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Development of Controlled Drug Delivery Systems for Bone Fracture-Targeted Therapeutic Delivery: A Review

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Abstract

Impaired fracture healing is a major clinical problem that can lead to patient disability, prolonged hospitalization, and significant financial burden. Although the majority of fractures heal using standard clinical practices, approximately 10% suffer from delayed unions or non-unions. A wide range of factors contribute to the risk for nonunions including internal factors, such as patient age, gender, and comorbidities, and external factors, such as the location and extent of injury. Current clinical approaches to treat nonunions include bone grafts and low-intensity pulsed ultrasound (LIPUS), which realizes clinical success only to select patients due to limitations including donor morbidities (grafts) and necessity of fracture reduction (LIPUS), respectively. To date, therapeutic approaches for bone regeneration rely heavily on protein-based growth factors such as INFUSE, an FDA-approved scaffold for delivery of bone morphogenetic protein 2 (BMP-2). Small molecule modulators and RNAi therapeutics are under development to circumvent challenges associated with traditional growth factors. While preclinical success has shown promise experimentally, drug delivery has become a major hurdle stalling clinical translation. Therefore, this review overviews current therapies employed to stimulate fracture healing pre-clinically and clinically, including a focus on drug delivery systems for growth factors, parathyroid hormone (PTH), small molecules,

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and RNAi therapeutics, as well as recent advances and future promise of fracture-targeted drug delivery.

Graphical abstract

1. Introduction

Although fractures typically heal following standard clinical interventions, approximately 10% of 7.9 million fractures annually in the US exhibit impaired healing, leading to delayed union and non-union. To date, bone morphogenetic protein-2 (BMP-2) and BMP-7 are the only FDA-approved therapies to specifically treat long bone fracture non-unions and recalcitrant long bone non-unions, respectively [1]. However, the limitations of BMPs are plentiful. BMP delivery requires surgical implantation at the site of fracture, which may result in local complications such as edema, infection, and heterotopic bone formation [2]. Furthermore, delivery of supra-physiological doses is often required to achieve therapeutic effects, leading to adverse side effects, clinical complications, and prohibitive costs [3–6]. Thus, development of alternative bone-acting therapeutics that can safely enhance fracture healing has great potential.

Innovative drug discovery strategies, such as high throughput functional screens, have led to the identification of many novel small molecule compounds and the repurposing of existing FDA-approved small molecule compounds. Small interfering RNA (siRNA), a new modality that a large number of pharmaceutical companies are evaluating, also has great potential to become the next-generation treatment for fracture healing. Delivering alternative therapeutic agents that target a variety of stages of healing, including inflammation, angiogenesis, and osteogenesis, augments fracture healing while avoiding the risks associated with growth factors. However, without targeting strategies, less than 1% of systemically injected therapeutics reaches bone, leading to sub-optimal therapeutic efficacy and side effects [7, 8]. These limitations highlight the need for targeting strategies and delivery systems to enable clinical success of bone-acting therapeutics.

With the goal of developing drug release systems to deliver therapeutics to bone fractures, research has been focused on local delivery and targeted delivery, which can alter drug biodistribution to augment fracture healing rate. This review highlights mechanisms of fracture healing and outlines current treatments and drug delivery platforms for next-

generation therapeutics for bone fractures. Drug delivery hurdles such as poor drug solubility, short half-lives, and off-target effects are addressed. Given the recent advances in biomaterial and drug delivery fields, combining potent therapeutic agents with ideal delivery systems will meet the challenges of fracture healing in the clinical setting.

2. Fracture healing mechanism and clinical significance

2.1 Mechanisms of normal fracture repair

Normal fracture healing is a complex, multistep process involving cellular events influenced and regulated by locally produced and systemically circulating factors (Figure 1). Hematoma and inflammation are the immediate reactions to injury, with soft tissue damage, disruption of vessels, and bone necrosis occurring simultaneously. In response to hematoma, inflammatory cells, such as macrophages and platelets, release cytokines to increase blood flow and cell migration to fracture sites [9]. Osteoclasts are activated to resorb bone debris, and mesenchymal stem cells (MSCs) stimulated by the cytokine release are recruited to the fracture. MSCs are multipotent stromal cells that are able to differentiate into multiple lineages (i.e. osteoblasts, chondrocytes, and adipocytes) based on soluble, matrix-associated, and biomechanical cues (Figure 1). The inflammatory response peaks within 24 hours of injury and is resolved after 7 days [10]. In the next stage, MSCs from the periosteum and endosteum proliferate and differentiate to chondrocytes, which contribute to the formation of a soft callus composed of cartilaginous tissue. Blood vessels also infiltrate into the callus to provide nutrients. As healing proceeds, the cartilage undergoes mineralization to form a hard callus that transitions to immature woven bone. Osteoblasts play a major role in this progressive stage by synthesizing type I collagen and directly generating calcified tissue. At the final remodeling stage, excess bone callus is resorbed by osteoclasts, and cycles of osteoblast and osteoclast activity ensue, restoring healthy bone morphology and mechanical properties.

2.2 Impaired fracture healing classification and diagnosis

The healing process fails in approximately 10% of fractures, culminating in a diagnosis of delayed union or non-union. The clinical evaluation of healing is based on both radiographic findings to identify bridging callus, the number of bridged cortices, and the disappearance of fracture lines, as well as clinical factors including absence of pain or tenderness at the fracture site during weight-bearing activity [11]. A fracture is considered delayed if it has not healed after 4 months [11], whereas a non-union is defined as a fracture that persists at least 9 months post-injury with no signs of healing for 3 months [12]. Non-unions are further categorized as hypertrophic or atrophic [13]. Hypertrophic non-unions have adequate vascularity and biological activity to progress to union but suffer from bony instability evident on radiographs with excessive callus in response to motion at the fracture site [13]. On conventional radiographs, the hypertrophic non-union displays a large, broadened callus towards the fracture gap, with a radiolucent area instead of bone bridging. Atrophic non-unions lack vascularity and sufficient osteogenic reaction and show no evidence of callus formation and healing. In radiological images, the atrophic non-union lacks callus tissue and exhibits narrowing of bone ends and a large radiolucent zone in the fracture gap [11].

2.3 Impaired fracture healing risk factors

Many physiological, pathological, or environmental factors can compromise fracture healing (Figure 1). These include fracture severity and location, concomitant use of many common medications, and disease comorbidities. For example, osteoarthritis, rheumatoid arthritis, and diabetes increase the risk of non-union by at least 40% [14]. In addition, a variety of other factors such as gender, age, alcohol consumption, and chronic inflammation affect healing. Defining patients at high risk of non-union early and accurately should result in more appropriate, patient-specific treatment interventions for successful healing outcomes.

2.3.1 Injury characteristics and related factors—The time to reach complete fracture healing is highly dependent on the extent of tissue damage and trauma [15]. More severe injuries with considerable tissue loss and serious soft tissue and vascular system damage are associated with delayed healing due to reduced blood flow to fracture sites. The reduced supply of nutrients and osteoprogenitor cells increases the potential for bone necrosis and inhibited repair [16]. Open fractures, which disrupt the hematoma and perturb initiation of the inflammatory phase of repair, can lead to impaired healing [17].

2.3.2 Fracture treatment-related factors—Initial cortical apposition in surgical treatment is crucial to ensure proper fracture healing. This is best highlighted in cases of severe trauma and osteoporotic fractures, where successful cortical apposition is difficult, directly leading to increased risk of impaired healing [17]. Even subtle fixation failure can prolong healing, demonstrating that fracture stability is critical for repair, as bone cells are sensitive to mechanical cues [16]. For example, hydrostatic forces promote pro-regenerative MSC chondrogenesis, whereas shear or tensile forces induce fibrogenesis, which is detrimental to fracture healing [13]. When fractures are not stably fixed, angiogenesis is initially increased [18]. However, instability also impedes cartilage replacement, diminishes angiogenesis, and prevents bone bridging [19]. Therefore, an optimal window of interfragmentary motion is needed for successful callus formation and bone bridging [18]. Moreover, great care must be taken during surgical stabilization, as open fractures with extensive necrotic tissue have a greater risk for infection, which can lead to non-union through hardware loosening and implant failure [17].

2.3.3 Age and gender effects—Internal factors such as age affect the progression of fracture healing. Non-union is rare in children. The immature skeleton has a robust periosteal layer of osteoblasts and plentiful vascularity, whereas the adult periosteum is partially fibrous and less cellular, leading to a slower periosteal response to fracture compared to children [20]. The decreased number of MSCs and osteoblasts leads to delayed cell differentiation and callus formation and lower osteoinductive activity of bone matrix, altogether contributing to impaired bone healing associated with aging.

Along with age, gender also plays a role. Females over the age of 55 years have a higher incidence of fracture non-unions versus males, likely due to estrogen deficiency. Androgens and estrogens both play important roles in bone healing, but estrogen levels are more strongly associated with bone mineral density (BMD), bone turnover, and bone loss than testosterone levels are in adult men [16, 21]. Serum estrogen levels in postmenopausal

women are lower than those in elderly men, underscoring the greater risk elderly women face to develop fragility fractures and impaired healing [16].

2.3.4 Comorbidities—Outcomes of fracture healing depend on comorbid diseases.

Osteoporosis, which is linked to aging, is another risk factor of impaired healing. Women aged 65 years and older account for 74% of all osteoporosis-related fragility fractures [22]. In the United States, the total costs of these fractures were estimated to be \$17 billion, and were projected to grow by almost 50% to \$25.3 billion per year by 2025 [23]. Osteoporotic bone differs from healthy bone in its reduced mass and deteriorated architecture, leading to bone fragility and increased fracture risk [10]. Osteoporosis is a consequence of the imbalance between osteoblast mediated bone formation and osteoclast mediated bone resorption, and this carries through to fracture repair. In the elderly, fracture healing is compromised by the functional decline of osteoblasts and increased activity of osteoclasts, which causes disrupted callus mechanical properties including decreased strength, peak failure load, and bending stiffness [24]. Clinically, the failure rates of fixation in patients with osteoporosis range from 10% to 25% [25].

Diabetes is often associated with delayed fracture healing, with an average of ~1.6-fold longer times for complete healing [26]. There is a reduction in collagen formation by osteoblasts in the callus in diabetic patients due to inadequate insulin production [17]. Diabetes-associated peripheral vasculopathy and neuropathy have also been linked to impaired fracture healing [27]. Furthermore, diabetes drugs such as thiazolidinedione have anti-osteogenic effects that are detrimental to fracture healing [27].

2.3.5 Alcohol and nicotine use—Alcohol consumption impairs fracture healing [28].

Acute alcohol exposure prior to fracture injury perturbs β -catenin signaling required in the critical stages of fracture callus formation (Figure 1), impairing the fracture healing process [29]. Excessive alcohol consumption in the post-trauma period inhibits new bone formation. Furthermore, newly formed bone exhibits reduced mineralization rates, which compromises bone mechanical properties [17].

Smoking also increases the risk of delayed unions. Nicotine, the main chemical in tobacco, inhibits the secretion of tumor necrosis factor alpha (TNF- α) by activating the cholinergic anti-inflammatory pathway, altering the maturation of macrophages and fibroblasts, and causing deficits in hematoma formation [30]. Nicotine has been reported to inhibit osteoblast alkaline phosphatase (ALP) and collagen production [13]. In addition, nicotine causes vasoconstriction leading to altered tissue perfusion and consequent hypoxia and ischemia, resulting in impaired healing [16].

2.3.6 Nonsteroidal anti-inflammatory drugs (NSAIDs)—NSAIDs are associated with delayed unions and non-unions, especially when administered acutely after fracture or chronically for longer than 2 weeks [16]. As discussed previously, inflammation is one of the first responses to bone fracture. Cyclo-oxygenase 2 (COX-2) is induced at inflammation sites, produces pro-inflammatory prostaglandins, and is essential for fracture healing [31]. However, NSAIDs inhibit the activity of COX enzymes, thereby affecting the synthesis of

prostaglandin signaling molecules [31]. Therefore, COX-2 selective NSAIDs should be avoided in patients who already have risk factors associated with impaired healing.

3. Clinical approaches to enhance fracture healing

3.1 Bone grafts

Several strategies have been developed clinically to enhance fracture healing. Bone autografts are the current gold standard for treatment of fracture non-unions. However, autografts have a risk of failure when the osteogenic potential of the available donor tissue is poor due to cell scarcity, abundance of fibrous versus collagenous periosteal tissue, or other impairments [11]. Autografts can also only be harvested in small volumes from non-load bearing regions of the skeleton, limiting their clinical utility. Alternatively, allografts, demineralized bone matrix, or synthetic scaffolds have been developed to fill the need for large volumes of graft material [32]. These materials eliminate donor site pain and morbidity, which are complications of autograft harvesting, but they suffer from incomplete healing as compared to autografts [33] and have major limitations associated with rejection, transmission of diseases, and cost [34].

3.2 Low-intensity pulsed ultrasound (LIPUS)

Low-intensity pulsed ultrasound (LIPUS) is an external stimulus that can be applied over the skin at the fracture site [13]. The use of LIPUS to accelerate fracture repair in humans was first reported in 1983 [35], leading to clinical trials and approval of EXOGEN™ system by the FDA for the accelerated healing of fresh fractures and also for use in fractures at risk for non-union [36, 37]. Mechanically, the emission of ultrasonic sound waves stimulates nano-movement, enhances the production of growth factors that expedite the removal of old bone, increases extracellular matrix (ECM) synthesis, and stimulates ALP production by osteogenic cells to increase mineralization of newly-forming bone [38, 39]. LIPUS has also been shown to activate the transcription of COX-2, which increases prostaglandin-E2 (PGE2), leading to greater mineralization by osteoblasts and enhanced endochondral ossification [40]. LIPUS is safe and noninvasive, and patients in a Level IV therapeutic study were highly compliant with 20-minute daily treatment sessions until bone healing was achieved, or up to the maximum 6-month study duration [38, 41]. One limitation of LIPUS is that it is only indicated for non-union patients who have achieved adequate fracture reduction (interfragmentary gaps <10 mm) with appropriate fixation stability [13, 38]. Additionally, LIPUS tissue penetration is only 1–5 cm, due to inverse proportionality to its low frequency, limiting its efficacy in patients with deeper injuries and greater subcutaneous fat [42]. With the ability to stimulate healing, the therapeutic potential of ultrasound can be further extended by introducing ultrasound-responsive drug delivery systems, including microbubbles, liposomes, emulsions, and micelles [43]. Drugs including small molecules, siRNA, DNA, and proteins have been loaded into these systems and delivered in response to ultrasound [43].

3.3 Bone morphogenetic proteins (BMPs)

Bone morphogenetic protein-2 (BMP-2) and BMP-7 are the most intensely studied growth factors for bone regeneration due to their abilities to promote MSC differentiation into

chondrocytes and osteoblasts [44]. Recombinant human BMP-7 (rhBMP-7) delivered via OP-1™ Implants is FDA-approved for repair of non-union long bone defects [45]. rhBMP-2 delivered via INFUSE™ collagen sponge bone grafts is also clinically approved for acute fractures and spinal fusions [1]. In clinical trials, diaphyseal tibia fractures were treated with either an autogenous bone graft or a combination of an allograft and rhBMP-2-INFUSE™ [46]. Healing rates were 67% and 87% in the autograft and rhBMP-2-INFUSE groups, respectively, suggesting BMP-2 use in fracture healing is advantageous compared to the gold standard. Although clinical data of these approaches are positive, the use of growth factors is limited by their pleiotropic nature [47]. Furthermore, delivery of supra-physiological doses is often required to achieve therapeutic levels at fracture sites, leading to adverse side effects, clinical complications, and significant costs [3–6]. Therefore, invasively placed, localized delivery strategies are often used for growth factor delivery. Although substantial progress has been made, rhBMPs are still not FDA-approved in the pediatric age group, in pregnant patients, or in tumor-bearing patients due to the supra-physiological doses and characterized long-term side-effects [48].

4. Investigating stem cells and their applications in fracture healing

Mesenchymal stem cells (MSCs) play a central role in fracture healing (Figure 1). MSCs are recruited and stimulated at the initial inflammation stage by growth factors, then differentiate into chondrogenic and osteogenic cell lineages to realize successful fracture healing. MSCs reside in the bone marrow, on endosteal surfaces, within the periosteum, and in circulation. There is evidence that MSC recruitment is aberrant in fracture non-unions [49]. In addition, MSCs isolated from non-union patients have reduced proliferation and lower osteogenic (but not chondrogenic or adipogenic) potential due to elevated secretion of dickkopf-related protein 1 (DKK-1), a Wnt/ β -catenin pathway inhibitor [50]. These data highlight the importance of osteogenesis, which contributes to bone formation and mineralization in fracture healing.

4.1 Stem cell delivery

In light of the critical role of stem cells in healing, transplantation approaches have been developed. These approaches rely on either cell homing after systemic delivery, a poorly understood process that is limited by the pulmonary first-pass effect [51], or direct delivery to the injury site by a scaffold. Scaffolds are implants or injectable formulations that form *in situ* and are typically three-dimensional porous matrices, nanofibrous matrices, or hydrogels. Scaffolds, cells, and external stimuli are the three major components in tissue engineering, where scaffolds serve as a template for cell growth. Drug delivery techniques can benefit from the use of depots due to sustained drug release, placement via minimally invasive procedures, and the ability match the shape of the cavity in which they are placed [52]. Ideal scaffold delivery systems for fracture healing should be biodegradable to avoid secondary surgeries and have limited inflammatory responses [53]. Biomaterials used for fabrication of scaffolds include natural polymers such as alginate, proteins, collagens, gelatin, fibrins, and albumin, or synthetic polymers such as poly(vinyl alcohol), poly(ethylene glycol), and poly(glycolide) [52, 54, 55].

4.2 Research applications of stem cells

Studies have identified approaches to increase MSC homing to bone [56]. A hybrid compound of LLP2A, which exhibits affinity for $\alpha 4 \beta 1$ integrin receptors on MSCs, and alendronate (Ale), which exhibits affinity for hydroxyapatite mineral in bone, was co-administered with MSCs in mice with closed transverse diaphyseal femur fractures. In this study, MSCs migrated to the bone marrow and callus, resulting in significantly greater maximal load and work-to-failure relative to control. Alternatively, hydrolytically degradable poly(ethylene glycol) (PEG) hydrogels have been developed to form a tissue-engineered periosteum to transplant and localize MSCs to allografts *in vivo* [33, 57, 58]. The hydrolytically degradable crosslinks allowed for tuning of degradation and subsequent persistence of cells [57]. The transplanted MSCs secreted myriad soluble factors critical for healing, resulting in 2.8-fold greater endochondral bone formation and 1.8-fold increased biomechanical strength of repair.

4.3 Future of stem cell therapy

Although MSCs improve regeneration, a limited number of MSCs directly contribute to mature repair tissues [59]. Instead, data suggest the paracrine effects of MSCs enhance the recruitment of macrophages and endothelial cells into the injury site, promoting angiogenesis and bone formation [60]. A better understanding of stem cell mechanism of action would be highly beneficial for development of translatable cell-based therapies for fractures. However, cell-based therapies are limited by the significant lead-time that is required to achieve adequate cell quantities and the batch-to-batch variability that exists in patient-derived cells [61, 62]. Moreover, patients suffering from fracture non-union may not have viable MSCs to harvest and expand due to risk factors discussed in section 2.3. To overcome these challenges, stem cell preconditioning and programming by physical, chemical, genetic, and pharmacological manipulations has shown promise in priming the cells to a “state of readiness” for therapeutic applications [63, 64].

5. Therapeutic agents and delivery strategies in development for fracture healing

Drug therapies have been investigated for their potential in harnessing bone fracture healing for long-lasting outcomes (Table 1). Growth factors remain the most studied therapeutics for fracture healing since they are intrinsically critical for bone regeneration. Parathyroid hormone (PTH), although only approved for treating osteoporosis, may also be adaptable as a treatment for fracture non-unions [5, 6, 65]. Novel bone regeneration strategies involving small molecule compounds have been explored to circumvent challenges of biologics. These small molecules, including statins [66], steroid hormones and prostaglandin agonists [67], and Wnt/ β -catenin agonists [68–70], are more stable, more affordable, and less immunogenic [71], thus overcoming many of the challenges associated with growth factors [72]. In addition, new modalities such as siRNA and miRNA have been explored to genetically manipulate regenerative cells to improve fracture healing [73, 74]. Although myriad drug candidates have been developed for fracture treatment, the major limitation of clinical translation of these therapeutics is the lack of appropriate delivery systems. It is

necessary to consider the route of delivery, the delivery vehicle, and the duration of treatment required to achieve effective outcomes while reducing off-target effects. Furthermore, to augment bone regeneration at fracture sites, there is a need to isolate delivery to bone to improve drug availability and reduce systemic toxicity due to aberrant stimulation of and crosstalk between signaling pathways. Note that therapeutic strategies for critical sized defects are not included in this review. However, these approaches may inspire novel and effective approaches to enhance fracture healing. For further reading, several recent reviews have been published on this topic [75–77].

5.1 Growth factors

Growth factors are native signaling proteins secreted by cells to promote migration, proliferation, differentiation, and a variety of other cell functions [78]. During early limb development, growth factors enhance maturation and function of differentiated cells (e.g. chondrocyte and osteoblast) by binding to serine/threonine kinase membrane receptors that modulate gene transcription [79]. As a means to emulate limb development and because growth factors are critical for various stages of fracture healing, they are commonly used clinically and experimentally to improve fracture healing. Growth factors such as BMPs [80], transforming growth factor-beta (TGF- β) [81], vascular endothelial growth factor (VEGF) [82, 83], fibroblast growth factor (FGF) [84], platelet-derived growth factor (PDGF) [85], and insulin-like growth factor (IGF) [86] have been investigated to improve fracture healing.

5.1.1 Growth factor delivery—Controlled release systems have been developed specifically for growth factors to provide protected and predictable delivery at fractures [62, 87]. Three-dimensional matrices and porous scaffolds are the most commonly investigated growth factor delivery systems. Biomaterial scaffolds or depots, which are aligned with BMP2-releasing INFUSE™, offer the potential to provide prolonged release through limited diffusion of encapsulated drugs. Several scaffold materials have been investigated for depot release of growth factors in orthopaedic applications including poly(L-lactic acid) (PLLA) [88], poly(propylene fumarate) (PPF) [83], and the biodegradable polyester polyurethane (PUR) [89, 90]. For example, rhBMP-7 encapsulated in poly(lactic-co-glycolic acid) (PLGA) nanospheres that were loaded into PLLA scaffolds and implanted subcutaneously exhibited *de novo* bone formation with mineralized matrix after 3 weeks due to sustained rhBMP-7 release [88]. Controlled delivery can also be achieved via release from hydrogels, where release is dependent on the physicochemical properties of the polymer structure, the bioactive factor, and the type and density of crosslinker. For examples, gelatin hydrogels have been used to deliver rhBMP-2 via subcutaneous implantation and showed enhanced osteoprogenitor cell recruitment and *de novo* bone formation [91].

Stimuli-responsive elements can be introduced to delivery vehicles as a biomimetic strategy to selectively trigger growth factor release. A pH-responsive and thermosensitive hydrogel was designed for BMP-2 delivery by adding pH-sensitive sulfamethazine oligomer (SMO) end groups to thermo-sensitive block copolymers made of poly(ϵ -caprolactone-co-lactic acid) (PCLA) and PEG units [92]. This system displayed functional BMP-2 activity over a narrow pH and temperature range, leading to elevated ALP activity and mineralization over

7 weeks *in vivo* following subcutaneous implantation. Pro-angiogenic peptides, including secreted protein acidic and rich in cysteine-113 (SPARC₁₁₃) and SPARC₁₁₈, and the VEGF mimetic, QK, were incorporated into PEG hydrogels by crosslinking norbornene-functionalized PEG with thiol-functionalized peptides flanked by matrix metalloproteinase (MMP) degradable substrates [54, 55, 93]. Enzymatically-responsive hydrogel degradation and peptide release was achieved [55, 93], and significant enhancement of angiogenesis was observed *in vivo* after implantation [54]. This system is versatile in that varied enzymatically-responsive linkers can be adapted depending on the target drug and delivery site.

5.2 Parathyroid hormone (PTH)

As an alternative to BMP, parathyroid hormone (PTH), which acts within the BMP pathway, has been used off-label for fracture healing. PTH and its peptide derivatives were approved by the FDA for treatment of osteoporosis as recombinant human parathyroid hormone peptide (rhPTH 1–34) (Forteo™) [94]. PTH is an endocrine regulator of calcium and phosphate homeostasis and induces an anabolic response in bone at intermittent low doses [95]. Anabolic effects plateau after two years of treatment, and catabolic responses can be triggered by continuous high doses of PTH [95]. PTH has been studied extensively in multiple animal fracture models and has been shown to improve rates of callus formation, callus mineralization, and bone remodeling [5, 6, 65].

5.2.1 PTH delivery—Clinically, PTH is administered daily by systemic subcutaneous injection, which is a limitation due to poor patient compliance associated with daily injections, motivating alternative delivery mechanisms. Local delivery of PTH to fractures has been explored to stimulate bone healing. An arginine–glycine–aspartic acid (RGD) modified PEG hydrogel with PTH 1–34 covalently bound to the PEG macromers was studied in osseointegration of canine dental implants [96]. Bone formation comparable to autogenous bone implants was induced by the RGD–PEG–PTH gel, whereas PEG hydrogel alone showed no more bone formation than the empty defect. Interestingly, the anabolic response observed in this study was significant, despite the use of continuous PTH release. These studies indicate that local delivery overcomes the challenges of maintaining a therapeutic window with systemic treatment. Nevertheless, PTH has been shown to degrade during storage, making delivery system development problematic [97]. Further studies in fracture patients are necessary to determine if results seen in animal models can be duplicated in humans.

5.3 RNA interference (RNAi)

RNA interference (RNAi) is a mechanism where gene expression is inhibited post-transcriptionally by a short RNA sequence, including small interfering RNA (siRNA) or microRNA (miRNA) molecules [98, 99]. The siRNA and miRNA pathways are similar, but siRNAs are designed to be 100% complementary to target sequences. Thus, siRNAs result in mRNA degradation, whereas miRNAs induce translational repression. As siRNA may be designed to specifically target a broad range of genetic sequences, siRNA-based drugs offer significant potential as a potent treatment for many diseases [100]. miRNA have also been shown to play a significant regulatory role as “molecular switches” to stimulate fracture

healing [101]. Therefore, RNAi therapeutics are advantageous when current techniques fail due to treatment inefficiency, complicated therapy schemes, or newly developed resistance drugs due to mutational escape [102].

For fracture healing, RNAi can be introduced to silence genes that negatively regulate fracture healing. Previous studies have reported promising results using RNAi to differentiate various stem cell types into musculoskeletal, cardiovascular, and neuronal lineages. For example, delivery of siRNA against DKK-1 [103] or guanidine nucleotide binding protein alpha stimulating activity polypeptide 1 (GNAS1) [104] resulted in enhanced osteogenic differentiation of MSCs. These results suggest that the application of siRNA to stem cells can be used to enhance tissue regeneration by promoting MSC differentiation, survival, and tissue production [105]. However, clinical translation of siRNA-based therapies has been hampered by delivery barriers, including siRNA susceptibility to nuclease degradation, cell and endosomal membrane impermeability at the target site, and transient gene silencing [106].

5.3.1 RNAi delivery—Numerous medicinal chemistry strategies have been explored to overcome siRNA delivery barriers [100]. For example, siRNA molecules have been conjugated to sugars [107] and cholesterol [108] for enhanced potency and stability. siRNA modified with cholesterol exhibits improved serum stability and interactions with cell membranes [109, 110]. Cholesterol-conjugated miR-29 mimic (miRagen Therapeutics, Inc.) is in clinical trials to treat scleroderma [111]. Responsive delivery of osteogenic miR-29b-3p using microbubble-ultrasound stimuli has improved fracture healing by increasing callus bone volume and mechanical properties [112]. Alnylam Pharmaceuticals developed N-acetylgalactosamine (GalNAc)-siRNA conjugates that selectively deliver siRNA to hepatocytes due to high affinity of GalNAc to asialoglycoprotein receptors expressed by hepatocytes [113, 114]. These chemical modifications improve the RNAi stability, which improves the potency and duration of knockdown.

Nanotechnology also has great potential for enhancing siRNA efficacy, particularly nanoparticle (NP) delivery systems. Cationic lipids (liposomes and lipid nanoparticles (LNPs)) are the most commonly used non-viral vectors for RNAi delivery, both in development and clinical trials [102, 115]. However, LNPs are well-known to be toxic at high doses due to compromised membrane integrity, resulting in cell lysis and necrotic death [116]. It has also been reported that LNPs induce immune responses [116]. Therefore, careful selection of lipids and controlled doses are important for translation of LNPs. Upon systemic delivery, liver dominates siRNA/carrier biodistribution due to reticuloendothelial system (RES) function (e.g., uptake by macrophages and liver Kupffer cells) [117]. While this is not favorable for some applications, it results in highly effective siRNA uptake in the liver, leading to several ongoing clinical trials [118]. For example, Patisiran, another Alnylam pipeline RNAi therapeutic that is mediated by LNPs, was developed to treat familial amyloidotic polyneuropathy. Subcutaneous administration resulted in 80% knockdown of the target gene, transthyretin, over 9 months and improved neuropathy scores in Phase II studies [119]. Recently, the Phase III study of Patisiran met its primary efficacy endpoint and all secondary endpoints, which is a milestone for RNAi therapeutics, as Patisiran might become the first commercially available siRNA medicine. Despite these

successes, LNPs have limited potential for siRNA delivery to target tissue other than liver [120, 121].

Cationic polymers have also been developed to overcome challenges in siRNA delivery. Polymers such as poly(ethylenimine) (PEI), dendrimers, poly-L-lysine, poly-L-arginine, chitosan, and their derivatives have been commonly used for siRNA nanocarrier development [100]. Among these, PEI has been the standard for nucleic acid delivery owing to favorable cell membrane interactions, high cellular uptake, and efficient endosomal escape [122]. Cationic linear PEI was conjugated into photocrosslinked dextran (DEX) hydrogels through a biodegradable ester linker to provide sustained release of siRNA. The degradation of the covalent ester linkages between the LPEI and hydrogels led to tunable release of LPEI/siRNA complexes over time [123]. However, PEIs exhibit significant *in vitro* and *in vivo* toxicity since PEI molecules can interact with negatively charged serum proteins and even red blood cell surfaces to form aggregates and lead to acute cell damage [122]. High molecular weight (HMW, >25 kDa) PEIs and branched PEIs have shown more cellular toxicity than low molecular weight (LMW, <10 kDa) PEIs and linear PEIs, respectively [122]. Yet in contrast to HMW PEI, LMW PEI exhibits low transfection efficiency. Therefore, strategies to lower PEI toxicity have been developed, primarily by chemical modification, to manage tradeoffs between cytotoxicity and delivery efficiency. For example, disulfide crosslinking of PEI, N-acylation, and grafting with PEG resulted in considerable gene delivery efficiency and mitigation of toxicity [124–126]. In addition, hydrophobic moiety substitution of amines within the polymer increases cellular uptake and gene silencing through more favorable cell membrane interactions [127, 128].

Cyclodextrin has also been used to form cyclodextrin-containing polycation (CDP) systems for siRNA delivery. The CDP system, CALAA-01, also incorporates PEG for stability and human transferrin as a targeting ligand for binding to transferrin receptors on solid tumors. This delivery system, developed by Calando Pharmaceuticals, was the first targeted delivery of siRNA to reach Phase I clinical trials [129]. However, the Phase I clinical trial was terminated in 2013 according to the U.S. Food and Drug Administration (FDA) [130]. PLGA, a copolymer of lactic acid and glycolic acid, has been used as a nanocarrier for siRNA delivery in recent years. PLGA-based siRNA delivery offers the advantages of high stability, biodegradability, low toxicity, and sustained release characteristics [131]. However, PLGA formulations have limited potential in siRNA delivery due to negligible electrostatic interactions between PLGA and siRNA [131]. To overcome these challenges, strategies have been developed to modify the surface of PLGA with various cationic NPs such as DOTAP, PEI, or polyamine [131, 132]. PLGA NPs functionalized with PEI using an emulsification-diffusion production method demonstrated optimized the delivery of siRNA [133].

In the past decade, polymeric micellar NPs for siRNA delivery have been developed [134–136]. Polymers that self-assemble to NPs offer the advantage of easy siRNA loading post NP formation, which allows for the construction of size-controlled and monodisperse nucleic acid loaded NPs, and display unique advantage for *in vivo* applications [137]. Complexed siRNA and cationic carriers interact electrostatically with negatively charged cell membranes for endosomal uptake [132, 138]. These polymers also exhibit pH responsiveness, which facilitates siRNA endosomal escape when the pH drops in the endo-

lysosomal pathway, via protonable block and hydrophobic membrane interacting monomers [139–141]. Cationic degradable micelles self-assembled from amphiphilic triblock copolymers composed of PEG-b-poly(ϵ -caprolactone)-b-poly(2-aminoethyl ethylene phosphate) mediated efficient siRNA delivery [142]. Additionally, NPs are able to load siRNA and hydrophobic drugs simultaneously to achieve potential synergistic effects [142, 143]. A poly(ethylene oxide)-block-poly(ϵ -caprolactone) (PEO-b-PCL) based micellar NP system was developed for co-delivery of siRNA, doxorubicin, and imaging probes, indicating the drug loading potential and versatile functionalization of polymeric micellar NPs [144].

Our lab has pioneered the development of a diblock copolymer NP delivery system that is designed to protect siRNA from degradation, improve cytoplasmic uptake, and realize endosomal escape [145–147]. The diblock copolymers contain a cationic block of poly(dimethylaminoethyl methacrylate) (pDMAEMA) and a pH-responsive endosomolytic block of DMAEMA, 2-propylacrylic acid (PAA), and butyl methacrylate (BMA) to achieve successful siRNA delivery to a variety of cell types [148–153]. The cationic pDMAEMA block contains tertiary amines that are 50% protonated at physiological pH to allow electrostatic complexation with anionic siRNA and facilitate cell membrane interactions. The second tercopolymer block composed of DMAEMA, PAA, and BMA enables pH-dependent endosomal escape, and owing to high BMA incorporation, it is also hydrophobic, which mediates self-assembly of NPs in aqueous neutral pH conditions [152, 154]. This diblock NP was used to complex with siRNA against WW domain-containing E3 ubiquitin protein ligase 1 (*Wwp1*), which is a negative regulator of bone formation, and then entrapped within poly(ethylene glycol) (PEG)-based hydrogels and implanted at sites of murine mid-diaphyseal femur fractures [73]. This hybrid NP/hydrogel system provided localized and sustained delivery via controlled release of entrapped siRNA/NP complexes. Results showed localization of hydrogels and controlled release of siRNA/NPs at fractures for 28 days. Fractures treated with siRNA/NP hydrogels exhibited accelerated bone formation and significantly increased biomechanical strength [73].

5.4 Small molecule therapeutics

Novel bone regeneration strategies involving small molecule compounds have been explored to circumvent challenges of growth factors. Small molecule drugs are appealing because they are affordable, are stable, and require a lower dose to achieve bone regeneration [77]. The small molecular weights (<1000 Da) also lower the possibility of inducing immune responses [155]. In the past decade, multiple small molecules with osteoinductive potential have been discovered [77, 156]. At the same time, some of these small molecules are already approved by the FDA for other diseases and have well-documented clinical bioavailability and toxicity data in humans. It is likely that these drugs, described in Table 1, could be repurposed for rapid translation to orthopaedic applications once further research establishes their osteoinductive abilities and optimizes the delivery route in pre-clinical animal models [156].

5.4.1 BMP signaling agonists—Statins, a family of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors originally developed to treat

hypercholesterolemia, have received significant attention due to their osteogenic potential [163]. Simvastatin reverses the suppressive effects of TNF and prevents BMP signaling inhibition mediated by Smad 1/5/8 phosphorylation [164]. *In vitro* treatment with simvastatin induced differentiation of murine embryonic stem cells (mESCs) into osteoblasts, as indicated by increased osteogenic gene expression and matrix mineralization [165]. A fluvastatin-releasing hydrogel delivery system was developed to release drug in a sustained manner and showed enhanced hMSC osteogenic differentiation [66]. *In vivo* studies utilizing simvastatin loaded gelatin sponge grafts have shown 240% more new bone than the control group in a rat mandibular defect model [166]. Delivery of statins using delivery systems such as collagen sponges and methylcellulose gels has also shown promising *in vivo* outcomes with enhanced bone healing by radiological and histological assessments [167, 168]. An injectable auto-forming alginate gel containing simvastatin with amorphous calcium phosphate increased bone mechanical strength at 12 weeks compared to simvastatin injection alone [169]. However, an *in vivo* study in rats also demonstrated that high doses of simvastatin (0.5–2.2 mg per site) might induce inflammatory responses or even impair bone healing [170, 171]. Therefore, further investigation is needed to establish drug carriers, therapeutic thresholds, and modes of delivery.

5.4.2 Wnt/ β -catenin signaling agonists—Wnt/ β -catenin signaling agonists have also been widely explored due to the crucial role of this signaling pathway in all stages of bone healing, including cell lineage determination, migration, proliferation, and matrix deposition [172]. The actions of Wnts are mediated through multiple pathways, of which the canonical pathway is the best characterized [67]. Through canonical Wnt signaling, intracellular β -catenin is stabilized and translocates into the nucleus to initiate osteogenic gene expression [173]. Canonical Wnt signaling plays an important role in fracture healing by upregulating RUNX2 and promoting the formation of osteoblasts and chondrocytes, the principal cell types involved in fracture callus formation [173]. Oral treatments of lithium chloride (LiCl), an FDA-approved glycogen synthase kinase (GSK)-3 inhibitor used to treat bipolar disorder, can activate Wnt signaling and accelerate fracture repair [174]. By inhibiting GSK-3 β , there is an increase in intracellular β -catenin, which promotes osteoblast differentiation and bone healing. However, this effect only occurred in the later phases of repair after mesenchymal cell osteogenic commitment [175, 176]. Small molecule GSK-3 β inhibitors have been tested pre-clinically, such as 603281-31-8 (Eli Lilly), which increased bone formation markers in ovariectomized rats [177], and AZD2858 (AstraZeneca), which enhanced osteoblast activity and fracture healing in rats [161]. However, the major concern of Wnt agonists is tumorigenic side effects resulting from poorly controlled doses at bone and off-target tissues [178–180]. Due to the broad expression of β -catenin, highly localized and controlled GSK-3 β inhibition would be advantageous from a fracture healing perspective to reduce risk of off-target effects [161, 180]. The GSK3 β inhibitor, 6-bromoindirubin-3-oxime (BIO), conjugated to a hydrophilic aspartic acid octapeptide assembled into micelle NPs exhibited prolonged circulation time and fracture-targeting abilities with accelerated healing [7, 181]. Small molecule GSK-3 β inhibitor AZD2858 was delivered with fracture-targeted poly(styrene-*alt*-maleic anhydride)-*b*-poly(styrene) (PSMA-*b*-PS) NP system [70]. A peptide with high affinity for tartrate-resistant acid phosphatase (TRAP), a protein deposited by osteoclasts on bone resorptive surfaces, was conjugated to the NP corona [182]. Targeted

NPs showed improved preferential delivery to fractured bone, with enhanced bone formation and microarchitecture.

6. Future directions for drug delivery systems for fracture healing treatment

A new realm of therapeutics and delivery systems are projected to improved fracture healing. Looking forward to newly developing frontiers is the combination of therapeutics to exploit the advantages of proteins, RNAi, small molecules, and stem cells. Further, to achieve safe and efficacious delivery of therapeutics to fracture, local and systemic delivery approaches can be tailored specifically to the therapeutics applied and the fracture environment.

6.1 Systemic targeted delivery

Currently, no systemically administered clinical treatments for fracture are designed to favor skeletal biodistribution. Systemically delivered, fracture-targeted systems have the advantages of simple dosing control, non-invasive administration, and fewer side-effects, which may make translation of therapeutics more feasible. A simvastatin prodrug micelle system [183] was designed to target fracture via tumor-like enhanced permeation and retention (EPR) effect, or “extravasation through leaky vasculature and inflammatory cell-mediated sequestration” (ELVIS) mechanism [183–185]. Passive targeting of NPs exhibited fracture localization for ~ 7 days. As active targeting mechanisms, bisphosphonates, such as zoledronate [186] and alendronate [187, 188], have been widely explored as effective strategies to target bone due to their high affinity to hydroxyapatite (HAp), which is the main mineral component of bone [62, 189, 190]. However, poor fracture specificity and inhibitory effects on osteoclasts limits the utility of bisphosphonates for bone targeting [190–192]. An osteoblast-specific aptamer, CH6, which was screened by exponential enrichment (Cell-SELEX), was used for osteoblast-specific siRNA delivery via LNPs [193]. Poly(aspartic acid) (pAsp) peptides have also been used to target drugs to bone tissue due to their affinity to high crystallinity HAp, which is characteristic of bone resorption surfaces [194, 195]. Oligopeptide (AspSerSer)₆ functionalized liposomes have shown efficient targeting to osteoblast-mediated mineralizing nodules at bone formation surfaces [196]. An oligo(ethylene glycol) copolymer system based on a peptide with high affinity to TRAP exhibited bone targeting effects, and the targeting was tuned by varying peptide orientations and polymer molecular weights [197]. The versatile system can deliver virtually any drug that maintains activity upon conjugation and/or release from the polymer. Although biodistribution is altered to favor bone delivery [147, 196, 198, 199], liver still remains as a major organ of biodistribution for these targeted carriers.

6.2 Hydrogels for sustained delivery of NPs loaded drugs

To overcome delivery challenges, a number of nanoscale drug delivery systems have been developed, although their small sizes result in dispersion *in vivo*, making long-term treatment of target tissues difficult and causing off-target effects. Hydrogels that are able to localize and sustain the delivery of NPs have great potential to enhance the therapeutic index of NP drugs. [200, 201]. As previously mentioned, our lab developed a localized strategy using PEG hydrogels with varied swelling and degradation profiles to control the release of

siRNA-loaded diblock NPs. Overall, our findings indicate that release of siRNA/NP can be controlled for short (1–3 days), moderate (7–14 days), or long (21–28 days) release by using varied PEG molecular weight. Higher molecular weight PEG results in larger mesh sizes and therefore exhibits greater release rates. PEG functionalized with PLA blocks within crosslinks exhibits expedited release with greater PLA length due to the increased probability for polymer chain scission, which leads to increased diffusion of NPs. We have also developed a systemic drug delivery strategy for small molecule Wnt agonists, GSK-3 β inhibitor AZD2858, as an alternative to local delivery [70]. The GSK-3 β inhibitor was loaded in TRAP-binding peptide-targeted PSMA-*b*-PS NPs [202, 203] at 15 wt% capacity, resulting in ~50 nm particles with ~–30 mV surface charge. The NPs are flexible platforms that can be easily integrated with other technologies and materials [204]. For example, PSMA-*b*-PS NPs can be loaded into aforementioned PEG hydrogels to realize sustained release of NPs (Figure 2). As shown in Figure 2A, PEG macromers with molecular weights of 2, 4, and 10 kDa were used to form hydrogels and incorporate PSMA-*b*-PS NPs, achieving NP release half-lives of 23 days, 13 days, and 6 days, respectively. Moreover, release profiles for all three types of hydrogels from day 4 through day 23 approximated zero order release (Figure 2B and C). Similar to previous findings, hydrogel mesh size controlled NP release, where NPs encapsulated in hydrogel networks with larger mesh sizes released NPs more rapidly. Due to greater NPs sizes (40–70 nm), the release of PSMA-*b*-PS NPs was sustained longer as compared to siRNA/NPs with smaller sizes (20–40 nm), where k' was calculated to be 0.18, 0.21 and 0.52 day^{–1} for 2 kDa, 4 kDa and 10 kDa PEG hydrogels, respectively [73]. These preliminary results are promising and warrant further investigation of using hydrogels to localize PSMA-*b*-PS-based NPs.

6.3 Co-delivery of siRNA with small molecule drugs

Co-delivery of siRNA with small molecule drugs was first investigated in cancer treatments [205]. Small molecule chemotherapeutic agents provide anti-cancer efficacy, and siRNA can overcome multidrug resistance (MDR) [205]. Promising results provide support to this strategy because combination delivery of siRNA and drug via NPs showed enhanced efficacy in inhibiting tumor growth compared to siRNA or drug alone [206]. This strategy can also be applied to bone-related diseases, especially for fracture healing, which involves multiple signaling pathways and mechanisms. To achieve successful fracture healing, synergistic effects from multiple therapeutic agents may be necessary. Nanoparticle systems are appealing for co-delivery because they are able to condense siRNA effectively via electrostatic interactions or chemical conjugation, and encapsulate drugs effectively via physical encapsulation, formation of an inclusion complex, or direct chemical conjugation [206]. For example, cyclodextrin-modified dendritic polyamine construct (DexAM) was developed to simultaneously deliver SOX9 siRNA and retinoic acid to direct stem cell differentiation to neurons [207]. The polyamine backbone complexes and protects siRNA, while cyclodextrins are cyclic oligosaccharides with a hydrophilic exterior and hydrophobic interior that enhance the solubility of hydrophobic drugs. A stearylamine/cholesterol (SA/Chol) sterosome system was developed to co-deliver hydrophobic small molecule phenamil and Noggin siRNA to enhance osteogenic differentiation of MSCs both in vitro and in a mouse calvarial defect model [208]. Phenamil is a small molecule BMP agonist, and Noggin is a BMP inhibitor that is expressed upon BMP stimulation. Delivering phenamil and

Noggin siRNA provided synergistic effects on BMP signaling activation. These promising results indicate that engineered multifunctional nanocarriers for co-delivery of siRNA and drug offer great promise in enhancing therapeutic efficacy of current treatments. The diblock NP for siRNA delivery developed in our laboratory can also be exploited for co-loading of hydrophobic drugs due to the stable hydrophobic core [209, 210]. For fracture healing, co-delivery systems can be used to deliver siRNA and drug that both stimulate bone formation. Alternatively, NPs can be used to deliver siRNA that stimulates bone formation, and small molecule antibiotics to prevent infection [210, 211]. Nevertheless, due to the distinct pharmacokinetic profiles of individual drugs, a deeper understanding of the optimal ratio of therapeutic agent (siRNA versus drug), as well as process development technologies that ensure stability of both payloads, are needed to achieve the maximum synergistic effect of co-administering siRNA with small molecule drugs.

6.4 Co-delivery of cells and therapeutics

As previously introduced, traditional regenerative strategies involve the combination of biomaterial scaffolds, regenerative cells, and factors to stimulate bone formation. The aforementioned PEG hydrogels encapsulating MSCs may be augmented by co-encapsulation of therapeutics to enhance MSC function. MSCs localized by these hydrolytically degradable PEG hydrogels also showed similar 'release' profiles in a murine segmental femoral graft model as compared to siRNA/NP incorporated in PEG hydrogels described in section 6.2, where hydrogels prepared from PEG macromers with longer PLA segments showed faster degradation and shorter persistence of cells [57]. Additionally, degradable PEG scaffolds loaded with human MSCs (hMSCs) and siRNA against Noggin and miRNA-20a delivered in combination showed controlled and sustained release and enhanced osteogenesis by encapsulated hMSCs [212]. Future directions can explore the fracture healing efficiency when treated by PEG hydrogels co-loaded with siRNA/NP and MSCs. siRNA/NP release studies with the presence of cells are needed to verify that release rates are comparable to hydrogels with no cells since cells endocytose siRNA/NP. At the same time, cell viability studies are needed to evaluate live MSC numbers in the hydrogels and investigate whether cell viability is affected by the addition of siRNA/NPs.

6.5 Thermo-responsive hydrogels

PEG hydrogels represent an efficacious yet simple depot for sustained delivery of NPs. However, when considering translation to patients, improvements are needed for greater ease of use. Thermo-responsive hydrogels, which can be formed spontaneously from polymer solutions in response to temperature changes, present an opportunity to improve localized drug delivery strategies. Thermo-responsive hydrogels can be injected directly into the defect cavity in impaired tissues at a low temperature and solidify at body temperature without any other physical or chemical initiators, representing highly non-invasive and convenient placement. Materials that have a low critical solution temperature (LCST) can form hydrogels when the environmental temperature is above their LCST [182]. For example, Pluronic F-127 is a thermo-responsive polymer that has been studied for decades in drug delivery applications [213]. F-127 is a triblock polymer consisting of poly(propylene oxide) (PPO) core blocks juxtaposed to two poly(ethylene oxide) (PEO) blocks that demonstrates an LCST gelling response [213]. Gelation of the PEO-PPO-PEO systems

relies on the self-assembly of hydrophobic propyl oxide groups versus hydrophilic ethylene oxide units [213]. Pluronic-based hydrogels combined with autologous MSCs have been shown to enhance bone formation in a goat tibia defect model [214]. However, these polymers are non-degradable, have high erosion rates and hindered sustained release capabilities [215]. Thermo-responsive hydrogels based on degradable poly(D,L-lactide-co-glycolide)-poly(ethylene glycol)-poly(D,L-lactide-co-glycolide) triblock copolymers (PLGA-PEG-PLGA) have also shown great potential for clinical applications due to their excellent biocompatibility and tunable gelation behavior [216]. For example, PLGA-PEG-PLGA hydrogels were loaded with simvastatin to provide sustained release of ~14 days and stimulated bone formation in a rat bone defect model [217]. Therefore, thermo-responsive hydrogels are appealing for fracture drug delivery because of the *in situ* forming property irrespective of fracture geometry, providing opportunities to treat challenging injuries with minimally invasive procedures.

6.6 Anti-fouling hydrogels

Foreign body reaction is one of the major challenges associated with implantable biomaterials. A foreign body reaction from implanted biomaterials can cause collagenous capsule formation and limit the performance of hydrogels by interfering with sustained drug release [218, 219]. PEG is one of the most widely used bio-inert materials, yet it elicits a foreign body response due to surface adsorption of inflammatory proteins [220–222]. Subsequently, macrophages recognize the protein coating and attempt to degrade the material. If the implant cannot be degraded, the macrophages fuse to form foreign body giant cells. These giant cells then secrete cytokines that induce fibroblasts to deposit a dense, avascular layer of collagen around the implant, forming a capsule [223]. To address this challenge, zwitterionic materials, including carboxybetaine, sulfobetaine, and phosphorylcholine chemistries, have emerged as an important class of robust ultra-low-fouling biomaterials [224]. The overall electrically neutral zwitterionic structures possess cationic and anionic residues that exhibit strong affinity for water, thereby giving rise to superhydrophilic surfaces that suppress the hydrophobic interactions known to denature proteins [225]. Zwitterionic hydrogels prepared from poly(carboxybetaine methacrylate) (PCBMA) were recently developed to resist the formation of a collagenous capsule in mouse models for at least 3 months and enhanced microvessel formation in the surrounding tissue [226]. Zwitterionic sulfobetaine hydrogels were used for controlled release of rhBMP-2 [227]. Results showed significantly higher sequestration of rhBMP-2 in zwitterionic gels compared to PEG gels. Temporally sequestered rhBMP-2 increased cell attachment and ECM deposition on the low-fouling zwitterionic hydrogel implants and promoted bone healing. These results indicate that zwitterionic materials are appealing in the field of drug delivery, with the advantage of avoiding foreign body formation when implanted for treatment of fractures.

6.7 Combination therapy using systemic and local strategies for fracture healing

Delivery systems that combine localized and targeted drug delivery can be highly versatile when facing clinical conditions that require enhancement of bone regeneration either locally or systemically [204, 228]. Depending on the extent of injury, healing potential, and specific requirements of each case, delivery systems can be combined to maximize healing.

Understanding the various stages of bone healing at the molecular level provides guidance when choosing specific therapeutics and drug delivery systems. For example, localized systems are favored for cell and therapeutic delivery at the initial stage of healing when patients are already undergoing surgical procedures. Scaffolds and implants can be easily placed or injected at the fracture site along with surgical fixation. When using degradable materials, no secondary surgeries are needed to remove the scaffold. In contrast, systemic targeted delivery carriers can be used at later stages as ‘boosters’ of bone formation. Based on longitudinal monitoring of healing progress, the type, dosage, and timing interval of drugs can be adjusted. Thus, systemic delivery is beneficial for later stages since it is more flexible, non-invasive, and likely to have high rates of patient compliance.

7. Conclusions

Skeletal diseases and injuries, such as bone fractures, affect a significant portion of the population. While several protein-based medications are currently approved, these protein-based therapeutics can cause significant and severe side-effects. The delivery systems discussed in this review are able to either localize therapeutics at the fracture site, or to selectively accumulate at fractures after systemic injection to increase local drug availability and efficacy while mitigating adverse effects related to off-target accumulation. The choice of local versus systemic delivery is dependent on the clinical situation and need. Local delivery systems are feasible when an open surgery is conducted and can also be used for mechanical support of the fractured bone. Alternatively, systemic administration could be employed at any time during the healing process without needing to directly access the fracture site. The dosage and interval for administration can be adjusted based on longitudinal radiological healing outcomes. Furthermore, a combinatorial approach can be used, in which a local depot is placed at the time of injury to augment early stages of healing, and systemic therapeutics are introduced later to improve osteogenesis and mineralization. Thus, it is beneficial to develop platforms for local and systemic drug delivery to advance the clinical management of fractures.

To date, no non-invasive therapy that improves fracture repair and reduces the risk of fracture-associated complications has been approved. New modalities, including RNAi therapeutics and small molecule compounds, are under development to circumvent challenges of traditional growth factors. Studies demonstrate that engineered delivery systems alter biodistribution of therapeutic agents to favor accumulation at fractured bone. Furthermore, the therapeutic efficacy of these systems enhances bone formation and bridging *in vivo* and reduces side-effects. As the development of biomaterials and drug delivery continues, incorporation of these technologies in the field of regenerative medicine will ultimately lead to new tools to enhance bone fracture healing.

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Figure 1. Schematic of fracture healing stages, various risking factors for development of non-union healing, as well as an overview of current clinical treatments

* indicate FDA-approved therapies for fractures. (Abbreviations: ACP5: Acid Phosphatase 5; ALPL: Alkaline Phosphatase; ANG: Angiopoietin; BGLAP: Bone Gamma-Carboxyglutamate Protein (Osteocalcin); BMP: Bone Morphogenetic Proteins; COL1A1: Collagen Type I, alpha 1; DKK-1: Dickkopf-related Protein 1; IL: Interleukin; LIPUS: Low-Intensity Pulsed Ultrasound; M-CSF: Macrophage Colony-Stimulating Factor; MSC: Mesenchymal Stem Cell; OPG: Osteoprotegerin; PPARG: Peroxisome Proliferator-Activated Receptor Gamma; PTH: Parathyroid Hormone; RANKL: Receptor Activator of Nuclear Factor Kappa-B Ligand; RUNX2: Runt-Related Transcription Factor 2; SOX-9: Sex Determining Region Y-Box 9; SP7: Osterix; TNFα: Tumor Necrosis Factor Alpha; VEGF: Vascular Endothelial Growth Factor; Wnt: Wingless-Related Integration Site)

Figure 2. Release of fluorescein-labeled PSMA-b-PS NPs from PEG-PLA-DM-based hydrogels (A) NP release was quantified by measuring fluorescence intensity using a microplate reader (470/515), mean $\pm$ SD, n=3. Dashed lines indicated release curves fitting into 1st order release model. (B) Release profile (day 4 to day 23) extracted from (A) and fitted into zero order release model. (C) Summary of rate constants for 2 models, half-lives from 1st order model and R^2 from zero order release model.

Table 1

Summary table of therapeutic molecules studied for enhanced bone healing

Therapeutic	Pathway	Delivery system	<i>In Vivo</i> Model	End-point analysis/Outcome	Ref.
rh-FGF-2	FGF-FGFR signaling	Hyaluronan gel percutaneously injected at fracture site	Primate fracture (1 mm-gap osteotomy)	Larger callus area and greater mechanical properties	[84]
rhPTH 1-34	PTH/PTHrP receptor	Arginine-glycine-aspartic acid (RGD) modified PEG hydrogel with PTH 1-34 covalently bounded.	Canine dental implant	Bone formation comparable to autogenous bone	[96]
Strontium Ranelate	Dual action bone agent	Systemic treatment	Ovariectomized (OVX) rats with fractured tibia	Promotes tibial fracture healing	[157]
Simvastatin	BMP/Smad	Poly(lactic-co-glycolic acid)/hydroxyapatite (SIM/PLGA/HAp) microspheres	Mouse osteotomy with necrotic bone graft	Increased callus formation, neovascularization, and significant cell ingrowth	[158]
Sclerostin monoclonal antibodies	Wnt/ β -catenin	Systemic injection	Femoral osteotomy	Accelerated fracture healing with increased bone mass and biomechanical properties in callus	[159]
Lithium Chloride	Wnt/ β -catenin	Systemic injection	Distraction Osteogenesis	Serum osteocalcin levels and complete bony union	[160]
AZD2858 (AstraZeneca)	Wnt/ β -catenin	Oral administration	Diaphyseal femoral fractures in rats	Enhanced osteoblastic activity and fracture healing	[161]
Lnk siRNA	RNAi	AteloGene atelocollagen gel	Femoral shaft fracture in mice	Intrinsic enhancement of angiogenesis and osteogenesis	[162]
miR-92a	RNAi	Systemic and local	Mouse femoral fracture model	Enhanced endochondral bone formation.	[74]