrinsic cues that induce bone regeneration. Here, we attempt to topically review the current trends and to suggest featured strategies for the design of therapeutically relevant bone scaffolds taking into account recent studies and applications.

Introduction

Tissue-engineered scaffolds have played a decisive role in the repair and regeneration of a diverse range of tissues, including bone. These scaffolds not only provide a supporting matrix for cells especially in bone tissue engineering, but also provide essential environments for cells to spread, migrate, multiply, and conform to differentiation into specific lineage. For this, bone scaffolds should be tuned physico-chemically, to successfully repair and regenerate bone.

Unlike conventional scaffolds that temporarily fill defects and need secondary surgery for their replacement and/or removal, promisingly therapeutic scaffolds have utilized a variety of biological actions that favor and trigger cells, especially stem cells, to carry out relevant therapeutic roles [1]. Bone repair or regeneration is a part of a complex dynamic event that involves many molecules and cells. After scaffold implantation, the therapeutic actions should thus be harmonized with the biological events and even facilitate a better healing process. Key events in the active healing process include mild inflammatory reactions with no tissue rejection, substantial angiogenesis to form blood vessels, recruitment of progenitor/stem cells, and driving these cells toward osteogenic lineage and finalizing matrix maturation.

Therefore, tailoring the scaffolds to aid and stimulate these biological processes is an important milestone for scaffold-based bone engineering. In this topical review, we highlight designs of therapeutically relevant scaffolds that trigger cellular functions that benefit the repair and regeneration of bone, which will lead to the development of ideal scaffolds for bone engineering.

Therapeutic actions in bone regeneration

While a series of events is involved in tissue repair and the bone healing processes, where multiple cells and signaling factors are engaged occurring (as summarized in Table 1), some key actions that need special consideration are described here which ultimately help in the design of scaffolds to enable favorable therapeutic actions in bone repair and regeneration. These actions include reducing tissue rejection/inflammation, homing of progenitor/stem cells, stimulating angiogenesis, improving cellular osteogenesis, maturation, and mineral formation, as schematically illustrated in Fig. 1.

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TABLE 1

Signaling molecules participating in bone healing; their targeted cells and major functions.

Signaling molecule	Targeted cell	Function
Cytokines [2] **(IL-1, IL-6, and TNF)**	Inflammatory cells	ECM synthesis, angiogenesis, endogenous cell recruitment
VEGFs [3,4]	Endothelial cells	Cell proliferation, bone formation
PDGF [5]	Mesenchymal cells, inflammatory cells, osteoblasts	Mitogenesis, chemotaxis
IGFs [6]	MSCs, endothelial cells, osteoblasts	Cell recruitment, proliferation, protein synthesis
FGFs [7]	Mesenchymal cells, epithelial cells, osteoblasts	Angiogenesis, mitogenesis
TGFs [8,9]	MSCs, osteoprogenitors, osteoblasts	Mitogenesis, Chemotaxis
BMPs [10,11]	Mesenchymal cell, osteoblasts, osteoprogenitors	Bone differentiation
PTH [12–14]	Systemic administration	Bone mineral-density
Vitamin D [15–17]		New bone formation
Calcitonin [18,19]		Osteoblast differentiation
Steroid [20]		

Tissue rejection/inflammation

In bone, tissue rejection is an innate immune response and is caused by the host's immune system. The immune system expresses pattern recognition receptors (PRRs), which are markers of tissue injury or damage-associated molecular patterns (DAMPS), to identify pathogen-associated molecular patterns (PAMPs) [21]. Local tissue damage generates many potential DAMPS binding to PRRs, which results in the potent activation of inflammation [22] and up-regulation of transcription of genes and micro-RNAs involved in inflammatory responses [23]. The final production of inflammatory mediators [24] include the inflammatory cytokines, interleukin (IL)-1, IL-6, and tumor necrosis factor α (TNF-α) [25], which can promote osteoclast differentiation and inhibit osteoblast activity. Oppositely, anti-inflammatory cytokines IL-4, IL-10, and IL-13 facilitate bone formation [26]. Bone also has immune cells, such as T cells, B cells, and natural killer (NK) cells, produced in the bone marrow. These immune cells play a crucial role in bone homeostasis by regulating a fine balance of the

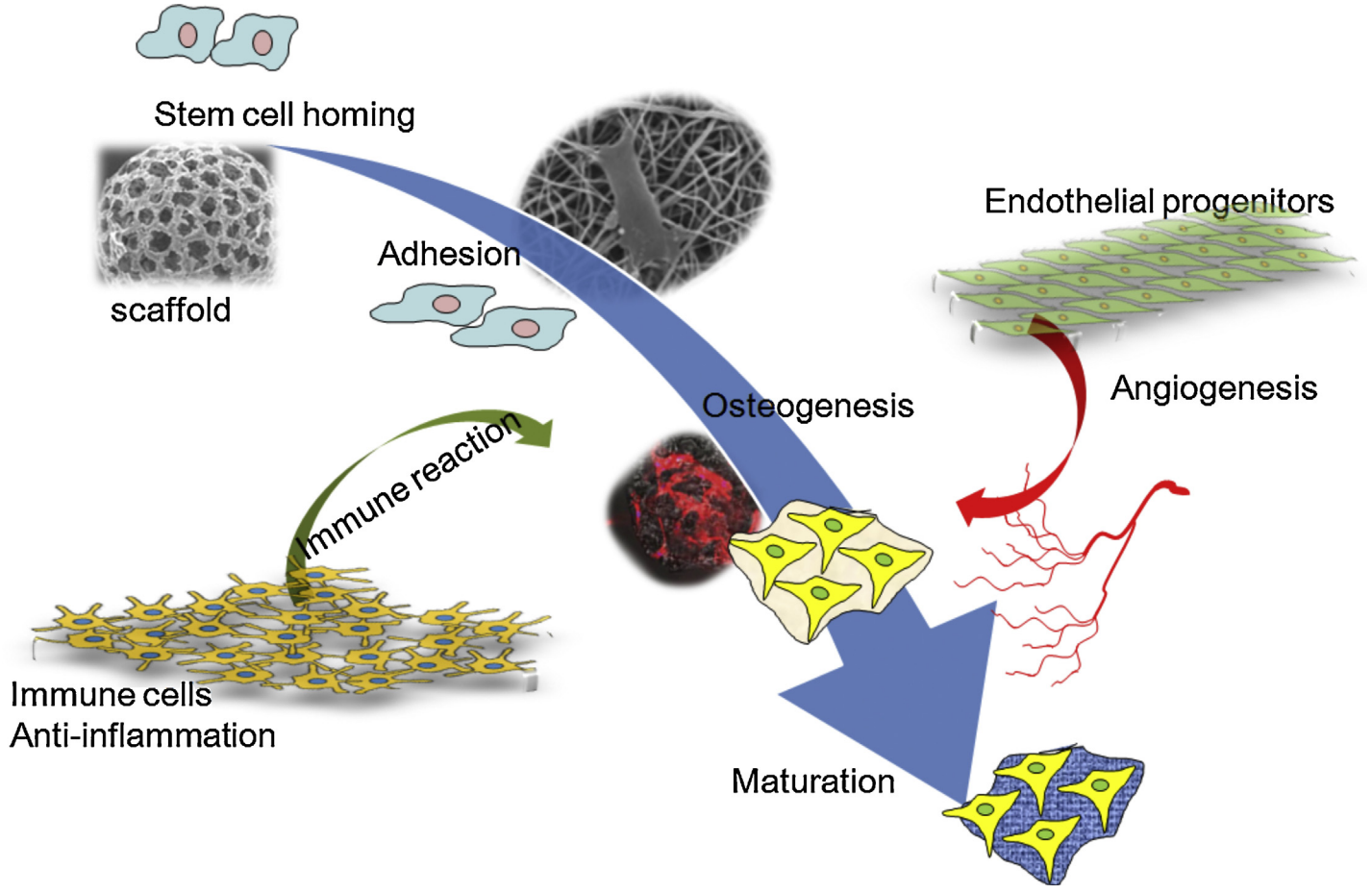

FIGURE 1

Therapeutically relevant events of different types of cells occurring in bone healing and regeneration processes, that ultimately governed by the scaffolds designed.

activity between osteoblasts and osteoclasts. Particularly, B cells act as the main inhibitors of osteoclastogenesis, and thus low numbers of B cells may result in microenvironment modification of bone tissue [27]. Meanwhile, macrophages are precursors of osteoclasts that play a major role in regulating the foreign material-induced immune response. Based on the characterization of macrophage phenotype, polarized macrophages are classified into classically activated macrophages (M1) and alternatively activated macrophages (M2) [28]. In general, M1 cells secrete inflammatory cytokines as well as toxic reactive oxygen and nitric oxygen intermediates, while M2 cells are involved in down-regulation of inflammation and improvement of tissue healing [29,30]. With this in mind, their polarization can be controlled by the scaffold design, i.e., by tuning the topography as will be discussed in following sections.

In most cases, transplanted scaffolds would be identified with an injury site and inflammation, and the immune system would produce acute phase proteins (e.g. complement factors) and trigger the recruitment of inflammatory leukocytes [31]. These inflammatory molecules spur secondary signals essential to stimulate angiogenesis, extracellular matrix (ECM) synthesis, and endogenous cell recruitment, consequently leading to improved bone healing. In recent studies, the presence of inflammatory signals in the injured site was highlighted to positively lead to bone healing while ameliorating tissue microenvironments [32]. Therefore, the inflammatory signaling is the process that is indispensable for the bone healing around the implanted scaffolds, and thus should be controlled not to reduce the expression of bone healing-associated growth factors, such as bone morphogenetic proteins (BMPs) [33].

Homing progenitor/stem cells

Stem cells are known to be involved in complex bone repair and regeneration processes, altering molecular, cellular, and biochemical metabolic changes. Amongst others, mesenchymal stem cells (MSCs) play an essential role in bone repair by differentiating into osteoblasts and chondrocytes under suitable conditions [34]. In basic research and the clinical field, MSCs have been delivered using a variety of techniques. However, in practice, their heterogeneity and off-target homing, especially lodging in the lungs [35], impedes the clinical use of MSCs. Therefore, the targeted delivery of the cells to the injured site is of critical importance. For the precise targeted delivery of the cells into the injured site, homing signaling factors such as stromal derived factor 1 (SDF-1) have been used, because they are known to drive recruitment of progenitor/stem cells. SDF-1, a member of the chemokine family, binds to the C-X-C chemokine receptor type 4 (CXCR4) receptors, and directs migration and homing of various types of stem cells. SDF-1 up-regulated at the injured site serves as a potent chemo-attractant to recruit circulating or residing CXCR4-expressing MSCs and participates in endochondral bone regeneration [36]. As such, the chemo-attractive bone scaffolds can provide significant benefits to progenitor/stem cells by eliciting their direct differentiation via paracrine effects.

Vascularization

Vascularization plays a critical role in bone healing and regeneration, as bone is a highly vascularized tissue. Early restoration of vasculature can have many benefits to bone regeneration allowing rapid access to inflammatory cells, metabolites, and stem cells [37]. A vascularization approach using delivery of angiogenic growth factors has aimed to improve early vascular support for bone [38]. Widely used angiogenic growth factors include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) [39]. Direct delivery of VEGF improved vascularity and bone repair, whereas its inhibition impaired bone regeneration [40]. In addition, VEGF indirectly affects osteoblasts through osteoblastic-forming (anabolic) factors produced by endothelial cells. Co-delivery of both growth factors, BMP-2 and VEGF, is often used to accelerate the early healing and bone formation [41]. Not only the growth factors, but also other molecules such as ions significantly alter the angiogenic processes. Because bone mineral contains many ionic components in the composition, ionic tailoring of scaffolds is a promising approach to improve vessel formation. Also, mechanical dynamics significantly alter vascularization and the regenerative potential of bone. For instance, collagen matrices containing micro-vessels showed increased *in vitro* angiogenesis under mechanical environments [42]. Collectively, angiogenesis can be improved by the scaffolds that deliver relevant growth factors and ions, which is also influenced by the mechanical dynamics of the cellular environments.

Osteogenic differentiation

Commitment of multipotent stem cells to an osteogenic lineage is crucial to attain specified functions in bone regeneration. In fact, a series of cellular processes during the initial phase, including cell homing, anchorage, and mitosis, are prerequisite for late lineage-oriented differentiation. As soon as the stem cells are recruited and anchored, they proliferate and sequentially change into osteogenic cells that can mature in the regenerating environments.

Many physico-chemical and biological factors have been implicated in osteogenic stimulation. Widely studied biochemical growth factors include FGFs, BMPs, PDGFs and IGFs [43]. For example, BMP-2 and BMP-7 play important roles in osteogenic differentiation, up-regulating alkaline phosphatase (ALP) and osteocalcin [44]. FGF family (FGF-2 and FGF-18) can also stimulate osteogenic differentiation [45]. FGFs are an effective mitogen for bone-derived cells and increase callus remodeling and fracture healing [46]. When MSCs were treated with FGF-2, the expression of osteogenic genes and proteins were substantially stimulated [47]. The FGF-18 treatment of MSCs also increased the ALP level and mineralization activity [45].

Along with biochemical growth factors, physical and chemical factors are implicated as inducers of stem cell differentiation. Surface topography at the micro/nanoscale and mechanical rigidity has largely driven MSCs toward the osteogenic lineage. Different functional chemical groups, having tunable charge properties, also dictate stem cell differentiation to osteogenic cells [48]. Therefore, scaffolds can be fine-tuned to provide chemical and physical environments for osteogenic stimulation of stem cells present in the body, which ultimately can develop into mature matrix and hard tissues.

Mineralization

Mineralization is a defining feature of hard tissues, and enables implanted materials to remain in place even when forcibly loaded.

The mineralization occurs at the final stage of bone matrix formation, after all the possible ECM proteins were secreted and matured. Therefore, the mineralization process is a result of cellular interactions with environments and the mineral deposition largely depends on the environmental conditions, such as pH, ionic concentration, and the matrix molecule types [2].

Cellular mineralization is a series of events, which are based on matrix vesicles. These matrix vesicles are small spherical bodies found in pre-mineralized matrix of bone composed of a lipid bilayer and have been shown to have small crystals of calcium (Ca) phosphate. The presence of the lipids allows the binding to Ca and is also able to form Ca channels through their membranes [49]. These vesicles contain several components that play regulatory and cooperative roles in the formation of inorganic phosphates [50]. On the other hand, the organic phase, primarily collagen fibrils, play an important role as a template for the inorganic mineralization [51]. The environmental factors, such as pH can also have a regulatory effect on the mineralization process [52].

The allocation of mineralized vesicles is primarily determined by ECM components, which can accelerate or inhibit the cellular mineralization rate. Alongside collagen, other non-collagenous proteins have been identified in the mineralization. For example, osteocalcin, a key non-collagenous protein present most abundantly in bone ECM, is expressed primarily during matrix maturation and mineralization [53]. Its amino acid sequences (aspartic acid and glutamic acid) specifically bind to Ca ions, which in turn capture phosphate ions, to induce apatite mineral crystals. In a similar way, osteopontin (OPN), bone sialoprotein (BSP), and matrix extracellular phosphoglycoprotein (MEPE) have also been shown to significantly affect mineralization. Furthermore, phosphorylated proteins, proteoglycans, and glycoproteins have also been identified in the regulation of the mineralization process. The extracellular phosphorylation of matrix proteins is an important step in biomineralization. In addition to the matrix molecules, soluble signaling factors such as IGFs are key modulators of local bone mineralization and remodeling.

The mineralization of the bone matrix is a key event to maintain the biological and mechanical functions of bone. Mineralized crystals provide strength and rigidity to the matrix to sustain mechanical dynamics. Moreover, cellular remodeling is the natural process of preserving bone mass controlled by balanced action of resorption of mineralized matrix and deposition of new matrix. In this way, the scaffolds that have the capacity to provide biochemical ingredients and physico-chemical cues that accelerate the formation of mineralized matrices are favored, where the cells can utilize the scaffold signals in the nucleation and growth of the mineral crystals.

Material intrinsic factors affecting bone regeneration

The factors that affect bone regeneration can either originate from properties of the materials such as chemical compositions and physical traits, or be derived from exogenous signals and conditions including the incorporation of therapeutic drug molecules and application of external mechanical cues. This section starts with the intrinsic material properties that affect bone regeneration.

Intrinsic chemical factors

The intrinsic chemical properties of scaffolds can be considered either on the surface or in the internal bulk. Because the biological responses primarily start on the surface ('interface') of the scaffolds, the chemical status of the surface is of particular importance in subsequent bone regenerative events. While the surface chemistry controls early biological events, the bulk chemistry primarily determines the long-term fate of scaffolds, such as mechanical functions.

Depending on the type of scaffolds, the bulk chemistry and the degradation behaviors are significantly different. For biodegradable polymers, degradation occurs either by hydrolytic or enzymatic processes [54]. Sometimes, the polymers are engineered to contain enzymatically cleavable chains to enable enzyme-specific degradation. The degraded products should not be cytotoxic. For bioactive ceramics, hydrolytic ion leaching is the most common type of degradation, where the ionic products have a significant influence on the cell behaviors, including cell mitosis, osteogenesis, and angiogenesis. Therefore, specific ions, including Ca, silicon (Si), zinc (Zn), strontium (Sr), titanium (Ti), manganese (Mn), cobalt (Co), iron (Fe), silver (Ag), and gallium (Ga), have been strategically incorporated within the chemistry of bioactive ceramics [55]. Detailed biological effects and case studies will be explored in the following part. In most cases, the degradation rate (ionic dissolution rate) is important; while such biologically active results are expected at therapeutically relevant doses, significant cytotoxicity is often accompanied when released at larger doses [56].

The change in bulk mechanical properties is deeply related with chemical factors especially when the scaffold is chemically degraded. Because one important function of bone is the load-bearing capacity in the body, mechanical weakness leading to premature failure is fatal for the successful bone regeneration. Therefore, the degradation rate of scaffolds needs to be optimized to balance with the tissue regeneration rate. Another aspect that is also closely related with the bulk chemistry and its change is the mechanical rigidity (stiffness) of the scaffolds which largely governs the fate of stem cells, such as the lineage specific differentiation. This area will be detailed in the following section.

Intrinsic physical factors

Physical factors that possibly govern the cell responses and bone regeneration can be considered in two ways; one is surface topology (or roughness) at the micro/nanoscale, and the other is matrix stiffness (or rigidity).

Surface topology can be the first physical cue where cells sense the scaffold. Recently biophysical studies have shown the important roles of the surface topology tailored at the micro- or nanoscale level [57,58]. The topological features that have been engineered and investigated include microgrooves, microislands, nanofibers, nanogrooves, nanodots, nanotubes, and complex shaped patterns, and mechanisms for their possible effects on the cellular responses are known to be 'mechano-transduction' [59].

Figure 2 depicts the exemplar studies on the influential roles of surface nano-/micro-topological cues. For examples, micropatterned/textured surfaces have profound effects on cell

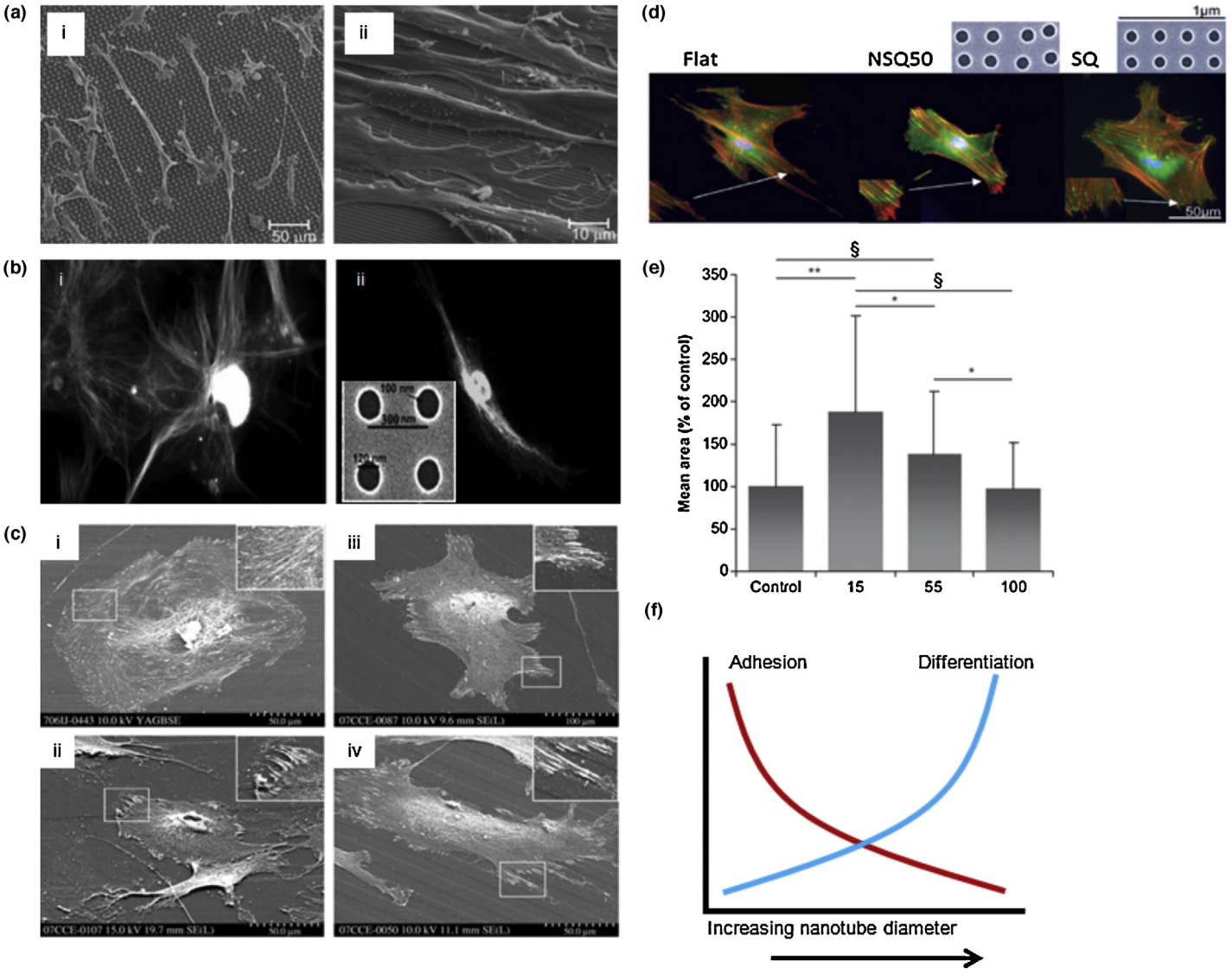

FIGURE 2

Influences of micro-/nano-topological features on cells. (a) MSC morphologies on (i) non-symmetric surface and (ii) micropatterned surface with 2 mm wide lines [60]. (b) Osteoblasts cultured on (i) planar surface and (ii) ordered nanopattern surface [63]. (c) Osteoblasts cultured on (i) planar surface, (ii) the 100 mm-groove, (iii) the 25 mm-groove, and (iv) the 10 mm-groove patterned surface [64]. (d) MSC spreading morphology on the flat control surface, the near square 50 (NSQ50) and the square50 (SQ50). For the SQ, center-center spacing of pits was 300 nm, whereas for the NSQ50, each pit had up to a 50 nm offset in X and Y [70]. (e) Cell spreading profiles for hMSCs cultured on Ti surfaces with different titinia nanopillar-like structures of 0, 15, 55, and 100 nm in height [72]. (f) Simplified drawing of the effects of nanotubular diameter on the hMSC responses after a 24-h culture. Solid red line indicates the change in cell adhesion and growth, while solid blue line indicates osteogenic differentiation [48].

adhesion, spreading and migration. Almost all cells seeded onto microgrooves aligned and elongated along the groove lines, changing the cell shape (Fig. 2a) [60]. Cell shape along the substrate wall reflects the cytoskeletal organization such as microtubules and actin filaments. These changes in cytoskeletal organization and cell mechanics are dependent upon integrin clustering which ultimately governs osteoblastic differentiation [61]. Therefore, focal adhesion assembly affecting cytoskeletal changes plays a critical role in undergoing osteogenic differentiation. Nanotopographical patterns also dominantly determine the focal adhesion and cytoskeleton organization [62]. On nano-gratings of polymer substrates, the expression of integrin subunits (α2, α6, α_V, β2, β3 and β4) in human MSCs was reduced compared to the unpatterned substrates. Common nano-topographical features include pits and islands arranged in an ordered or random way, where cells are also able to sense the differences in the concave and convex surfaces. The nano-craters and nano-islands have been shown to alter osteoblast behaviors [63]. As shown in an example (Fig. 2b), compared to those on the planar surface, human osteoblasts on ordered nanopatterned surface showed an elongated morphology with less cell spreading, which indicated decreased microtubule organization. To further understand, an exemplar study (Fig. 2c) showed osteoblast spreading on groove-patterned surface with varying in the groove size. Osteoblasts tended to show an increased adhesion in the larger groove whereas the smaller nano-groove reduced adhesion because of increased contact guidance [64]. Furthermore, osteoblasts showed the highest motility on grooves with a ridge to groove ratio (1:3) compared to other

ratios of 1:1 or 3:1 [65]. Nanoparticles were also used to control cell adhesion and spreading behaviors. In a study, the surface was coated with positively charged silica nanoparticles with differing spacing. The higher nanoparticle spacing increased the cell density and allowed better allocation of spaces to cells for integrin clustering and focal adhesions with stronger actin cytoskeletons [66].

Nanoscale-tailored surfaces can have a more profound and significant impact on the fate of cells, particularly stem cells. Initial adhesive events, including focal adhesions, filopodia growth and spreading have been identified to significantly lead to favored engagement in subsequent osteogenic differentiation [67–69]. Interesting studies [70,71] have recently shown the effects of nanotopological 'ordering' on the multipotent stem cell differentiation behaviors. When the nanotopology was arranged in a highly ordered manner, the MSCs underwent down regulation of canonical and metabolic signaling, only slightly altering their phenotype. On the other hand, when the MSCs were cultured on disordered nanotopography, they were stimulated to an osteogenic differentiation. An exemplar study showed the contrasted MSC spreading behaviors on the surface with ordered and disordered nanotopography (Fig. 2d). The height of the nanotopography in the form of nano-pillars has also affected osteogenesis of MSCs, where 15 nm-sized nano-pillars were optimal [72]. The comparison of the cell area cultured on the titania with pillar-like topographic features of various heights up to 100 nm revealed the significant influence of the nanotopological height (Fig. 2e). Vertically arranged nanotubular topology significantly affects osteogenic [48]. Interestingly, small nanotubes (30 nm in diameter) allowed enhanced adhesion of MSCs without any significant differentiation, whereas larger nanotubes (between 70 and 100 nm) showed considerable selective osteogenic differentiation. The effect of the nanotubular size on the MSC responses is simplified.

A simple scheme of these overall nanotopographical effects on the MSC responses after a 24-h culture is illustrated (Fig. 2f). Pluripotent stem cells have also been cultured on the nanotopology, altering their phenotypes toward the osteogenic lineage. Embryonic stem cells were cultured on the polycarbonate substrate with nanotopological cues without any external chemical factors [73]. The cells displayed enhanced expression of mesenchymal markers as well as expression of early osteogenic progenitors, and the changes in adhesion proteins and the epithelial to mesenchymal transition were possible reasons for the phenomenon. Surface topography can also be tuned to polarize the macrophage behavior into an M2 phenotype and therefore control bone related processes. Taking into account that M1 polarized cells present a rather round morphology, whereas the M2 polarized cells present a rather elongated shape, by varying the groove width on a patterned substrate (from 20 to 50 μm), could alter the morphology of macrophage and ultimately the phenotype [74].

However, almost all the above-mentioned nanotopological features can only be examined on flat substrates, with limited evidences of the nanotopological cues on the 3D complex scaffolds. The nanofibrous matrices are, on the other hand, promising 3D matrices with nanotopological cues. Cells settle down well on the nanofibers and recognize the nanofiber guidance with preferred extensions along the fiber, conforming to the nanofiber topological and mechanical cues, and ultimately change their fate to an osteogenic route [75,76]. Largely, the nanofibrous structures have shown better osteogenic differentiation of stem cells *in vitro* than the flat control structures [77]. Furthermore, the fiber size and alignment have also shown significant effects, either on cellular shape, migration and/or osteogenic differentiation [78,79]. In a similar way, the physical properties of nanofibers have also been used to guide the functional polarization of macrophages, showing that increase in the fiber and pore dimensions significantly enhanced the M2 phenotype, and hence enhanced bone regeneration [80].

The pore geometry of a scaffold is also of great importance in governing cell behaviors and the overall bone regeneration process. It has been established that pores that allow cell penetration as well as nutrient and oxygen diffusion are necessary in scaffolds, and the pores over 300 μm have been shown to allow enough cells to penetrate and populate the scaffolds [81]. Along with pore size, the pore interconnectivity is also considered to be important. When the scaffold was designed to have higher pore interconnectivity, the cell colonization was higher and the cell distribution was more homogenous, although the cell differentiation decreased because of the reduced cell-to-cell contact [82]. On the other hand, in the *in vivo* conditions, the higher pore interconnectivity led to two-fold increase in bone growth rate compared to poorly interconnected scaffolds, thanks to the enhanced colonization [83]. Furthermore, the pore architecture has also shown to play a key role. The randomly oriented spherical pores enhanced osteogenic differentiation *in vitro*, which is related with better cell accumulation and therefore closer cell-to-cell contact. Nevertheless, when columnar and directional pores were used, the *in vivo* cell infiltration to the scaffolds was improved, allowing higher levels of angiogenesis [84]. It seems that not all the *in vitro* results can be extrapolated to the *in vivo* phenomena in terms of cell proliferation, osteogenesis and angiogenesis, and thus more systematic studies are needed. Overall, the pore size, interconnectivity and pore geometry should be designed carefully to achieve 3D scaffolds that favor specific cellular processes and bone regeneration.

Along with the surface nano/microtopology and porosity, the matrix stiffness (rigidity) governs the capacity of scaffolds for osteogenic control. Many recent bio-mechanical/-physical studies have explicit results on the role of matrix stiffness in dominating stem cell behavior, even from the very early stages to late osteogenic differentiation [85,86]. Cell behavior closely links to the elastic properties of the underlying substrate; in general, when the substrate has low stiffness, stem cells tend to change their fate into soft tissue lineages, such as nerve and cartilage. On the other hand, when the substrate is stiff, they sense the contractile force and tend to differentiate into osteogenic lineage [86]. It is because of the phenomenon known as 'mechanotransduction', in which cells are able to convert their mechanical stimulus into biochemical responses [87].

Over the past few years, several landmark studies have elucidated the decisive roles of matrix stiffness on the cellular fate control [88]. For example, an interesting study has touched on the role of matrix stiffness in cellular memory. The MSCs were first directed into two different lineages upon different matrices, that is, neuron on 0.5 kPa soft substrate and osteoblast on 40 kPa rigid substrate [89]. When the MSCs were then transferred to the opposite stiffness substrate, the neurogenic-lineage was changed to osteogenic,

however, the osteogenic MSCs still maintained high levels of osteogenesis markers, suggesting a degree of irreversible activation [89]. The polyacrylamide hydrogels coated with collagen were prepared with different elastic moduli (7 and 42 kPa). Osteogenic differentiation increased in the stiffer matrix and the phenomenon was through the signaling pathways of integrin alpha 2–FAK–ROCK [90]. In general, the scaffolds with stiffness over 30–50 kPa, which is similar to the stiffness of natural (uncalcified) osseous matrix, have the elasticity potential to drive osteogenic stimulation of stem cells [91,92].

The stiffness-matched scaffolds are thus considered at least as a standard matrix platform for bone regeneration. Designing a scaffold to possess the stiffness that matches to the native uncalcified osseous matrix or that is appropriate for directing stemness of cells into an osteogenic lineage may require careful tailoring of the 3D elastic properties at the nano/micro-levels (Fig. 3a). Recently, an interesting study reported the important role of the density of cell anchorage points. Although cells behave differently depending on the stiffness of hydrogels (0.5 kPa vs. 20 kPa), when the anchoring points of adhesive collagen were varied via concentration of cross-linker (high vs. low) in the matrices with the same stiffness, the cell behaviors were determinant as if the stiff hydrogel with low concentration anchoring points was similar to the soft hydrogel (Fig. 3b,c) [93]. However, the relationship between the anchoring points and stiffness needs to be further examined. Some strategic tools to tailor the stiffness levels matching to osteogenic matrices will be detailed in the next part.

Exogenous/external cues to bone regeneration

Tethered and delivered signaling molecules

The surface of the scaffolds plays a key role in the cell behavior, as it is the interface through which cells interact with the materials. Therefore, it is of great importance to tailor this surface composition to allow control over cell behavior. Regardless of the composition of the scaffolds, the commonly used approach is to tether

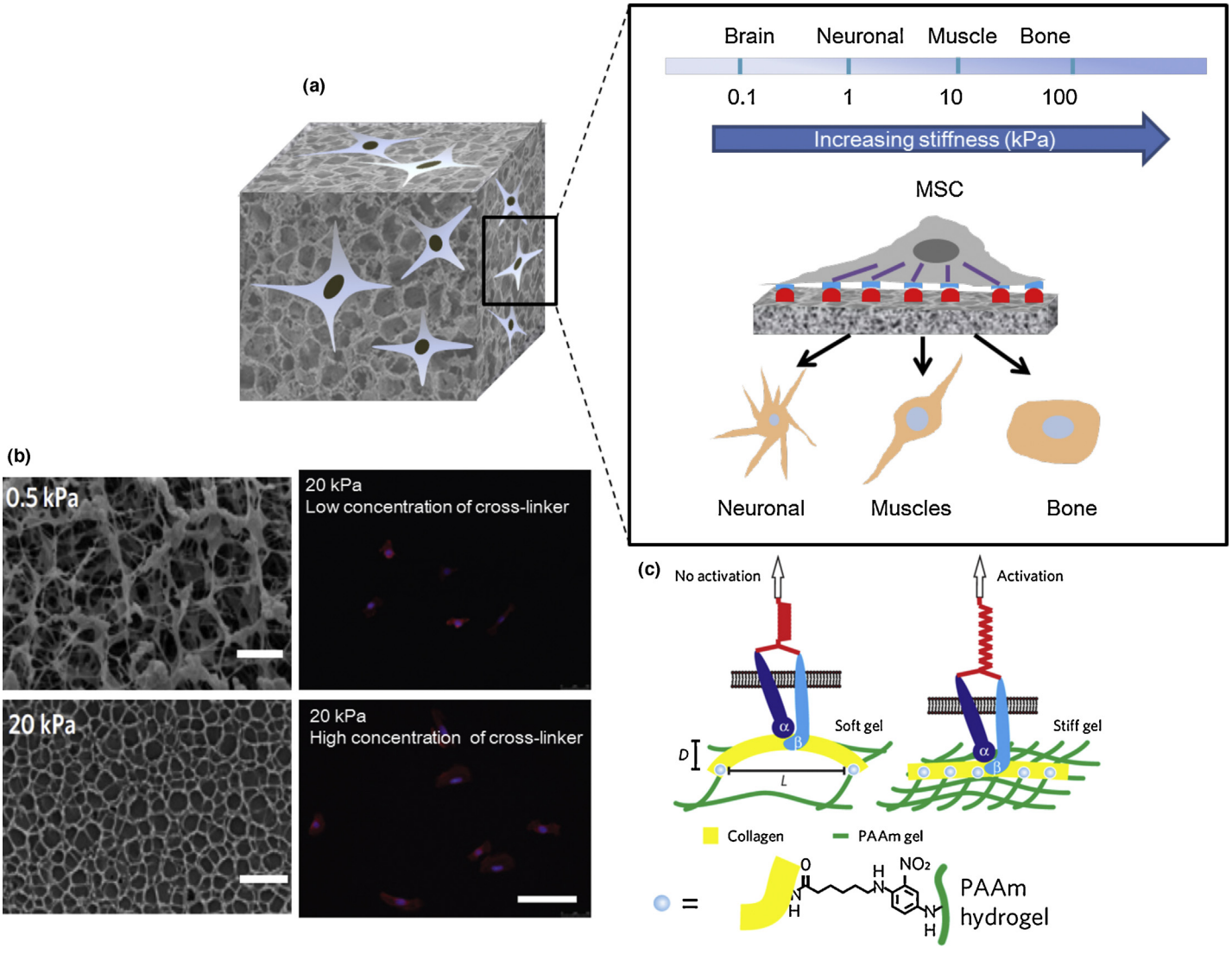

FIGURE 3

(a) Stiffness effects of the scaffolds on the differentiation behaviors of MSCs down to different lineages. Importance of cellular anchoring points as well as stiffness levels of the matrices that determine the cell fate; (b) hydrogel matrices typically with two different stiffness levels, and the higher stiffness gels implemented with two different concentrations of cross-linker, where the cells on the low concentration gel behaved as if they were in the low stiffness gel, (c) illustration showing the role of anchoring points of adhesive protein collagen in determining cellular behaviors [93].

ECM molecules. For example, cell-adhesive proteins, including fibronectin, vitronectin, collagen, laminin, and osteopontin, are the choice of ECM proteins which satisfy initial cell anchorage, and possibly subsequent processes. Not only with native form, but also with short domains of these proteins with similar functionality, was it possible to achieve the functions offered by native ECM proteins [94]. In a similar way, other relevant bioactive molecules, such as growth factors and drugs, have also been tethered on the surface of scaffolds to allow direct interaction between the molecules and cells [95].

An important issue in surface tethering is how to link the molecules to the surface of scaffolds. Although non-covalent links to a scaffold surface is possible because of hydrophobic or ionic interactions between surface chemical groups and protein molecules, the adsorbed proteins may easily desorb. Thus, for the biopolymer scaffolds, the linking is generally made by covalent bonding after the activation of the polymer surface chemistry. While the ECM proteins can tightly adhere to the surface with good physical stability, the protein activity can also be damaged because of the substantial conformational change and nonspecific bonds [96]. Therefore, a better approach is the affinity-binding, which enables relatively tight bonds while preserving the protein's biological activity. Specific binding domains can be engineered to the recombinant form of proteins or nature-inspired binding affinity can be introduced [97]. Recently, biopolymer bone scaffolds were developed to link bi-functional proteins through the specific binding of osteocalcin to HA crystal components mineralized on the surface, as illustrated in Fig. 4 [98,99].

In addition to surface tethering, the incorporation of biological molecules within the structure of scaffolds has been considered as a facile tool to give appropriate cues for the surrounding cells to alter their proliferation or differentiation. The commonly used biological molecules are drugs, growth factors, and genes that need to be released at desired times. For the case of ECM adhesive proteins, this incorporation may not be optimal as they can mediate cell interactions at the surface. On the other hand, osteogenic drugs including bisphosphonates and statins as well as many growth factors involved in bone regeneration can be incorporated within the scaffold networks [100]. The key issues are how to load the therapeutic molecules safely within the scaffolds and how to deliver them in a controlled manner. For example, use of nano/microcarrier materials for loading cargo molecules is effective in safely integrating the complexes into the scaffold structures.

Controlling the release profiles of cargo molecules is possible by tailoring physico-chemical properties of the scaffolds. Swelling, degradation, and mineral deposition that may occur in bone scaffolds can significantly alter the release pattern of the incorporated molecules. Particularly for the delivery of certain growth

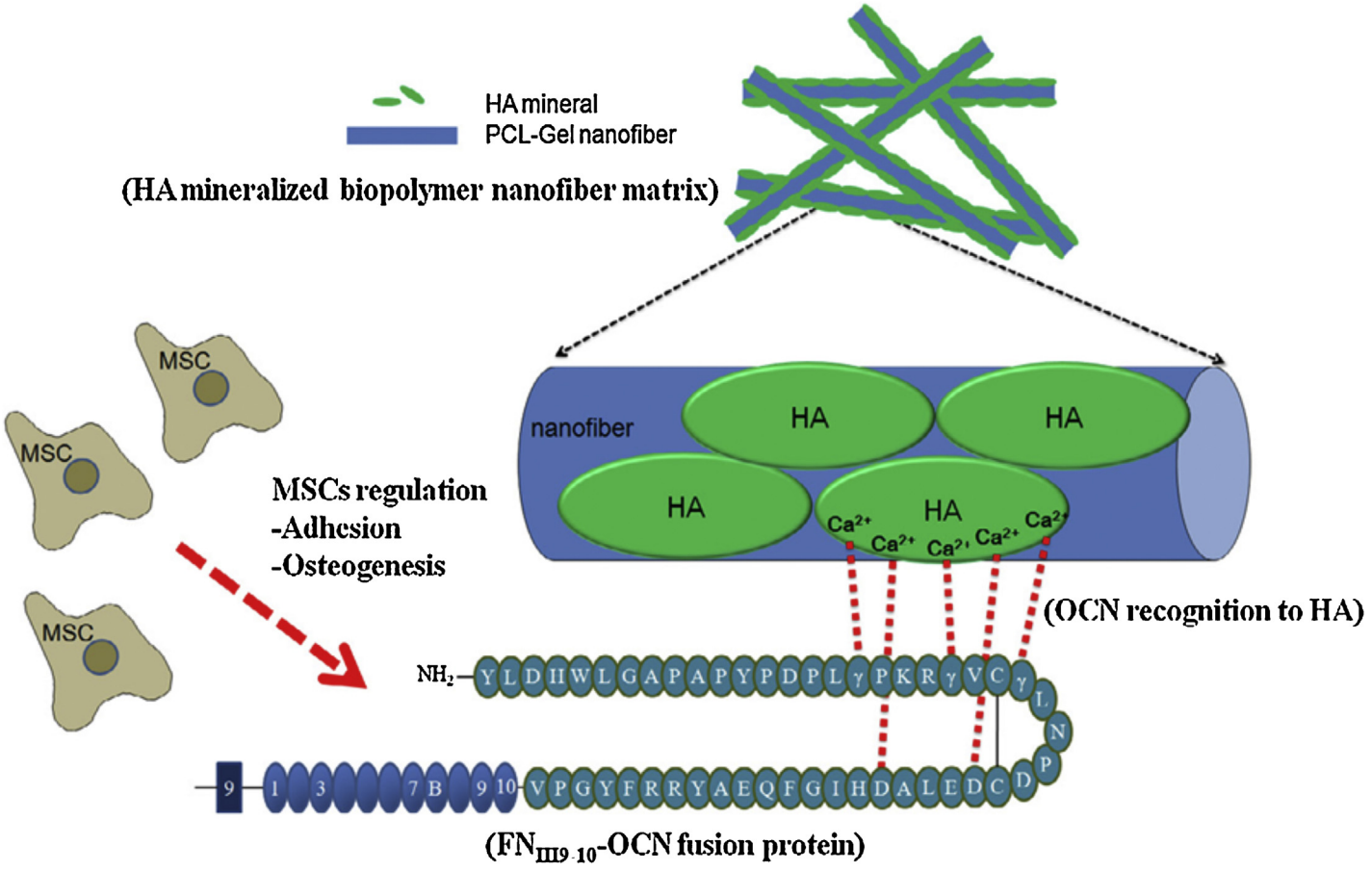

FIGURE 4

Example approach of affinity-driven protein tethering onto scaffolds. FN-OCN fusion protein tethered to HA-mineralized surface of biopolymer scaffolds through OCN molecular recognition to HA crystals (Ca ions).

factors, like BMPs, highly sustained release for periods of weeks to months is required, which can function at the late stage of bone maturation and mineralization. On the other hand, relatively rapid release of growth factors, such as pro-angiogenic or anti-inflammatory factors, is required initially in bone healing. After all, the action period and doses of released molecules should be carefully designed in the drug delivering scaffolds to enable optimal biological reactions, including anti-inflammation, cell homing, angiogenesis, osteogenesis, and bone maturation. Although the growth factors can also be directly tethered onto the surface of scaffolds, their actions will be largely limited to a short time frame; therefore, the incorporation within scaffold networks is possibly a more favored strategy.

Compared to the drug and protein molecules, the delivery of genes generally needs a nanocarrier which can protect the genetic molecules and allow cellular entry. The nanocarrier loaded with drug molecules can be combined with scaffolds either on the surface or within the bulk, which then are released to travel into the intracellular compartments, where the genetic information will be modified to alter the cell fate favorable for bone regeneration, as depicted in Fig. 5. Therefore, for the gene delivery, the nanocarriers that are endocytosed to cells and interact with intracellular organelles should be carefully designed to improve the gene transfection efficiency.

External stimuli

External stimuli can be applied to scaffolds to induce responsive actions of the scaffolds. The most common stimuli include light, electricity, magnetism, pH, temperature, or enzymatic reaction. The responsive actions to these stimuli are the change in size and temperature increase of scaffolds, which consequently enables potential drug delivery applications as well as hyperthermia treatment. There are a wide range of polymers that represent this responsiveness to external stimuli [101–107]. As the bone bioactivity of the stimuli-responsive polymers is relatively poor, for bone tissue engineering they are often combined with bioactive ceramics which can also contain bioactive molecules.

Among the different stimuli sources, temperature or pH-responsive scaffolds can be used for the treatment of bone tumors because cancer cells have slightly lower pH than normal cells and they can be killed by localized temperature increases. Often the temperature rise is linked with external magnetic fields. Scaffolds incorporating superparamagnetic nanoparticles are responsive to external alternating magnetic fields, with a temperature increase of a few degrees, which is effective for hyperthermia treatment of bone cancer. Furthermore, the deformation and volume change in scaffolds accompanied with the magnetic responsiveness gives rise to pumping out of drug molecules, enabling on-demand magnetic-responsive drug delivery systems (Fig. 6a). Another merit of external magnetic fields is that they have been shown to exert significant influence on the cell proliferation and osteogenic differentiation (Fig. 6b) [108–110]. Also, the magnetic scaffolds enable magnetic resonance imaging, which potentiates their therapeutic and diagnostic purposes (Fig. 6c) [111,112].

Design of therapeutic scaffolds: exemplar studies and applications

As mentioned above, the therapeutic actions of scaffolds can be diverse by intrinsically tailoring the properties or through utilizing exogenous factors and stimuli. While many different methodologies have been exploited thus far, here we describe some landmark approaches that have significantly improved the therapeutic potential of scaffolds for bone regeneration. These include fine-tuning the surfaces and bulks of scaffolds, control of ionic releases, sequential delivery of multiple drugs, and enabling stimuli-responsiveness.

Fine-tuning of surfaces and bulks

Tailoring the chemical and physical properties of the scaffold surface is a key approach to trigger initial cell sensing mechanisms,

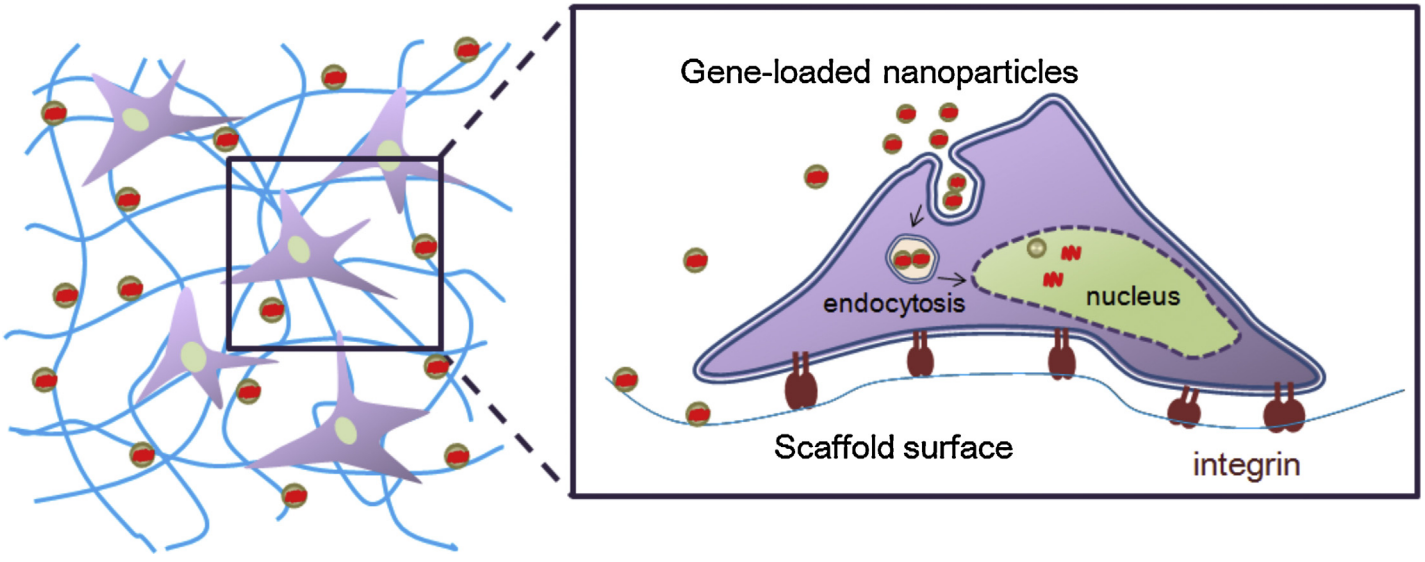

FIGURE 5

Strategy to non-viral gene delivery through scaffold systems. Nanocarriers complexed with genetic molecules hybridized to the scaffolds are endocytosed to travel through the intracellular pathways to genetically modify the cells.

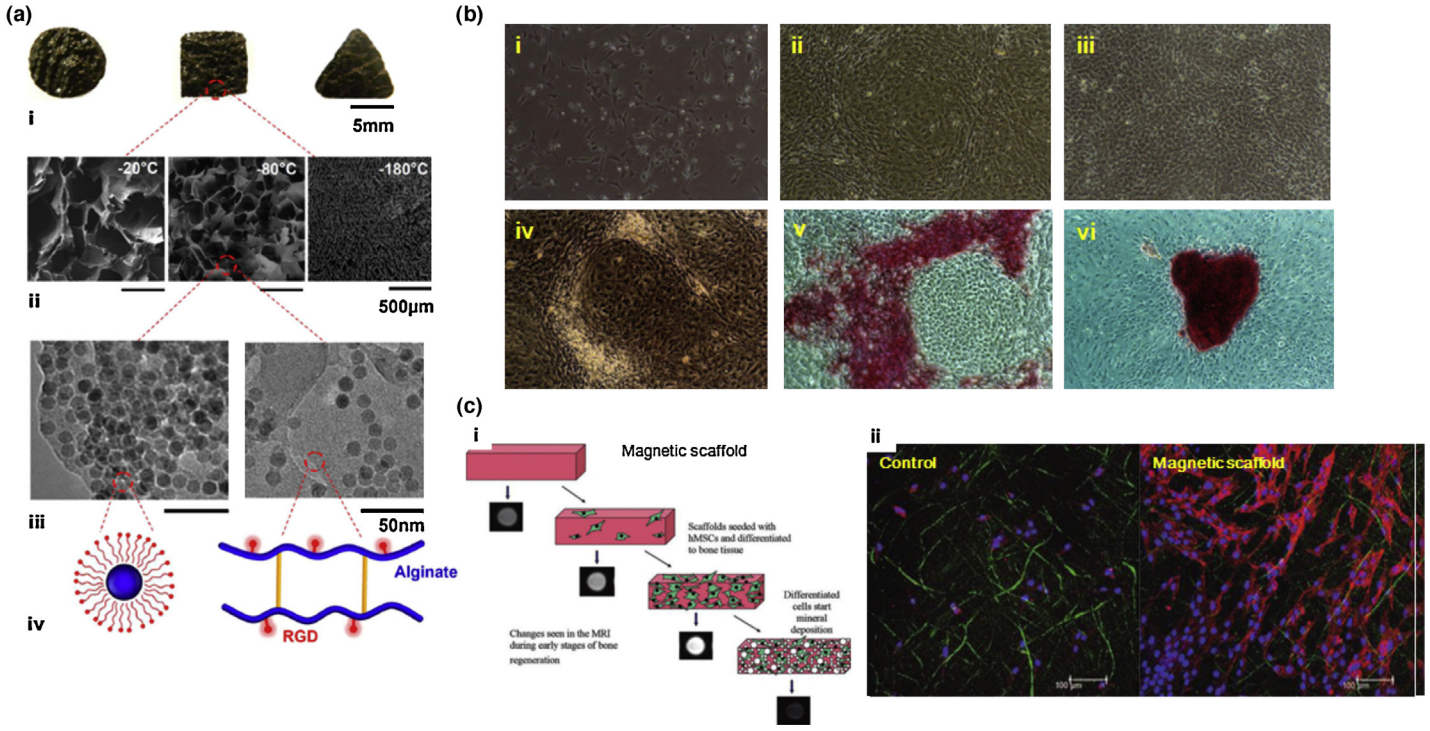

FIGURE 6

Potential applications of magnetic scaffolds for bone. (a) Photographs of magnetic scaffolds with various shapes (i). SEM images of scaffolds (average pore diameter from left to right: 700, 300, and 20 μm) (ii). TEM images of the scaffolds containing iron oxide nanoparticles at predetermined concentrations (*Left*, 13 wt%; *Right*, 4 wt%) (iii). Schematic plots of the nanoparticles coated with Pluronic F127 (*Left*) and covalently cross-linked alginate coupled with RGD sequences (*Right*) (iv) [113]. (b) Osteoblasts influenced by the magnetic fields: (i) fibroblast-like after 48-h culture, (ii) whirlpool-like circle after a 5-d culture, (iii) control after 5-d culture without SEMFs, (iv) calcified nodules formation after 8-d culture, (v) Alizarin red staining after a 10-d culture, and (vi) untreated control after 10-d culture (100×) [114]. (c) Magnetic scaffolds enabling magnetic contrast imaging; (i) schematic illustration of the different stages of changes in the MRI contrast during osteogenesis *in vitro*, (ii) confocal images of hMSC morphologies on the magnetic scaffold after 24-h culture stained for actin (red), nucleus (blue), and nanofibrous scaffold (green) [115].

which in turn potentiates further cellular fate like osteogenesis of stem cells. More than anything else, control over surface with nano-/micro-topology is a facile way to determine stem cell behaviors favorable for bone regeneration.

While nano-/micro-topological tailoring is yet to be widely realized on 3D scaffold systems, some pioneering studies have applied 2D approaches to 3D scaffolds [116]. Although not providing precise and ordered control over nano-/micro-topology, surface decoration with bone mineral crystal HA enables nano-roughness and excellent biological properties. For this, the surface of scaffolds was first activated chemically and then subsequently soaked in calcium phosphate ionic media. Porous foam and nanofibrous biopolymers could thus be tailored with apatite-mineralized surface [117]. The mineralized surface increased the hydrophilicity, protein adsorption capacity, and the cell adhesion processes. In fact, biological apatite minerals have been shown to impart excellent compatibility with many adhesive proteins and key bone-associated proteins. The mineralized PCL macrochanneled scaffolds increased the protein adsorption 5-fold higher than non-mineralized PCL [98]. Furthermore, the mineralized scaffolds released the protein molecules for a long period. For instance, the sustained release pattern of VEGF from Ca phosphate-mineralized poly(lactic acid) (PLA) scaffold was measured over a month [118]. As to the cellular responses, the mineralized surface enhanced the stem cell anchorage, spreading and differentiation into an osteogenic lineage [119]. The initial cell adhesion events may be a result of the rapid and selective adsorption of adhesive proteins, and the subsequent osteogenic differentiation mechanism would be triggered by the adhesion-related mechanotransduction [120]. Not only this anchorage-related mechanistic event, but the innate Ca and phosphate ionic sources can also be beneficial for the late stage of cellular mineralization on the secreted protein matrices.

For the case of bioactive ceramic scaffolds, the nanotopological feature can be primarily achieved by the nano-grain morphology. The HA scaffolds with different surface roughness from submicrometers to micrometers were produced [121]. The results showed increased osteoblast attachment and differentiation on the micro-roughened HA surface compared to the smoother surface. On the other hand, osteoclasts presented higher values of tartrate resistant acid phosphatase on smoother surfaces, and there was no inhibition of resorption on the micro-roughened surfaces because of the disruption of the actin-filaments. This was an interesting finding that reported on the nanotopology effects on not only osteoblastic responses, but also the osteoclastic cells. Because bone is consistently remodeled in the repair process by the interactive roles of osteoblasts and osteoclasts [122], the effects of scaffolds on both types of cells need to be considered carefully.

Calcium phosphate cements, an essential class of inorganic injectables, can also be tailored with different nano/micro-crystallized structure. By changing the starting powder size, the dissolution-and-reprecipitation process can be controlled. With fine powder, a more rapid process induces higher numbers and

smaller-sized nuclei of crystals, which consequently leading to nano-grained crystalline morphology of the cements [123]. Otherwise, some extrinsic nanomaterials can be coated on the bioactive ceramic scaffolds, such as carbon nanotubes. Bioactive glass scaffolds have been coated with carbon nanotubes to have nanotubular networked surfaces [124]. The nano-textured scaffolds showed excellent bone bioactivity similar to the native bioactive glasses. While those previous works have explored some possible ways to tailor the bioactive ceramic scaffolds with nano-roughened/-textured surfaces (as summarized in Table 2), there have, however, been no systematic exploration of texture or patterns on the surface of the bioactive ceramic scaffolds, which remains an area for further research.

For the metallic scaffolds, because of their electrical properties, many electrochemical methods can be utilized to achieve nano/micro-textured surfaces. Anodizing has been applied to many medical grade metals. In particular, nanotubular structures have recently attracted great interest. The nanotubular size is easily controlled by the anodizing parameters. Stem cell behavior on the nanotubular-structured Ti oxide surface have shown interesting results, with certain sizes revealing optimized osteogenic behavior [48]. Based on the 2D anodizing-texturing, the foam scaffolds have also been tailored to have nanotubular surfaces. Larger (~70- to 100-nm diameter) nanotubes induced dramatic cell elongation indicating differentiation into pseudo-osteoblasts, but there were no significant changes of stem cells on the smaller (~30-nm diameter) nanotubes.

While the surface of scaffolds can be fine-tuned with nano/micro-structures, the bulk properties of scaffolds can also be tuned to control and trigger stem cell fate favorable for bone regeneration. Above all, control of the stiffness of the scaffolds is of special importance. Because the different compositions generally lead to different mechanical stiffness, the examples may be huge; however, here we focus on the representative studies that aimed at exploiting this stiffness change of scaffolds.

For instance, glycosaminoglycan was used as an effective enhancer of the stiffness of collagen hydrogel matrices, resulting in an increase in cell population and activity [135]. Similarly, a chemical crosslinker, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), also enhanced the structure of gelatin scaffolds. As a result, chondrogenic differentiation was favored at early time points, while both chondrogenic and osteogenic differentiation occurring at later time points [136]. It indicated that mechanically strengthened scaffolds promoted osteogenesis by enhancing endochondral ossification. As another example, gelatin scaffolds linked to transglutaminase increased in their mechanical properties with increasing gelatin concentration [137]. Stiffer scaffolds promoted osteogenic differentiation by facilitating focal contact formation, while softer scaffolds promoted cell proliferation. Using silk-based composites, the combined effects of surface topography and stiffness on the myogenic or osteogenic differentiation of C2C12 cells and MSCs, respectively, have also been reported [138]. The MSCs cultured on polyacrylamide gels with higher stiffness showed preferences to osteogenesis [85]. On the stiffer substrates made of PEG-based polymers, the osteogenic differentiation could also be enhanced [139].

An important approach to tailor surface and bulk properties of scaffolds that are favorable for bone regeneration is through the biomimicry concept, which employs bottom-up methodologies to produce scaffolds with compositions and structures mimicking those of native bone. One of the biomimicry modifications of scaffolds is possible by the nature-inspired surface tethering which primarily aims to enable bio-recognition at the nature-inspired molecular level, and thus introduces specific interactions of enzyme–substrate, antibody–antigen, and ligand–receptor [140–142]. The most issued biomimicry design has been on the mineralization step over the organic templates to mimic the native mineralized bone structure. One representative study performed by Hartgerink *et al.* developed peptide amphiphile nanofibers that contain highly phosphorylate serine residue that is designed to interact strongly with calcium ions and help direct mineralization of hydroxyapatite. The alignment of mineral phase was found to be the same as that observed between collagen fibrils and hydroxyapatite crystals in bone [143]. The peptide amphiphile gel matrix containing phosphorserine residues in the sequence has also shown significantly higher bone formation at 4 weeks relative to controls lacking phosphorylated residues and comparable bone formation to that observed in animals treated with a clinically used allogenic bone matrix [144]. On the other hand, Spoerke *et al.* utilized enzyme-mediated bone mineral induction over the peptide amphiphile nanofiber assembly [145]. Likewise, the fibrin nanofibers, when immobilized with alkaline phosphatase, an enzyme involved in bone mineralization, have shown enhanced mineral deposition and gene expression of osteoblast markers with respect to the enzyme-absent nanofibers [146]. While the chemically driven mineralization processes at the molecular level over

TABLE 2

Methods to create nanotopological features on the surface of bioceramic-based bone scaffolds.

Method	Pros	Cons	Topographical feature
Electron beam lithography [125]	Precise geometry Easy patterning	High-cost Need of high energy electrons	10–100 nm
Colloidal lithography [126,127]	Easy patterning, low-cost Rapid coverage of large area	Only simple features	10–100 nm
Photolithography [128]	Precise geometries and patterns	High-cost Limited wavelength of the light source	100–200 nm
Simulated body fluid (SBF) treatment [129,130]	Similar to human body blood plasma Easy apatite coating	Weak apatite coating	Concentration and time-dependent
Composite apatite coating [131]	Little flaking off apatite coating	Difficulty in control over surface roughness	600–800 nm
Polymer demixing [132,133]	Simple, fast, inexpensive, control over height	Only simple features Limitation in pattern organization	10–100 nm
Chemical etching [134]	Simple, fast, low-cost	Only simple features	<100 nm

the organic templates have shown some significance in largely mimicking native bone structure, the cellular-processed mineralization in the physiological conditions and the resultant mineralized matrices can be, strictly speaking, more mimic to native ECM of bone.

Control of therapeutic ions

Many ions are trace elements of bone, and the deficiency of certain ions causes significant dysfunction and diseases related with bone, such as osteoporosis [147]. In bone formation, many biological ions play essential roles in up-/down-regulating cellular functions and biological processes [148]. Therefore, the exogenous utilization of ions is an effective way of tailoring scaffolds toward therapeutic purposes in bone repair and regeneration, as illustrated in Fig. 7. Here we cover the ions that have shown beneficial effects on bone biological processes including cell mitosis, osteogenesis, angiogenesis and antibacterial properties. The candidates are Ca, Zn, Cu, Sr, Si, Co, Ag and Ga. These ions can be incorporated into the scaffolds to be released at therapeutically relevant doses in an appropriate time frame to elicit beneficial cellular responses.

General incorporation of the ions is achieved by an addition of ionic precursors within the chemical structure of bioactive ceramics scaffolds, such as in the crystalline form or in the glassy phase [149]. For example, representative calcium phosphate bioceramic scaffolds (HA and tricalcium phosphate) can contain cations (Zn, Sr, Si, Co, Cu, among others) in partial replacement of Ca without significantly altering the crystalline chemical structure [150]. In a similar way, bioactive glasses (phosphate-based or silica-based) can easily incorporate those relevant ions within the random networks as intermediates or modifiers [151]. The different ions can be structured during the thermal (sintering or quenching) processes of the ceramic scaffolds, direct incorporation of ionic compounds can be obtained for the biopolymer scaffolds. Some pioneering works have shown the release of different levels of Ca ions from collagen gels, showing different effects on cell behaviors [152]. Sr ions were also incorporated into carboxymethylcellulose hydrogels as a bone scaffold to enable ionic stimulation of cells for bone formation.

Most studies have incorporated the inorganic phase in the form of composites and hybrids where the inorganic phase provides ion sources while polymeric part supports scaffold frame. The most

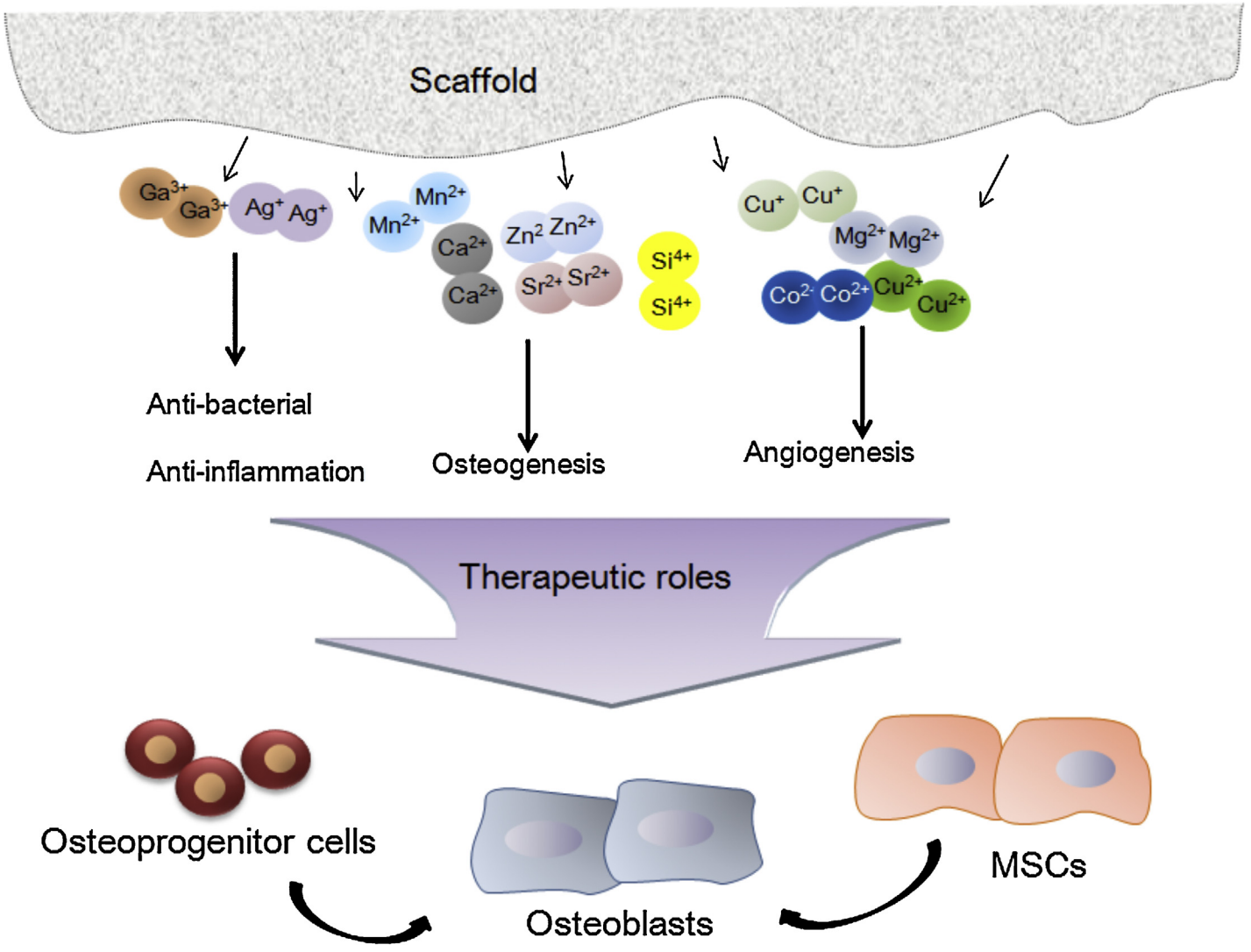

FIGURE 7

Biologically therapeutic ions control the multiple functions of cells (osteoblasts and stem cells).

widely studied ion is Ca, which mediates multiple cellular responses, including cell proliferation and osteogenesis [153]. While many *in vitro* studies have demonstrated the proper ionic doses favorable for cell proliferation and osteogenesis, a limited number of studies have examined the functions in 3D scaffolds. Different Ca concentrations incorporated into a collagen gel showed that low concentrations (2–4 mM) promoted osteoblast proliferation, medium concentrations (6–8 mM) induced cellular differentiation and mineralization, whereas concentrations greater than 8 mM induced cytotoxicity [154]. On the other hand, high Ca content incorporated within silica based microspheres showed enhanced cell proliferation [155], while the increased Ca level was shown to be related with lower levels of ALP activity [151]. Therefore, the released dose of Ca ions from the scaffolds is of great importance.

Another interesting phenomenon where Ca ions play a key role is the ability to polarize the phenotype of macrophages. As previously introduced, the switch of macrophage phenotype from M1 (inflammatory) to M2 (regeneration) has been shown to enhance bone regeneration. In this sense, β-TCP extracts were shown to induce this polarization of macrophages although the activation of the Ca-sensing receptors which in turn enhanced the BMP2 expression. Interestingly, these macrophages were then able to induce osteogenesis of MSCs, suggesting the importance of ionic extracts and the macrophage-regulated osteogenesis [30,156].

Si also plays a key role in bone growth and has shown excellent *in vitro* and *in vivo* osteogenic potential [157]. Several biomaterials have been developed that incorporate Si into their structure. For instance, β-tricalcium phosphate and dicalcium phosphate were prepared incorporating Si and showed excellent *in vivo* ability to form bone [158]. Similarly, Si-substituted HA granules and porous scaffolds were implanted in a rabbit model to show increased bone ingrowth and bone implant coverage [159]. Another report used small particles of Si-doped vaterite which were combined with PLA to obtain electrospun microfiber scaffolds [160]. The fibers were shown to release Si ions over 7 days and enhanced osteoblast proliferation *in vitro*, as well as having higher levels of new bone formation *in vivo*.

Another promising ion is Zn, which is known to be essential for the formation and mineralization of bone [161]. Zn-doped Ca phosphate scaffolds [162] or biopolymer scaffolds [163] showed improved osteogenic differentiation. When incorporated into phosphate-based glasses, bone cell adhesion and proliferation were also enhanced [164]. Sol–gel derived bioglasses after Zn incorporation increased ALP activity in osteoblastic cells [55].

Similarly, Sr is a key ion found in natural bone, and its deficiency is known to be related with osteoporosis [165]. Sr was incorporated within phosphate glasses, and the release increased cellular proliferation [166]. When incorporated within mesoporous bioactive glass scaffolds, Sr stimulated osteoblastic proliferation and differentiation of MSCs and osteoblastic cells [167,168]. Furthermore, *in vivo* increased bone formation was enhanced by Sr ions released from the mesoporous bioactive glasses scaffolds [168–170] and Ca phosphate scaffolds [171]. Recently, Sr-substituted bioactive glass-incorporated PCL nanofibers were developed [172]. The results showed that the dissolution of the Sr ions into the culture media promoted the precipitation of CaP layer on the nanofiber structure, stimulating pre-osteoblastic cells to express high levels of ALP. Sr ions have also been shown to play a key role in suppressing inflammatory response. The Sr-containing CaP stimulated the macrophage phenotype shifting toward M2 [173,174].

Along with the osteogenic stimulation of ions, the angiogenesis has also been stimulated by the use of ions. Co has shown to be a powerful regulator of initial angiogenesis responses, inducing hypoxia conditions and stimulating the production of VEGF and up-regulation of angiogenic-related genes, as illustrated in Fig. 8 [175]. Some recent works have shown not only their angiogenic stimulating effects, but also their osteogenic roles, making it a promising candidate for bone regeneration purposes. One recent work elaborated bioactive glass scaffolds incorporating Co at low quantities (<5%), and showed up-regulation of the VEGF production and bone-related genes of MSCs [176]. Cu has also been shown to be an angiogenic stimulator. Cu ions incorporated into mesoporous bioactive glasses enhanced initial angiogenesis followed by an improved osteogenesis [177]. Similarly, very small doses of Cu have also simulated blood vessel formation. When added to macroporous brushite scaffolds at a low dose (56 ng) the vascularization was substantially enhanced. However, a higher dose (560 ng) enhanced wound tissue ingrowth [178]. Here again shown was the significant dose-dependent role of trace elements in biological outcomes, and the use of therapeutically appropriate doses to gain required functions.

Bone scaffolds have also been designed with antibacterial properties through the incorporation of antibacterial ions, such as Ag and Ga. Ag binds to the bacteria's DNA, preventing its replication. The release of Ag from phosphate and silicate based glasses has been shown to be effective against bacteria [179]. Ga has shown not only antibacterial effects, but also therapeutic effects in bone cancer treatment. The Ga-doped phosphate-based glasses showed antimicrobial activity against planktonic *P. aeruginosa* by Ga release [180]. Furthermore, the Ga-containing glass polyalkenoate anti-cancerous bone cement showed anti-inflammatory and immunosuppressive activity in the *in vivo* studies [181].

While the series of works reporting the ionic stimulatory effects on cellular and biological responses have implicated the potential utility of those ions, systematic studies have yet to be carried out, in terms of the controlled release of those ions at appropriate doses

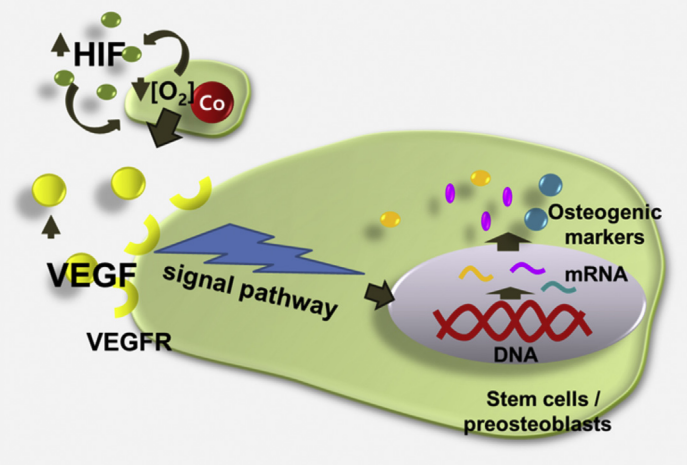

FIGURE 8

Illustration showing the Co ion-induced angiogenic effects on cells.

and time frames from the bone scaffolds, which needs to be studied.

Multiple and sequential drug/protein delivery

Drug delivery has become of great interest to stimulate cell differentiation while maintaining high rates of cell proliferation during short periods. One approach has been the elution of two different biological molecules at different defined times. For instance, initial proliferative stimulation or stem cell homing with molecules such as FGF or SDF1, while allowing a proper differentiation at longer time points with BMP-2 or dexamethasone among others has been recently explored. Furthermore, this mimics the biological process that takes place *in vivo*, where tissue regeneration is not merely based on a single molecule, but rather a combination of several molecules that are sequentially delivered. For this phenomenon to take place, the scaffolds need to be designed to allow the release of the molecules at specific time points. A common approach is the use of layered scaffolds, where two well defined layers can be obtained, having different properties in terms of compositions and thus degradation rates that can allow the release of molecules at different times. In a similar way, coating on the scaffolds has allowed a multiple and sequential release pattern. More sophisticated designs involve the use of micro- or nano-particulates incorporated in the scaffold matrix that is able to hold cargo molecules.

Bone has attracted considerable attention because of its intrinsic regeneration properties which involve a cascade of several growth factors. For instance, bone regeneration is known to start with an initial angiogenesis process followed by proper bone maturation. Therefore, several studies have focused on the design of scaffolds that are capable of releasing angiogenic factors over short periods while releasing osteogenic growth factors over longer periods, as presented in Fig. 9. The combinations of VEGF or FGF and BMP-2 or dexamethasone become good examples of molecules contained in these scaffolds. An example was the layered scaffolds made of polyelectrolyte films prepared by a layer-by-layer technique [182]. The sandwich structured allowed the entrapment of the desired growth factor within the layers, and led to a rapid release of VEGF for up to 8 days and a sustained release of BMP-2 for over 2 weeks. This design stimulated the proliferative capacity of HUVEC and the differentiation of pre-osteoblasts, as well as enhanced *in vivo* ectopic bone formation in a synergistic way. On a similar note, FGF and BMP-2 also showed a synergistic effect *in vivo* when these were sequentially released from collagen scaffolds [183]. In another approach, sequential delivery of VEGF/BMP-2 was also achieved in the VEGF-loaded alginate fibers embedded in BMP-2-loaded PLA scaffolds [184]. The VEGF was released for 7 days and BMP-2 release was sustained up to 28 days, performing excellent bone regeneration ability ascribed to the sequential release. Core–shell fibrous scaffolds consisting of alginate and α-TCP were developed to effectively delivery dual factors, where the components loading each protein were extruded via a dual concentric nozzle into a $CaCl_2$ solution, generating fibrous scaffolds for *in situ* controllable delivery [185]. Micro-/nano-particulates are often incorporated into scaffolds to sustain the delivery period and enable multiple factors delivery. In an example, BMP-2 loaded PLGA microsphere composite with polypropylene scaffold was surrounded with gelatin hydrogel containing VEGF. The BMP-2 release was sustained

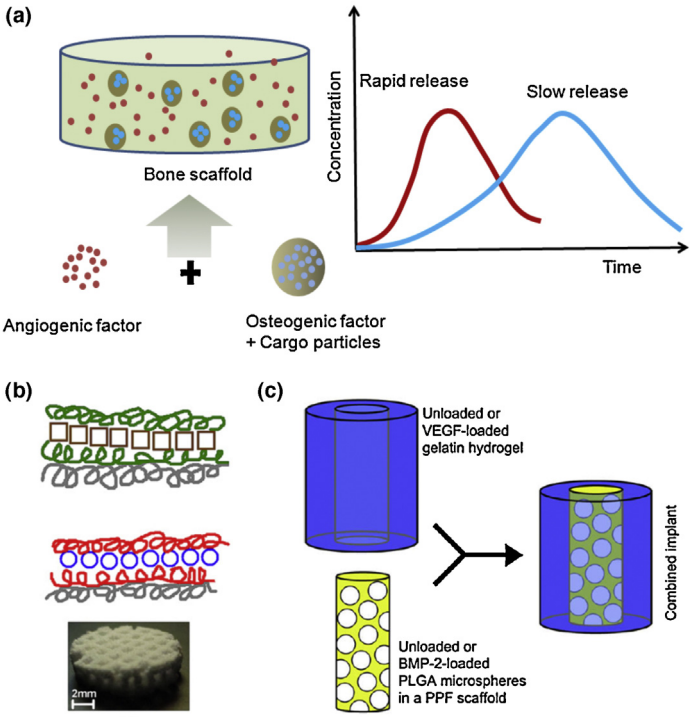

FIGURE 9

Schematic showing the designs of therapeutic bone scaffolds with sequential delivery potential of bioactive signals. (a) Sequential release of angiogenic and osteogenic molecules enabled by the particles-incorporation. (b) Polyelectrolyte tetralayer film assembly loading positively charged rhBMP-2 and rhVEGF165 [182]. (c) Composite core–shell scaffolds for sequential delivery of VEGF and BMP-2 [186].

over the full 56-day implantation period whereas VEGF release ended up within the first 3 days [186]. The mesoporous bioactive nanospheres that incorporate one type of drug molecule were embedded in PCL-gelatin nanofibrous matrix that was loaded with the other molecule to show sequential delivery profile [187]. In a similar design, the osteogenic FGF18 was pre-loaded onto bioactive nanoparticles, which were incorporated in the core–shell electrospun fibers that also contained cell proliferative FGF2. The sequential delivery patterns of FGF2/FGF18 were proven, and the capacity to stimulating cellular responses and the *in vivo* bone formation was well demonstrated. Collectively, the strategy of sequential delivery of more than two different molecules from the specified matrix is achievable in a controllable manner, and depends on the use of matrix materials with inherently different physicochemical properties, which become promising for effective bone regeneration.

Stimuli-responsive scaffolds

Stimuli-sensitive materials are generally used to deliver drugs on demand. Materials responding to changes in the surrounding environment can provide a potential to regulate cell responses, as illustrated in Fig. 10. However, most of the stimuli-sensitive materials are inherently bioinert with poor biological properties and low biomechanical functionality. For this reason, the stimuli-sensitive materials are often combined with biocompatible and natural materials.

Modifying the scaffold surface with temperature-responsive polymers, such as poly(*N*-isopropylacrylamide) (pNIPAAm) [188]

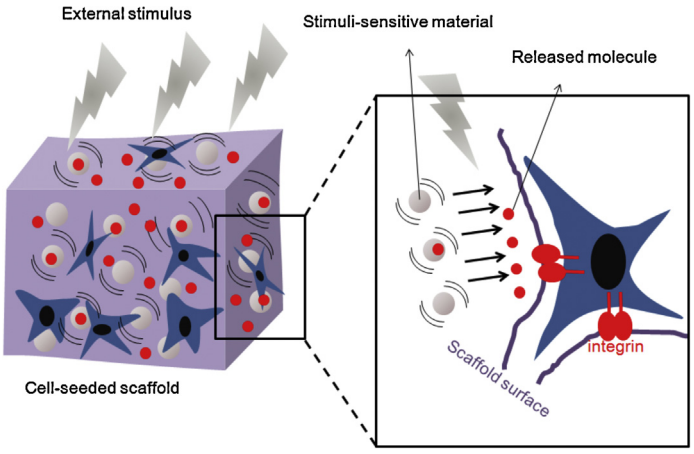

FIGURE 10

Illustration showing the stimulation of cell responses exerted by the stimulus-sensitive scaffolds.

and pluronics [189] changes wettability by surface-bound molecular motions, ultimately leading to promoted responses of bone-associated cells [190]. Hydrogel forms or microspherical and nanofibrillar structures are some of the applications of the stimuli-responsiveness materials for on-demand drug delivery. The alginate microspheres coated with pNIPAAm showed the transition at near-body temperature [191–193]. Similarly, nanofibrillar synthetic polymers based on PEO and pNIPAAm showed excellent on demand drug eluting properties [194]. The release profiles could be adjusted by changing the ratio between PEO and pNIPAAm. Similarly, drug-loaded thermo-sensitive liposomes were also employed by crosslinking to the surface of collagen/HA scaffolds [195]. A paste made of temperature-sensitive PLGA/PEG particles and growth factors entrapped could harden at 37°C owing to the temperature-induced PEG leaching [196]. In another view, the electrolytic surface of scaffolds is effective for pH-dependent drug release. Recently, a protein-eluting polyelectrolyte complex of poly(L-histidine) and poly(methacrylic acid) coating on the titanium surface showed the pH-dependent release pattern; rapid release at pH = 5–6, but sustained release at neutral pH (7–8) [197]. Dextran hydrogels hybridized with silica xerogels with the conjugation of doxorubicin drug also showed pH-responsive drug release [198].

Magnetic scaffolds that incorporate magnetic nanoparticles (MNPs) present magnetism-responsive properties, where the MNPs can change the shape of scaffolds as well as can deliver bioactive molecules, upon the application of an external magnetic field [199,200]. The presence of MNPs within an alginate matrix was effective for on-demand release of cells and bioactive molecules. The alternating magnetic field allowed significant volume change (~70%) of scaffolds, triggering the release of not only proteins and genes, but also cells [113]. MNP aggregation occurs when the external magnetic field is applied, shrinking the structure of the matrix, and allowing on demand release [199,200]. Crosslinked gelatin hydrogels were also fabricated to incorporate MNPs which were able to control the release of the cargo under the external magnetic fields [201]. Similarly, PVA hydrogels containing MNPs were able to contract upon applying magnetic field, which allows drug accumulation around the hydrogel [202].

Concluding remarks

Significant progress has been made exploring scaffolds that are therapeutically relevant, for the repair and regeneration of bone. Therapeutic actions that recruit and trigger cells after the scaffold implantation must be harmonized with the dynamic biological events involved in bone healing, including anti-inflammatory reactions, angiogenesis to form blood vessels, homing of progenitor/stem cells, and driving them toward osteogenic lineage and matured state. Thus, a variety of intrinsic and extrinsic factors have been considered in designing therapeutic bone scaffolds.

For instance, chemical intrinsic factors such as surface composition influences initial cellular events while the bulk degradability is an important parameter for mechanical degradation and the bone regeneration rate. In particular, the ionic components of scaffolds have been highlighted to determine some of the key events in angiogenesis, cell mitosis, and bone maturation. On the other hand, intrinsic physical properties, primarily matrix stiffness and nano-/micro-topology, have been tailored to regulate a series of cellular reactions in the cell anchorage and osteogenic differentiation. However, those physical factors have been mainly studied in 2D conditions, while the translation into 3D scaffold conditions requires further exploration.

While the tailoring of the intrinsic properties of scaffolds is a simple approach, more extensive and powerful therapeutic actions can be allowed through extrinsic factors. Many cell-adhesive ECM proteins, including fibronectin, vitronectin, collagen, and osteopontin, have been tethered to the surface of scaffolds to enhance initial cell anchorage and subsequent cellular events. An important issue in the surface tethering is how to link the protein molecules to the surface of scaffolds. Above all, the affinity-binding of engineered proteins to the scaffold surface has recently been highlighted as an effective technique. On the other hand, osteogenic drugs and many potent growth factors have been incorporated within the scaffold networks. In this case, scaffolds that load the therapeutic molecules safely and in large quantities and deliver the molecules in a controlled manner need to be designed. Compared to drug and protein molecules, the delivery of genes generally needs nanocarriers which can secure genetic molecules and allow cellular entry, to be interactive with intracellular organelles, and to enable gene transfection. Therapeutic control over the scaffold for bone regeneration is also possible by developing the scaffolds to be stimuli-responsive. The responsive actions to stimuli, like light, electric fields, magnetic fields, pH, temperature, or enzymatic reaction, can alter the status of scaffolds that are ultimately useful for on-demand drug delivery and hyperthermia therapy.

In fact, many recent approaches of developing scaffolds have featured these design strategies, which include fine 3D tuning of nano-/micro-topology of scaffolds to dictate cellular fate, control over the incorporation and delivery of ions with therapeutic potential in bone cell stimulation and mineralization, and the loading and delivery of growth factors in multiple or sequential manners to harmonize angiogenesis and osteogenesis. Although these scaffolds might not exactly replicate the structure and properties of native bone ECM, their performance in specific tasks can be potentiated when designed properly to exert therapeutic actions in the curing of bone diseases and the healing and regenerating of damaged/dysfunctional tissues.

Acknowledgement

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