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## Delivery of small molecules for bone regenerative engineering: preclinical studies and potential clinical applications

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### Abstract

Stimulation of bone regeneration using growth factors is a promising approach for musculoskeletal regenerative engineering. Common limitations with protein growth factors are high manufacturing costs, protein instability, contamination issues, and unwanted immunogenic responses of the host. New strategies for bone regeneration that obviate these problems can have a significant impact on the treatment of skeletal injury and diseases. Over the past decade, a large number of small molecules with the potential of regenerating skeletal tissue have been reported in the literature. Here, we review this literature, paying specific attention to the prospects for small molecule-based bone-regenerative engineering. We also review the preclinical study of small molecules associated with bone regeneration.

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## Keywords

growth factor; small molecule; orthopedic; osteogenesis; tissue engineering; regenerative engineering

Regenerative engineering is an emerging interdisciplinary field at the convergence of life sciences, engineering technology, and physical sciences. Laurencin *et al.* defined regenerative engineering as 'the integration of tissue engineering with advanced material science, stem cell science, and areas of developmental biology' (Figure 1) [1]. Developmental biology research has uncovered pro-regenerative biological protein-based factors that have the capabilities to modulate stem cell activity towards a final outcome of regenerating injured, damaged, or otherwise impaired tissue [2]. The use of growth factors, such as bone morphogenetic protein-2 (BMP-2), for bone repair and regeneration has been widely researched [3–7]. However, growth factors have significant drawbacks that have so far hindered their practical applications [6,8–12]. Small molecules with osteoinductive potential have been proposed as promising alternatives because they are able to minimize or overcome many of the problems associated with protein-based growth factors [11–18]. For instance, in general, small molecules are often too small in molecular size (<1 000 Da) to induce unwanted immune responses in the host [19]. In addition, unlike protein-based growth factors, structural integrity is usually not required for the bioactivity of small-molecule compounds [10,11,20]. With the advent of high-throughput screening (HTS), a large number of small molecules with osteoinductive potential have been discovered over the past decade [21–26]. A literature survey for osteogenic small molecules based on a search of electronic databases over the past 10 years (Figure 2) clearly indicates the increasing interest in the application of small molecules for bone repair and regeneration: a total of 80 relevant publications appeared in electronic databases from January 2013 to October 2013 versus only one article in 2003.

Considering the growing number of osteoinductive small molecules that have been reported in the literature, some of them might represent the next generation of therapies for clinical bone repair and regeneration. In this review, we focus on the prospective future of small-molecule delivery to bone tissue as well as on current preclinical studies associated with small molecules for bone repair and regeneration.

## Delivery of small molecules

Despite the fact that emerging small molecules show promise in various orthopedic applications, their use is limited by their nonspecific adverse effects on nontarget tissues and organs [11,27]. The key to success with utilizing small molecules for bone regeneration is designing suitable delivery systems to localize and sustain the controlled release of small molecules to target sites. Although many types of biomaterial, from biologically derived constructs to those of synthetic origin, have been developed to address this need, constructs that are biocompatible and biodegradable are of the utmost interest. Biodegradability is of particular importance because small molecules can be entrapped during construct fabrication and released during scaffold degradation [28–33].

Scaffolds have been used as vehicles for the controlled delivery of small-molecule drugs, proteins, and nucleic acid for engineering various musculoskeletal tissues, such as bone, skin, nerve, cartilage, ligament, and muscle [34]. For bone tissue-engineering applications, scaffolds are usually biocompatible, 3D, and highly porous, as well as being able to mimic the extracellular matrix of bone in both physical architecture and chemical composition [34]. Such scaffolds provide an elegant system for osteoblasts or stem cells to adhere to the implant surface and respond to small molecules loaded within 3D matrices that initiate the cascade of osteogenic molecular signaling [35]. Many natural and synthetic materials have been used for scaffolds, but within the realm of small-molecule delivery, calcium phosphate (CaP) ceramics have garnered much attention because of their inherent properties that make them attractive as osteoinductive materials [36,37]. There are several types of CaP compound, namely hydroxyapatite (HAp), tricalcium phosphate (TCP), amorphous calcium phosphates (ACPs), and biphasic calcium phosphates (BCPs), which differ slightly in physical and chemical properties. Properties such as surface roughness, crystallinity, solubility, and surface charge stimulate varying effects on cells *in vitro* and the treatment of osteoporosis and healing of long bone fractures, non-unions, and spinal injuries *in vivo* in preclinical animal models. However, the overall chemical nature of these materials is such that they all mainly comprise calcium and phosphate ions, the same ions that make up the bulk of natural bone mineral. Thus, when implanted in bone, these materials are capable of participating in calcium phosphate solid–solution equilibrium at their surface, wherein the requisite calcium and phosphate ions needed to establish this equilibrium can be derived from the implant or surrounding bone, or both. This process has been shown to enhance bone regeneration at defect and injury sites in several preclinical orthopedic applications.

In addition to CaP compounds, polymeric scaffolds have emerged as prime candidates for the delivery of small molecules because of the ease of their processibility and tailorability towards specific chemical and physical properties that, among other design considerations, lead to adjustable pharmacokinetic release profiles [38]. For instance, synthetic polymers, such as poly(lactic-co-glycolic) acid (PLGA), have been among the most attractive candidates used for drug delivery device fabrication [39]. Natural polymers, such as chitosan, have also been used as drug delivery platforms because of the simple preparation methods for their encapsulation of drugs [40]. Furthermore, there are several 3D scaffold fabrication and encapsulation techniques, including but not limited to microspheres, nanospheres, and nanofibers [36,41]. Such scaffolds are favorable because they have more design flexibility because of their inherent ‘bottom-up’ fabrication method.

There are several types of incorporation technique, including covalent bonding, physical adsorption, and entrapment (Figure 3) [42]. Each method has inherent advantageous depending on the biomolecule chemical structure, intended scaffold fabrication method, and desired drug release profile. Given that small molecules are usually highly thermally stable and soluble in various organic solvents, small molecules can be conjugated or loaded into polymeric scaffold by a variety of methods that are not feasible with more fragile full-length protein growth factors [11,13]. Nuttelman *et al.* covalently grafted small-molecule dexamethasone, a supplement used in osteoinductive culture medium, to poly(ethylene glycol) (PEG) through a degradable lactic acid linker using the cross-linker di-isopropyl

carbodiimide. Dexamethasone was released from the polymer upon degradation of the lactide bone, which was shown to be biologically active because it enhanced the osteogenic differentiation of human mesenchymal stem cells *in vitro* [43].

## Preclinical studies and potential clinical applications

Typically, drug development can be divided into three major steps: drug discovery, preclinical animal studies, and clinical trials in humans [44]. Recently, several preclinical studies detailing the use of small molecules in bone regeneration have been reported in the literature. Here, we summarize the recent literature in the area of preclinical evaluations of osteoinductive small molecules over the past 5 years. These preclinical studies focused on animal models of osteoporosis, long bone fractures and non-unions, and spinal fusion (Table 1).

### Osteoporosis

Osteoporosis is the most common form of metabolic bone disorders. In the USA, nearly half of American Caucasian women over the age of 50 have osteoporosis [45]. Although it is often thought of as a disease that affects women, osteoporosis also significantly affects men [46]. Recent US Food and Drug Administration (FDA)-approved medications for osteoporosis, such as recombinant human parathyroid hormone peptide (rhPTH 1-34) (trade name: Forteo<sup>®</sup>), have been shown to be effective in treating osteoporosis [47]. However, they can be problematic because, for example, rhPTH 1-34 can degrade during its shelf life [48]. These observations suggest that small molecules could be utilized as alternative novel bone anabolic agents for the treatment of osteoporosis. In recent years, statins, a family of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors originally developed to treat hypercholesterolemia, have received significant attention in the area of osteoporosis treatment because of their osteoinductivity shown in osteoblasts and bone marrow stromal cells via the bone morphogenetic protein (BMP) signaling pathway [49]. Although statins are the most prescribed medication in western countries, recent evidence from an epidemiological study demonstrated an association between long-term statin use and a reduced risk of low-energy hip fractures in middle-aged and older women [50]. Sustained-release drug delivery systems for statins have been proposed as an effective strategy for osteoporosis. For instance, Ito *et al.* fabricated an injectable delivery device containing the small-molecule simvastatin encapsulated in CaP nanoparticles [51,52]. This novel drug delivery construct was shown to reduce simvastatin cytotoxicity significantly in osteoclast-like MLC-6 cells.

Moreover, implants with local delivery of small molecules have been studied in animal osteoporotic models. The therapeutic efficacy of the simvastatin-loaded CaP coated delivery vehicle observed in an osteoporotic mice model resulted in increased body weight, bone mineral content, and bone mechanical strength compared with controls [52]. Additionally, the therapeutic effect of small-molecule simvastatin on osteogenesis around titanium implants was demonstrated in an ovariectomized model [53,54]. CaP-coated implants releasing bisphosphonate osteoporotic drug increased periprosthetic bone density and implant pull force, compared with control implants in an osteoporotic sheep model [55].

Taken together, these observations indicate that local delivery of small-molecule drugs has great potential to heal osteoporotic bones.

### Long bone fractures and non-unions

In the event of a bone fracture or break, the osseous tissue has the unique repair ability to remodel mineralized tissue to heal the site internally. However, in instances of trauma, congenital defects, or bone tumors, resulting large bone defects often cannot heal and require bone replacement to restore mobility and function [56]. Although bone replacements have traditionally been from autologous or allogenic sources, both types are usually associated with myriad issues, such as donor site morbidity, risks of infection, immune response, and lack of adequate supply [57–59]. Therefore, alternative synthetic options, which are more readily accessible, easily modified, and cost effective, have been explored [1,59]. Tissue-engineered scaffolds loaded with small molecules have been proposed for long bone fracture and non-union applications [11]. For instance, FTY720 is an osteoinductive small-molecule analog of sphingosine-1-phosphate (S1P) that targets the receptors S1P1 and S1P3-5, and has been shown to increase new bone and microvascular formation within a rat cranial defect model [60–62]. To demonstrate the potential of FTY720 to enhance current clinical repair practices for non-unions, investigators from the Botchwey research group coated devitalized bone allografts with a continuous phase of small-molecule FTY720-loaded PLAGA polymer. Results showed that, within a massive rat tibial defect model, the osseointegration, mechanical stability, and smooth muscle cell recruitment of the coated allograft was enhanced by the local delivery of FTY720. At 6 weeks, FTY720-treated groups demonstrated higher bone density at the allograft–implant interface, superior mechanical properties in both elastic modulus and compressive strength, and increased smooth vessel incorporation compared with unloaded controls [60]. The results of this study warrant further investigation into the localized delivery of S1P agonists for improved incorporation of porous, mechanically relevant bone allografts.

Small-molecule purmorphamine has been shown to induce *in vitro* osteogenesis by activation of the Hedgehog signaling pathway [63]. Recently, CaP-bound purmorphamine was fabricated to determine whether the local administration of this small molecule could accelerate bone growth and repair in an *in vivo* chick embryo chorioallantoic membrane (CAM) assay. Purmorphamine was adsorbed to CaP and then injected into a defect site made within the donor femurs. After 7 days, the proportion of trabecular bone area to overall bone area was significantly higher than in the control [64].

Small-molecule BMP-signaling activators have been recently discovered and their *in vitro* and/or *in vivo* effects on osteogenesis in the presence of low dosages of exogenous rhBMPs have been reported [26,65–67]. It is believed that application of these small molecules can significantly lower the dosages of exogenous rhBMPs required or eliminate the need for exogenous rhBMPs, thus significantly reducing overall treatment cost [67]. In one study, Wong *et al.* demonstrated that a single dose of the small-molecule BMP activator, SVAK-12, could accelerate fracture healing in a rat femoral fracture model without the need for exogenous rhBMPs [67].

Several small molecules extracted from traditional Chinese herbal products have shown potential clinical effects. Interestingly, Wong *et al.* evaluated several of these herbally derived small molecules in a rabbit critical-sized bone defect model. In each instance, the authors first created 5 mm × 10 mm critical defect sites in parietal bone in rabbits and the defects were then grafted with collagen matrix carriers preloaded with various small-molecule candidates. Their results revealed that, in each case, delivering small molecules extracted from herbs such as buguzhi [68], psoralen [69], daidzein [70], genistin [71], and quercetin [72], increased bone formation in the critical defect site compared with the control groups. These interesting results indicate the promising effects of herbally derived small molecules to heal bone defects.

As described in the previous section, simvastatin has been investigated extensively for its ability to induce osteogenesis in osteoporotic models. It is believed that simvastatin also has an important role in healing bone fracture. In a recent study, Tai *et al.* incorporated simvastatin in PLAGA/HAp microspheres to induce bone formation in a mouse fracture gap model bridging with a necrotic bone graft (i.e., dead bone). More specifically, at 2 and 4 weeks, it was shown that simvastatin-loaded PLAGA/HAp significantly increased callus formation around the implanted area and increased blood vessel formation and cell ingrowths in the necrotic bone graft, thereby substituting the dead bone [73].

Last but not least, other recent preclinical studies that focus on small-molecule delivery, such as octahydrochloride hydrate (AMD3100) [74], osteogenic inducible compound-active 006 (OIC-A006) [75], and *N*-acetyl cysteine [76], highlight the exciting preclinical developments of small molecules. Such work has the potential to revolutionize strategies for long bone and non-union treatment and repair.

### Spinal fusions

Intervertebral discs (IVDs) give flexibility to the spine and enable the body to twist and bend into a wide range of postures. Approximately 80% of the human population has back problems at some point during their lives [77]. IVD degeneration leads to chronic back pain, is a major health problem in the USA, and causes substantial disability [78]. Spinal fusion is an effective surgical approach following the removal of degenerated IVDs to treat spinal disorders [77]. It is estimated that approximately more than 300 000 lumbar spinal fusions occur each year in the USA [79]. Osteoinductive small molecules are expected to impact on spinal fusion surgeries. Several studies have investigated various small molecules to determine their capacity to aid in spinal fusion. In a recent study, a single dose of simvastatin (120 mg/kg/day) was administered orally to rats and the results showed significantly improved spinal fusion grades in rats through histological examination. More specifically, the rats administered with simvastatin had a mean of 9.30 ± 0.949 Newtons fusion mass compared with a mean of 6.82 ± 2.044 Newtons fusion mass of untreated rats. The histological data were further confirmed by radiographic examination [80]. Interestingly, an earlier study reported by Yee *et al.* revealed that rabbits that were orally administered simvastatin did not exhibit a statistically significant increase in spinal fusion mass compared with the control group [81]. These contradicting studies suggest that there are interspecies differences in the effect of simvastatin on rats and rabbits. Future studies are

needed to determine the effectiveness of small-molecule simvastatin on a larger animal model to provide further evidence of its potential as a spinal fusion agent.

Two novel analogs of oxysterols, Oxy 34 and Oxy 49, have recently been shown to induce bone formation in a rat spinal fusion model. This study revealed that rats receiving collagen implants loaded with Oxy 34 or Oxy 49 showed comparable osteogenic efficacy to BMP2/collagen implants as evaluated by manual inspection, micro-computerized tomography, radiography, and histological analysis [82]. More recently, a similar study conducted by Stappenbeck *et al.* demonstrated that three novel analogs of oxysterols, Oxy 4, Oxy 18, and Oxy 21, were able to stimulate bone formation in a rat spinal fusion model [83]. These observations demonstrate the promise of using small-molecule oxysterols in spinal fusion.

To our knowledge, literature reporting the delivery of small molecules in animal spinal fusion models is limited. Therefore, further research is needed to investigate which specific spinal fusion model, delivery method, and dosage, as well as choice of small molecule, could enhance the result of spinal fusions and, thus, provide the foundation for future clinical usage.

## Concluding remarks and future directions

The use of small molecules for bone regenerative medicines has been largely overlooked. There are several rate-limiting factors for progress in the field of small molecule-based bone regeneration. In general, small molecules are small enough to penetrate nontarget cells and trigger unwanted signaling cascades [84]. Thus, the major concern associated with small-molecule therapeutics is their nonspecific adverse effects [10,11,85–87]. In addition, a lack of an effective delivery strategy for small molecules is another issue, given that one of the objectives of small molecule-based bone regenerative engineering is to develop an engineered scaffold system that provides adequate doses of the small molecule and/or acts as a structural support for infiltrating cells [11,17,60]. Sustained release from ceramic and polymeric carriers has been proposed as a viable strategy for numerous biomolecules, including protein and small-molecule drugs [88–91]. However, the key problem with using ceramic and polymeric carriers for drug delivery is the difficulty in controlling sustained delivery rates [92]. This is particularly true for low-molecular-weight drugs because the diffusion rate is faster and most of the drug diffusion occurs during the first 24 h [93]. More sophisticated drug delivery systems will be valuable for imparting spatiotemporal control over small-molecule release kinetics. Although small molecule-based bone-regenerative engineering holds tremendous promise in various orthopedic applications, several questions for small-molecule researches need to be addressed before clinical translation, including: what is the signaling mechanism of these small molecules in cells? How can one minimize the nonspecific adverse effects of small-molecule drugs? How can one combine stem cell sciences and/or advanced materials for small molecule-based bone regenerative engineering?

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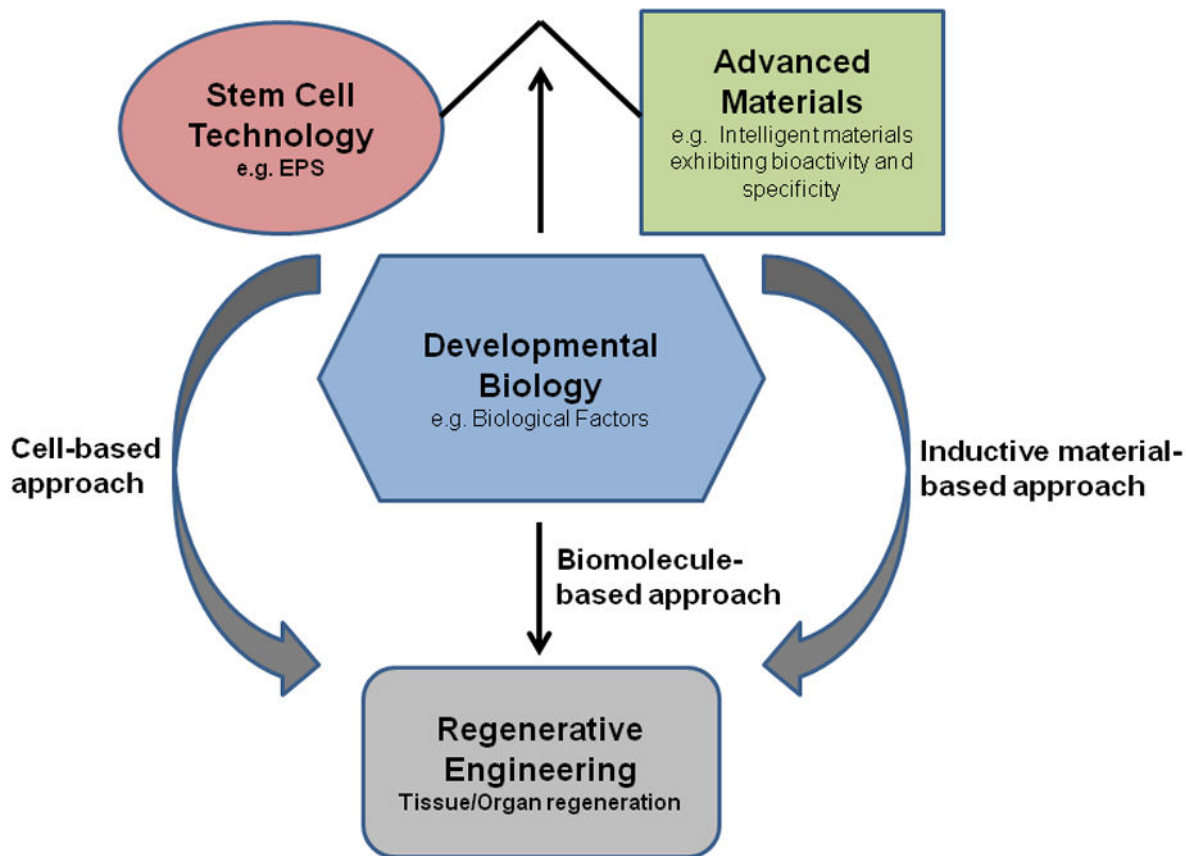
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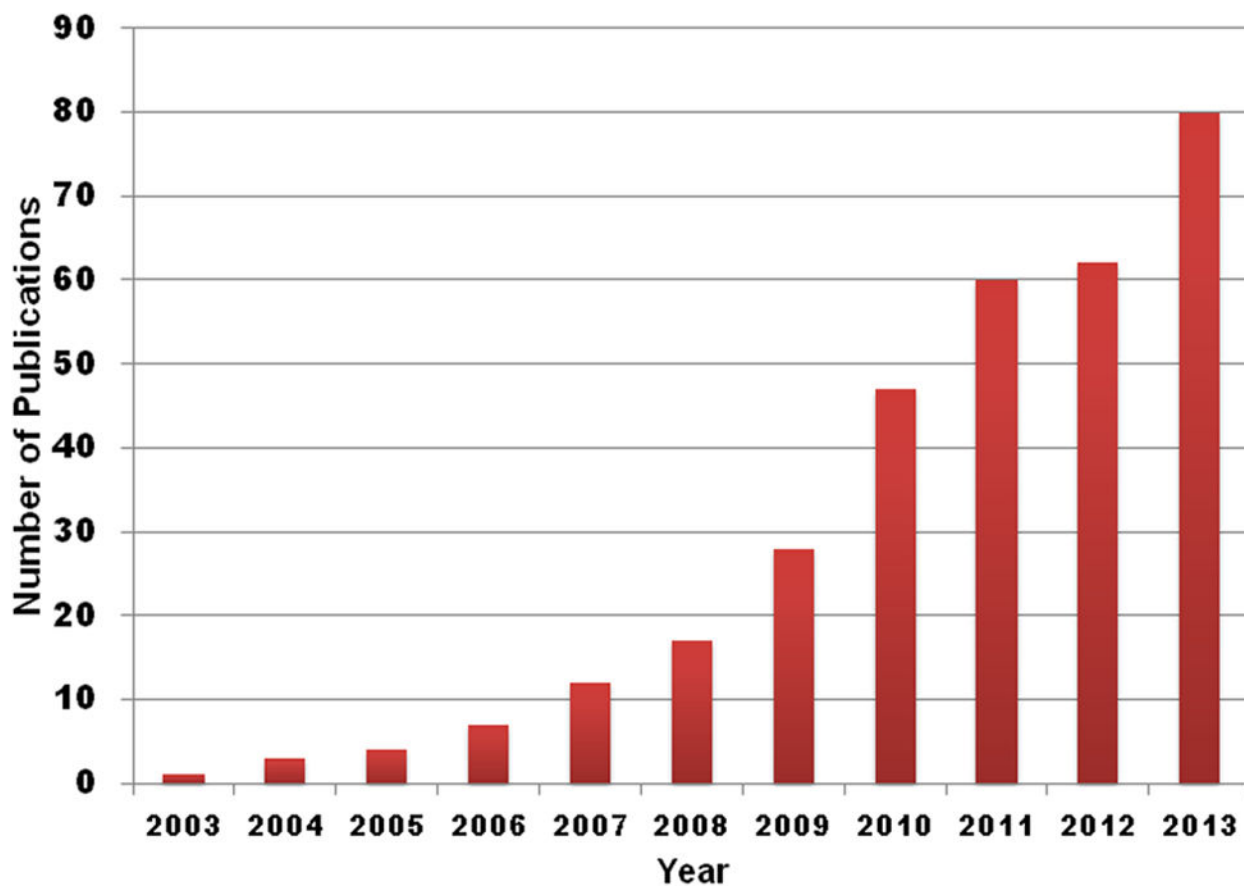
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### Highlights

- Regenerative engineering has been recently proposed to regenerate skeletal tissue
- Regenerative small molecules play a vital role in bone regenerative engineering
- Numerous small molecules have been recently discovered for bone regeneration
- Polymeric scaffolds play an important role to delivery small molecules *in vivo*

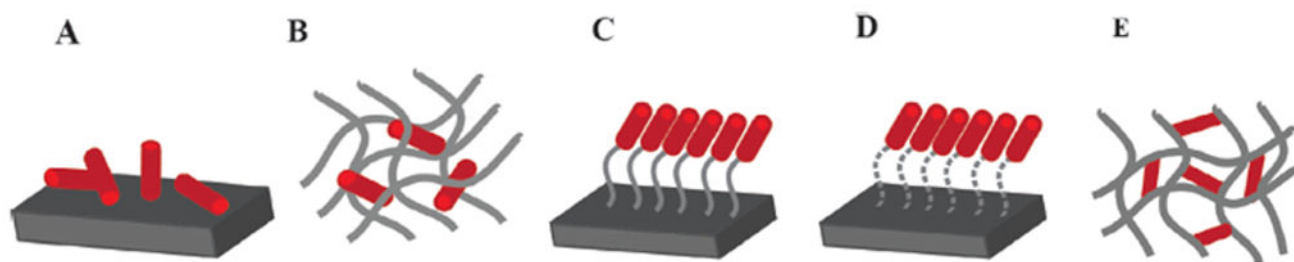


**Figure 1.** A schematic representation of the emerging field of ‘regenerative engineering’. Advanced materials, stem cells, and biological factors alone or in combination have important roles in regenerating tissue. Abbreviation: EPS, XXXXX. Adapted, with permission, from [18,94].



**Figure 2.**

A research survey conducted using different keywords, such as ‘osteogenic small molecules’ or ‘small molecules and bone tissue engineering’ shows an increasing number of publications relating to small-molecule application for bone regenerative engineering over the past 10 years.



**Figure 3.** Schematic depictions of physical adsorption (**A**) and entrapment (**B**), or chemical covalent binding (**C,D**), and crosslinking (**E**) as strategies to incorporate small molecules onto biomaterials. Adapted, with permission, from [42].



**Table 1**  
Small molecules that have been investigated in preclinical bone regenerative engineering studies

Small molecule	MW (Da)	Pathway	Delivery system	Loading	Animal model	Refs
<b>Osteoporosis</b>						
Simvastatin	418.6	BMP/Smad	CaP nanocapsules, including deoxycholate micelles containing simvastatin	1:3 (v/v)	Ovariectomized mouse	[52]
Bisphosphonate	270	Mevalonate	Hydroxyapatite plasma-coated titanium alloy cylinders	Soaked in $2.25 \times 10^{-5}$ mol/l bisphosphonate solution for 48 h	Ovariectomized sheep	[55]
<b>Long bone fractures and non-unions</b>						
FTY720	343.9	S1P	50:50 PLAGA-coated demineralized allograft	1:200 (wt/wt)	Critical-sized rat tibial defect	[60]
Purmorphamine	520.6	Hedgehog	HAP beads	Soaked in 200 mM purmorphamine solution for 24 h	Fetal chick femur	[64]
SVAK-12	137.14	BMP	Not applicable	200 to 250 mg SVAK-12 percutaneously injected	Rat femoral fracture model	[67]
Simvastatin	418.6	BMP/Smad	Calcium sulfate/simvastatin/MSC sheet	CS mixed with 2.5 ml of 200 mg/ml simvastatin solution, then 0.5 mg simvastatin was applied in each scaffold	Rat tibia osteotomy	[95]
Simvastatin	418.6	BMP/Smad	Simvastatin/PLGA/HAP microspheres	77.7% $\pm$ 10.3% (encapsulation efficiency)	Mouse model of gap fracture bridging with a graft of necrotic bone	[73]
Lovastatin	404.5	BMP/Smad	Lovastatin microparticle-loaded polyurethane scaffolds	25 or 100 mg LV-MP in PBS injected into PUR scaffolds	Femoral plug defects and critical-sized segmental defects	[96]
Rosuvastatin	481.5	BMP/Smad	Adsorbable collagen sponge	Soaked in 0.1 mg/ml	Critical-sized rabbit cortical bone defect	[97]
Helioxanthin derivative (TH)	348	BMP/Smad	Tetrapod-shaped alpha tricalcium phosphate granules (Tetrabone®)	Soaked in 1 mM TH solution in DMSO overnight	Rat femur bone defect model	[98]
N-acetyl cysteine	163.19	Unknown	Commercial collagen sponge (Teruplug®)	Soaked in 5.0 mM NAC solution	Critical sized rat femur cortical bone defect	[76]
<b>Spinal fusions</b>						
Simvastatin	418.6	BMP/Smad	Not applicable	Orally administered at a dose of 120 mg/kg/day	Rat spinal fusion	[80]
Oxysterols	420	Hedgehog	Adsorbable collagen sponge	Soaked in 50 ml Oxy34 or Oxy49	Rat spinal fusion	[82]
Oxysterols	420	Hedgehog	Adsorbable collagen sponge	Soaked in 40 ml Oxy4, 18, or 21 for 1–2 h	Rat spinal fusion	[83]
Rolipram	275.4	PKA	Alginate-microfibrous patch	25 or 50 g/ml rolipram-loaded hydrogels	Rat hemisection spinal cord injury	[99]