Biomimetic hydrogels for controlled biomolecule delivery to augment bone regeneration

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Abstract

The regeneration of large bone defects caused by trauma or disease remains a significant clinical problem. Although osteoinductive growth factors such as bone morphogenetic proteins have entered clinics, transplantation of autologous bone remains the gold standard to treat bone defects. The effective treatment of bone defects by protein therapeutics in humans requires quantities that exceed the physiological doses by several orders of magnitude. This not only results in very high treatment costs but also bears considerable risks for adverse side effects. These issues have motivated the development of biomaterials technologies allowing to better control biomolecule delivery from the solid phase. Here we review recent approaches to immobilize biomolecules by affinity binding or by covalent grafting to biomaterial matrices. We focus on biomaterials concepts that are inspired by extracellular matrix (ECM) biology and in particular the dynamic interaction of growth factors with the ECM. We highlight the value of synthetic, ECM-mimicking matrices for future technologies to study bone biology and develop the next generation of ‘smart’ implants.

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1. Introduction

A substantial fraction of bone defects do not heal properly using standard treatments [1]. The incidence of such ‘non-unions’ correlates with the severity of an injury, the extent of soft tissue damage, inadequate
internal fixation, advanced age, or co-morbidities such as diabetes [2-7]. Despite decades of intensive research, the grafting of autologous bone derived from the iliac crest remains the gold standard for treatment. Unfortunately, the amount of autologous bone is limited and surgical intervention is associated with donor site morbidity [8,9]. The use of allogeneic bone tissue from cadavers can overcome this problem but is complicated by possible infections and immune responses of the host tissue [1,10].

These clinical challenges have been a strong impetus for the development of alternative approaches to regenerate bone based on strategies promoting the inherent (limited) capacity of bone to regenerate. Approaches based on gene therapy, for example via the transduction of genes encoding osteogenic factors, stem/progenitor cell-based therapy, or by example by the transplantation of autologous bone marrow-derived stem cells, and the therapeutic delivery of osteoinductive biomolecules have been tested. Although gene therapy and stem/progenitor cell transplantation have shown high promise in small animal models, significant safety and efficacy concerns will need to be overcome first before these approaches become clinical reality. For further information on these two approaches the reader is referred to other reviews and references therein [11,12]. Here we focus on the last approach, the controlled delivery of osteoinductive factors by means of biomaterial matrices to elicit a highly localized tissue response.

The discovery of factors that induce bone growth such as bone morphogenetic proteins (BMPs) has raised tremendous hope that bone could be readily regenerated by their local application [13,14]. Unfortunately, the promising outcomes of studies in small laboratory animals [15] could only partially be translated to the human situation [16-18]. A major limitation to growth factor therapy is that enormous quantities of growth factors, largely exceeding physiological levels, are needed to induce the formation of bone. For example, 3.5 mg of recombinant BMP-7 used for the treatment of a bone defects corresponds to 2-fold of the entire amount of BMP-7 found in a human being [1]. This discrepancy leads to very high treatment costs and a significant risk for adverse side effects such as ectopic bone formation or osteoinduction [19]. It is not entirely clear what causes the very low efficiency of growth factor (e.g. vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), BMP) treatment [20,21]. It has been suggested that an inadequate presentation of the growth factors, resulting in their fast inactivation and clearance, could be responsible [20,21]. Alternatively, negative feedback mechanisms, that is, the induction of growth factor antagonists or inhibition of agonists, and the limited functionality or availability of responsive (bone progenitor) cells could play a role. Irrespective of the underlying mechanisms, it is clear that better delivery strategies must be developed to improve growth factor therapy.

Not surprisingly, the development and application of novel biomaterials for the controlled delivery of growth factors has stimulated considerable interest in the (bone) tissue engineering community [13,22,23]. In particular, ‘biomimetic’ scaffolds emerged whose design was inspired by the role of the natural extracellular matrix (ECM) in regulating tissue regeneration [24,25]. Such scaffolds are conceived to take advantage of the bodies’ inherent capacity to heal, essentially using the body as bioreactor to regenerate bone. Here, we will discuss this fascinating approach. We begin with a brief review of the biology of bone regeneration and the dynamic role of the ECM in this process. We then focus on strategies to engineer ECM-mimicking biomolecule delivery systems, using hydrogel materials as examples. Finally, we provide an outlook on the combination of growth factor binding and delivery strategies with other technologies such as microfabrication to build more realistic in vitro platforms to experimentally model the biology of bone regeneration.

2. ECM components in growth factor dynamics and signalling

Throughout tissue development, maintenance and regeneration, the ECM provides critical instructive cues to control the behavior of embedded cells that constantly remodel their microenvironment by proteolytic processes [26]. Thus, cells secrete proteases such as matrix metalloproteinases (MMPs) that degrade ECM components. At the same time, novel ECM components are secreted and deposited by cells to build up new ECM. During this process, the composition of ECM signals modulates the expression and synthesis of new ECM, establishing a “dynamic reciprocity” [27,28]. Furthermore, the dynamic change in ECM composition results in the release and presentation of ECM-derived ‘cryptic’ fragments such as collagen type IV- or XVIII-derived canstatin [29], tumstatin [30] or endostatin [31], that actively contribute to the regulation of angiogenesis [32]. Additionally, the change in matrix composition leads to a shift in ligand spectrum and consequently modification of integrin receptor signalling. Finally, the ECM is crucial for tissue dynamics through its ability to store and release growth factors as discussed below.

2.1. ECM-growth factor interactions

The organic fraction of the bone ECM is an osteoblast-deposited scaffold consisting of mostly fibrillar proteins (e.g. collagen type I), glycoproteins (e.g. osteonectin, fibronectin, and thrombospondin), sialoproteins (e.g. bone sialoprotein and osteopontin), and proteoglycans, consisting of a core protein with covalently attached glycosaminoglycans (GAG) (e.g. chondroitin sulfate, dermatan sulfate, heparan sulfate, and heparin) [33]. It is well accepted that many growth factors interact with GAG components [34] and that this interaction is critical for morphogenetic processes during tissue formation and tissue regeneration [35,36]. The interaction between growth factors and proteoglycans is mediated by electrostatic interactions, hydrogen bonds and van der Waals interactions [37]. Depending on the growth factor type resulting affinities range from $K_D$ values of 1 to 100 nM for VEGF, fibroblast growth factor-2 (FGF-2), and transforming growth factor beta (TGF-β) [38-40]. Localized growth factor binding by ECM components results in the formation of a growth factor ‘sink’, which, depending on the affinity for the respective growth factor, can result in a stable growth factor gradient. The contribution of gradients to tissue morphogenesis has been impressively shown with the angiogenesis inducing VEGF. Dependent on the isoform, VEGF does (VEGF$_{121}$) or does not (VEGF$_{165}$, VEGF$_{189}$, VEGF$_{206}$) contain a C-terminal heparin binding domain, resulting in differential distribution in the cell surrounding ECM [41-43]. While heparin-bound VEGF$_{185}$ formed a stable gradient and resulted in directed migration of endothelial cells towards the highest concentration, soluble VEGF$_{121}$, distributed by diffusion control, was unable to recruit endothelial cells and eventually stimulated endothelial cells of distant capillaries. Mice expressing only one of the respective VEGF isoforms had signs of vessel malformation and only the coordinately expressed expression of all VEGF isoforms leads to the formation of properly branched vascular network [44]. This is only one of many examples that illustrate the role of ECM components in establishing stable growth factor patterns that are key to proper development. Similar findings were reported for ECM components such as fibronectin, vitronectin, tenasin, thrombospondin, fibrillin and collagen type II, which specifically bind platelet derived growth factor (PDGF), VEGF, hepatocyte growth factor (HGF), TGF-β and BMP [45].

2.2. Interactions between matrix-immobilized ligands and growth factor receptors

Matrix molecules are composed of multiple and often repetitive domains with different functions. For example, several ECM proteins including laminin, tenasin, thrombospondin, and versican were described to contain epidermal growth factor (EGF)-like repeats that were shown to interact and activate EGF receptor (EGFR) [46-49]. Such domains, also named matrikines, are exceptional, as they are matrix-immobilized and might result in long-term binding to cell surface receptors normally binding cytokines, chemokines or growth
factors. In analogy to integrin-mediated ECM interactions, the binding affinity of matrikines is relatively weak compared to growth factors—receptor interactions. Nonetheless, as such ligands are presented as multiple repeats, a strong avidity is achieved resulting in significant binding strengths. These examples illustrate that ECM do not only contribute to growth factor binding and presentation of tethered signals, but also act as repository of signalling molecules [45].

There is only relatively limited data available on growth factor receptor interactions with ECM-bound growth factors. One such example is binding dependence of FGF to a heparan sulfate to effectively stimulate FGF receptor (FGFR) [50]. Interestingly, Marfan syndrome, a genetic disorder caused by mutations in the fibrillin gene, appears to affect the skeletal system by impaired sequestering and availability of the latent form of TGF-β [51,52].

2.3. ECM/growth factor co-signalling

Adhesion molecules such as integrin ligands and cell adhesion molecules (CAMs) actively participate in the regulation of growth factor-mediated signalling [53]. The integration of integrin and growth factor signals seems to be achieved by formation of supramolecular complexes containing integrins and growth factor receptors (GFR) [54]. Such laterally segregated complexes allow the sensing of positional cues and the linking of growth factor functions to sites of adequate adhesion. Furthermore, ECM ligand—integrin interactions have been suggested to induce ligand-independent partial activation of GFR, resulting in minimal cell survival and migration signals. Such activation could also sensitize GFR, whereby subsequent stimulation through the growth factor ligand can potentiate the GFR's activity of matrikines. In analogy to integrin-mediated ECM interactions, the binding strength of integrin ligands and cell adhesion molecules (CAMs) is relatively weak compared to growth factors—receptor interactions. Nonetheless, as such ligands are presented as multiple repeats, a strong avidity is achieved resulting in significant binding strengths. These examples illustrate that ECM do not only contribute to growth factor binding and presentation of tethered signals, but also act as repository of signalling molecules [45].

Since the co-ordinated signalling of GFR and integrins is only beginning to be understood, not much effort has been spent on presenting growth factors with signalling promoting cell adhesion ligands. Only in a very recent study biomaterials schemes have been developed which allow the evaluation of growth factor/integrin co-signalling [63]. This study provides evidence that the growth factors efficiencies could be dramatically increased by co-presentation of fibronectin (FN) fragments with PDGF, VEGF or BMP.

3. Bone tissue remodelling and regeneration

During bone tissue formation, homeostasis and regeneration, which is the recapitulation of the initial developmental process [64], the immobilization and release of growth factors is likely dependent on respective microenvironments. For example, during osteoclast-mediated bone remodelling, BMP-binding hydroxyapatite is resorbed by the low pH in the resorption pits, while the remaining organic matrix is digested by MMPs and proteinase K [65,66]. The tightly regulated coupling of osteoclast-mediated bone resorption and osteoblast-mediated bone formation today is thought to be controlled by ECM-derived signals such as immobilized growth factors [67], osteoclast secreted signals or even mechanical stimuli [68]. Also, the healing of fractures and large bone defects appears to be largely established by temporally coordin-ated anabolic and catabolic processes orchestrated by growth factors and cytokines [69].

Healing can either occur via direct intramembranous bone formation, if fractures are well apposed and stabilized or via endochondral bone formation if non-stabilized or large defects need to be bridged [64,70]. Endochondral bone formation goes through four histologically distinguishable stages (inflammation, soft callus formation, hard callus formation, and remodelling), leading to the reestablishment of functional bone [64,71]. By disruption of the tissue integrity and disintegration of vessels a hematoma is formed immediately, which is a rich source of platelet-derived, inflammation-inducing cytokines and growth factors (Fig. 1a) [72]. Macrophages and other inflammatory cells infiltrate the hematoma and release cytokines and growth factors [64,71,73]. In this provisional fibrin matrix rich in FN, growth factors eventually bind to FN and regulate the ingrowth of capillaries and the formation of a granulation tissue. The myriad of released growth factors attracts additional leucocytes, which in turn recruit fibroblasts and mesenchymal stem/progenitor cells (termed here ‘MSC’) from adjacent periosteum, bone marrow, or circulation. MSCs proliferate, condense, and differentiate to form chondrocytes that, together with fibroblasts, form a callus that adds to the stabilization of the fracture site (Fig. 1b). Cartilage formation occurs until the granulation tissue is entirely replaced. The formed matrix rich in collagen type II and GAGs likely stores growth factors, which in the later stages of bone healing can be released by ECM-degrading proteolytic enzymes and heparin lyses (removal of heparan sulphate), together modulating the availability of GF [74]. During this phase, the ingrowth of capillaries is initiated by pro-angiogenic factors. In order to further stabilize the fracture site, the soft callus is gradually remodelled and osteoprogenitors give rise to osteoblasts that form a proteinaceous and mineralized bone matrix starting from the peripheral callus (Fig. 1c) [64,71,72]. The de novo formed woven bone is remodelled into cortical or trabecular bone at later stages.

Taken together, bone remodelling and regeneration is a highly dynamic process governed by complex cellular and molecular mechanisms. The resulting temporal growth factor composition, availability, distribution and activity will likely provide multiple possibilities for therapeutic interventions.

4. Growth factors involved in bone regeneration

The discovery of BMP has initiated the search, characterization and cloning of growth factors involved in bone formation [75,76]. BMP-2 to BMP-9 are the most potent growth factors known to induce ectopic bone formation [77] and are therefore strongly dominating today’s bone tissue engineering landscape. However, fracture healing is a multistep process, involving various cell types, such as inflammatory cells, vascular cells, mesenchymal progenitor cells, and osteocytes. Their behavior is orchestrated by a specific set of growth factors, which can be grouped into inflammatory growth factors and cytokines, pro-osteogenic growth factors, and angiogenic growth factors [72]. Inflammatory growth factors and cytokines are involved in the first stage of fracture healing and mainly stimulate the recruitment of additional inflammatory cells. They include FGF-2, interleukin-1 (IL1), interleukin-6 (IL6), macrophage colony stimulating factor (M-CSF), and tumor necrosis factor-α (TNF-α). Correlating with the formation of a fracture callus, high concentrations of pro-osteogenic growth factors, such as PDGF and TGF-β, that stimulate the recruitment of mesenchymal progenitor cells, can then be observed. Subsequently, progenitor cell proliferation and differentiation is induced by the pro-osteogenic growth factors TGF-β, FGF-1, insulin-like growth factor (IGF), and members of the BMP family. Pro-angiogenic growth factors, such as VEGF, BMPS, FGF-1, and TGF-β, recruit endothelial cells, which invade the soft callus and re-establish the vasculature network.

Since multiple cell types and biomolecules need to perform their allocated function to orchestrate bone regeneration, growth factor delivery for effective bone tissue engineering should consider more than just a single step in the process and involve growth factors belonging to all classes. Hence, although BMPs might be the most ‘prominent’ growth factors, only by combining them with signals that trigger other cascades in regeneration, can we expect to fully enlist the body’s capacity to regenerate bone.
5. Biomimetic hydrogels for cell-demanded release

The increased knowledge on growth factor biology in bone regeneration led to high expectations for clinical exploitation. What is more, the possibility of large-scale production of recombinant growth factors in prokaryotic or eukaryotic cells, as well as the possibility to flexibly modify by recombinant technology was expected to result in fast advances in therapy. Indeed, preclinical trials have shown promising results. However, first clinical trials with a number of promising growth factors including VEGF and BMP for neovascularisation and bone formation, respectively, have either not shown significant effects or were of limited efficiency [16,20,78]. The generally poor outcome of first-generation recombinant growth factors could be attributed to their mode of delivery, which usually was the injection of pure growth factors as bolus, as probably would be appropriate for hormones rather than for growth factors [79]. Such uncontrolled delivered growth factors results in systemic rather than localized responses. Furthermore, in addition to their distribution within the body, the relatively short half-live of GFs in the circulation might translate to initial excess levels, which are quickly followed by a deficit of growth factors [80-82]. Importantly, a relatively narrow therapeutic window of growth factor dose is expected to lead to regeneration, whereas too low or too high growth factor doses will most likely lead to severe side effects, as shown in animal models [83,84]. Since for the generation of constant growth factor levels, multiple injections or even continuous infusion of high doses would be necessary, alternative routes involving biologically inspired growth factor delivery are sought.

5.1. Hydrogels for growth factor immobilization

In an ideal situation, the localized delivery of growth factors should present physiologically relevant doses and preserve its activity for prolonged periods of time. Moreover, carrier materials should facilitate the communication with cells of the host so as to be actively involved in the process of regeneration. Synthetic hydrogels, highly swollen three-dimensional (3D) networks of macromolecules, have emerged as powerful candidate biomaterials to fulfil these requirements [85,86]. Hydrogels for growth factor delivery applications

Fig. 1. Bone fracture healing. The healing of non-stabilized fractures is a complex process which, by a series of catabolic and anabolic processes, leads to the establishment of functionally and morphologically intact bone structures. The initial healing can by histological appearance be divided in (A) inflammatory, (B) soft callus, and (C) hard callus phase. Each phase is regulated by a myriad of soluble, matrix immobilized, and matrix cues as well as different cell types.
have either been generated from natural derived biomolecules such as alginate, collagen, and fibrin or from synthetic polymers employing chemical or physical crosslinking reactions \[87\].

For growth factor administration, the growth factor can either be freely embedded in the hydrogel or bound to it. In the former approach, growth factor release is driven by passive diffusion or coupled to material degradation. Release kinetics can be varied by altering material degradation rate or by changing growth factor quantity. For further information on such materials and delivery strategies the reader is referred to other reviews and references therein \[22,81\]. Here, we focus on the latter approach and below we will briefly review covalent and non-covalent strategies for growth factor immobilization in bone regeneration.

### 5.2. Covalent tethering of growth factors to hydrogels

To allow covalent tethering to synthetic or biologically derived hydrogels, GF were chemically modified or genetically engineered to contain functional groups such as thiol, acrylates, azides, and Gln tags. Table 1 summarizes the different approaches that have already been employed to attach growth factors to hydrogels.

Initial approaches to chemically conjugate growth factors to collagen matrices were based on the use of homobifunctional poly(ethylene glycol) (PEG)-based cross-linkers containing terminal and primary amine selective succinimidyl groups \[88,89\]. The use of such linkers then expanded to simultaneous cross-linking of collagen matrices and covalent tethering of growth factors (Fig. 2A). The sustained delivery of tethered TGF-ß2 and VEGF \[90\], resulted in an enhanced and prolonged response in vivo, compared to the unmodified growth factors. In another chemical coupling strategy, growth factors were incorporated into synthetic PEG networks using hetero-bifunctional N-hydroxysuccinimide (NHS)-PEG-acylation linkers. Growth factors such as TGF-ß1, FGF-2 \[91\], EGF \[92\], and ephrin-A1 \[93\] were modified in a first step by reaction with the amine-specific NHS group. In a subsequent step, the acylated growth factors were covalently conjugated into PEG-diacrylate (PEG-DA) networks by photopolymerization (Fig. 2B). Recently, ‘click’ chemistry was reported as promising technique to fabricate bioactive PEG hydrogels having tethered biomolecules \[94\]. Azide-functionalized BMP-derived peptides (Az-PEG-BMP) were formed by reacting PEGylated BMP with 4-carboxybenzenesulfonazole. A click reaction then allowed for forming hydrogels and simultaneous tethering of Az-PEG-BMP. The immobilized BMP peptide was used to induce osteogenic differentiation and mineralization of bone marrow stromal cells.

The described strategies are based on adding a chemically functionalized linker to the native protein. This can be achieved by a cross-linking reaction between the growth factor amines from any accessible lysine and the N-terminus and a functional group of the linker. While these strategies have the advantage of being applicable to a broad spectrum of proteins, the exact site and the number of modification are difficult to control and may interfere with the growth factors bioactivity \[95,96\]. Therefore, strategies were sought that allow for site-specific modification. Since reduced (i.e. unpaired) cysteines are of relatively low abundance in proteins, proteins can be recombinantly engineered with additional cysteines. In one study, an exogenous cysteine was added to the C-terminus of VEGF \[97\] and VEGF \[97\]. Positively charged amino acids in close proximity to the exogenous cysteine lowered the pK\(_{a}\) of the cysteine residue's thiol rendering it more susceptible to undergo Michael-type addition reaction with vinylsulfone groups of PEG macromers (Fig. 2C). Release of covalently bound VEGF was mediated by MMP- or plasmin-mediated gel degradation.

To couple growth factors to fibrin hydrogels in a highly site-specific fashion, at the end of the 1990s Hubbell and colleagues developed an elegant enzymatic technique for the covalent incorporation of bioactive polypeptides and growth factors \[98-101\]. This strategy relies on mimicking the transglutaminase FXIII-mediated incorporation of \(\alpha\)-plasmin inhibitor as it occurs during the natural cross-linking of the physically associated fibrin fibres in the blood clot during coagulation. Any desired peptides or proteins could be produced as fusion peptide/protein containing the eight-amino acid substrate sequence NQEQVSP (Gln) of \(\alpha\)-plasmin inhibitor's N-terminus such as to be incorporated into the forming fibrin matrix by the enzymatic activity of factor Xllla (Fig. 2D). Fibrin matrices that are functionalized with growth factors in such a way can serve as excellent growth factor delivery vehicles, releasing the growth factor cargo through proteolytic activity of invading cells in a release that is ‘in tune’ with tissue morphogenesis. By recombinant engineering the Gln-sequence was for example grafted to the N or C-terminus of beta-nerve growth factor (\(\beta\)-NGF) \[99\], VEGF \[101-104\], truncated angiopoietin-1 (\(\Delta\)Ang-1) \[105\], ephrin-B2 \[106\], sixth Ig-like domain of L1 (L1Ig6) \[107-110\], parathyroid hormone (PTH) \[111\], IGF-1 \[112\] and BMP \[113\]. Fibrin gels containing these fusion peptides were fabricated and validated in various models of tissue regeneration. The resulting growth factor delivery matrices were for example shown to elicit angiogenic and osteogenic responses in \(in vivo\) and \(in vitro\) settings. The tethered growth factors can be released by bulk matrix degradation whereas the speed of gel degradation is dependent on the localized cell-mediated proteolytic activity of recruited cells. This cell-demanded release is highly sustained and requires only low-doses of growth factors. In the case of VEGF, the strategy was shown to induce significantly more and morphologically intact blood vessels as compared to fast released VEGF that induced the formation of malformed, leaky vessels \[102\]. Through the introduction of a plasmin-sensitive

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**Table 1**

<table>
<thead>
<tr>
<th>Biomolecule</th>
<th>Engineering</th>
<th>Linker/reactive group</th>
<th>Matrix</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-ß2</td>
<td>Chemical</td>
<td>SG-PEG-SG</td>
<td>Collagen</td>
<td>[88]</td>
</tr>
<tr>
<td>VEGF [100]</td>
<td>Chemical</td>
<td>SS-PEG-SS</td>
<td>Collagen</td>
<td>[89]</td>
</tr>
<tr>
<td>TGF-ß1, VEGF [105]</td>
<td>Chemical</td>
<td>NHS-PEG-Acryloyl</td>
<td>PEG-DA</td>
<td>[90-93]</td>
</tr>
<tr>
<td>PDGF-BB, FGF-2</td>
<td>Chemical</td>
<td>NHS-PEG-Acryloyl</td>
<td>PEG-DA</td>
<td>[90-93]</td>
</tr>
<tr>
<td>BMP- peptide</td>
<td>Chemical</td>
<td>PEG-azide</td>
<td>PLEOF</td>
<td>[94]</td>
</tr>
<tr>
<td>KGF</td>
<td>Chemical</td>
<td>SMCC-Gln</td>
<td>Fibrin</td>
<td>[114]</td>
</tr>
<tr>
<td>SDF-1c</td>
<td>Chemical</td>
<td>BTC-PEG-BTC</td>
<td>Fibrin</td>
<td>[156]</td>
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<tr>
<td>IFN-γ</td>
<td>Chemical</td>
<td>IDC/sufo-NHS</td>
<td>MAC</td>
<td>[157]</td>
</tr>
<tr>
<td>VEGF [100]/VEGF [105]</td>
<td>Recombinant</td>
<td>Recombinant Biocomposite-Cys</td>
<td>PEG-VA</td>
<td>[97,158]</td>
</tr>
<tr>
<td>((\gamma)-NGF, BMP, VEGF [105], (\Delta)Ang-1, ephrin-B2, PTH, L1Ig6, IGF-1)</td>
<td>Recombinant</td>
<td>Recombinant Cln-Biocomposite</td>
<td>Fibrin</td>
<td>[99,101-113]</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>Recombinant</td>
<td>Recombinant Cln-Biocomposite</td>
<td>PEG-TG</td>
<td>[116]</td>
</tr>
</tbody>
</table>

**Abbreviations:** SG-PEG-SG, disuccinimidyldiglutaratepolyethyleneglycol; SS-PEG-SS, disuccinimidyldisuccinatepolyethyleneglycol; NHS, N-hydroxysuccinimide; DA, diacrylate; SMC, succinimidyl carbonate; PLEOF, poly(lactide-co-ethylen oxide-co-fumarate); SMCC, succinimidyl trans-4-[(maleimidyl)methyl] cyclohexane-1-carboxylate; Gln, NQEQVSP- peptide; Cys, cysteine; BTC, benzotriazole carbonate; MAC, methacrylamide chitosan; VS, vinylsulfone; \(\Delta\)Ang-1, truncated version of angiopoietin-1; PEG-TG, transglutaminase FXIII formed PEG hydrogel reactive group interacting with growth factor is underlined.
linker domain between the growth factor and the Gln-containing peptide, release relies on the cleavage of the immobilization domain and can therefore be accelerated [103]. Thus, the ability to engineer differential release patterns offers an almost unprecedented opportunity to tailor biomaterials that sequentially release waves of growth factors over extended periods of time.

A modified version of the above enzymatic coupling strategy was presented by the coupling of the Gln-sequence to keratinocyte growth factor (KGF) via a two-step chemical cross-linking mechanism (Fig. 2E) [114]. Hetero-bifunctional succinimidyl-PEG-maleimide was employed in a first step to form maleimido-KGF, which in a second step was reacted with Cysteine-functionalized Gln-peptides. The in vivo effectiveness of KGF-modified fibrin was demonstrated in a wound healing model, showing that the gradual KGF release and prolonged persistence in the wound results in advantageous wound healing.

Recently, the FXIII-based coupling strategy was conferred on a fully synthetic FXIIIa cross-linkable PEG hydrogel system (Fig. 2F) [115], consisting of equimolar mixtures of precursors that are terminally functionalized with either the FXIIIa-specific substrate sequence AcFKG or the Gln-sequence. Thus, simultaneous to the FXIIIa-controlled covalent hydrogel formation, the growth factors of peptides bearing Gln tags can be incorporated [116]. In this completely synthetic scheme, growth factor release can be flexibly and reproducibly controlled by variation of the matrix cross-link density, engineering the matrix sensitivity towards proteolytic remodelling, and using different immobilization strategies.

Fig. 2. Biomimetic hydrogels. Biomimetic hydrogels with different covalent and affinity based immobilization strategies for growth factors have been developed in the past decade. (A–F) Covalent strategies generally rely on (A, B, E) chemical (carboxyle, amine, or cystein selective) or (C, D, F) genetic (addition of functional tags) modifications of growth factors. (G–J) Non-covalent strategies employed naturally occurring (G–I) heparin or (J) fibronectin affinities to immobilize growth factors via affinity interactions within biomaterials.
5.3. Non-covalent tethering of biomolecules to hydrogels

Since the covalent immobilization of growth factors relies either on chemical modifications or genetic engineering of growth factors these strategies, in addition to being rather time- and labor-intensive, might compromise the growth factor activity [117]. The development of hydrogels that bind growth factors by affinity interactions is an attractive alternative to covalent GF incorporation [118]. Taking advantage of affinity interactions that naturally occur between growth factors and components of the ECM has been pursued extensively in the past decade (Table 2). Since this strategy does not require the direct chemical or genetic modification of the protein, it offers a higher grade of flexibility. Furthermore, since no proteolytic activity is required to release growth factors from reversible affinity interactions, they offer an elegant way to tune the delivery of growth factors from hydrogels [117]. Therefore, much effort was devoted to the modification and incorporation of hydrogels by naturally derived growth factor binding components such as heparin, chondroitin sulfate, hyaluronic acid or fibronectin.

An early example of this strategy is the covalent incorporation of EDC/NHS-activated heparin into collagen matrices using NHS-chemistry (Fig. 2G) [119]. Heparin-modified collagen matrices were employed in multiple experimental paradigms to trap and deliver VEGF [120–122], FGF-2 [119,123,124], and stromal cell-derived factor-1 α (SDF-1α) [125]. Since heparin is a widely used anticoagulant, immobilization of heparin on collagen matrices reduces the thrombogenic activity of collagen and may therefore prevent platelet adhesion and blood coagulation [124]. A similar strategy was employed for the modification of purely synthetic hydrogels with heparin. To this end, amine-functionalized star-PEG was modified with heparin by EDC/sulfo-NHS chemistry [126]. Interestingly, heparin was also used as the main component of the hydrogel matrix and as a growth factor adhesion site by functionalizing it with maleimide groups. Upon reaction of heparin-maleimide with thiol terminated 4-arm star PEG, hydrogels could be formed [127]. FGF added to the reaction mixture was immobilized in the assembled hydrogels via heparin binding and the release was a function of matrix erosion. The same group also reported on a strategy for hydrogel formation via the cross-linking of heparin functionalized PEG by dimeric, heparin binding growth factors, as shown for VEGF [128]. An interesting outcome of hydrogels produced with this strategy is the fact that they can be degraded via receptor-mediated gel erosion and thus gels can specifically target cells that express the receptor corresponding to the growth factor used for crosslinking. One disadvantage of strategies relying on chemical conjugation of heparin is perhaps the fact that they preclude the simultaneous addition of growth factors and cells during crosslinking.

In order to overcome this limitation and to functionalize fibrin matrices with heparin, a bifunctional linker-peptide was designed, consisting of a heparin binding peptide (HBP) derived from antithrombin III (ATIII) and a Glu acceptor substrate [98]. The covalent tethering of the linker-peptide to fibrin matrices during crosslinking confers heparin affinity to the fibrin matrix. Thus, heparin and growth factors with a natural affinity for heparin are preferentially retained within this modified fibrin leading to a slow and continuous delivery modality (Fig. 2H) [129]. This strategy was employed for example for the delivery of a variety of growth factors such as β-NGF [130], neurotrophin-3 (NT-3) [131–136], FGF-2 [129], and PDGF-BB [137,138]. In vitro an in vivo data demonstrated the ability of such growth factor depots to promote nerve regeneration, angiogenesis, and tendon healing.

Along similar lines, the development of an injectable hyaluronic acid (HA)-based hydrogel crosslinked with PEG-diacylate and consisting of thiol-modified heparin (heparin-DTPH) was reported (Fig. 2I) [139,140]. The hydrogel network could also be modified with chondroitin sulphate or gelatine using chondroitin sulphate (CS-DTPH) or thiol-modified gelatine, respectively and has been used to deliver several proteins, including VEGF, FGF-2 [141,142], Ang-1, HGF [143], KGF, and PDGF and combinations thereof [139,140,144]. Recently, a very interesting growth factor immobilization scheme was presented that is based on functional domains of the ECM protein fibronectin [63,145]. Bifunctional fusion proteins of the 12th–14th type III fibronectin repeats and the Glu acceptor peptide were incorporated into fibrin matrices. This fibronectin domain was demonstrated to bind various growth factors with Kd values in the nanomolar range (Fig. 2I). For example, PDGF-BB that was immobilized via the 12th–14th type III domains was shown to stimulate sprouting of smooth muscle cell spheroids in vitro [145].

### Table 2

<table>
<thead>
<tr>
<th>Strategy for affinity based immobilization of growth factors.</th>
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<tr>
<td><strong>Biomolecule</strong></td>
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<tr>
<td>FGF-2, β-NGF, PDGF-BB, NT-3</td>
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<tr>
<td>VEGF, FGF-2, SDF-1α</td>
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<td>PDGF-BB, EGF, BDNF</td>
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**Abbreviations**: Gln-HBP, bifunctional NQEQVSPF-heparin binding peptide; NHS, N-hydroxysuccinimide; EDC, N-(3-dimethylaminopropyl)-N'-ethylenediamine; PEG-NH₂, PEG-amine; PEG-SH, PEG-thiol; DTPH, (3,3′-dithiobis(propanoic hydrazide)); HA, hyaluronan; CS, chondroitinsulfate; GEL, gelatine; ADH, adh; hydrazide functionalized heparin; PEG-SBA, N-hydroxysuccinimimidyl ester PEG-bis-butanolic acid; PF4 ZIP, heparin binding, coiled-coil peptide; HIP, peptide sequence from heparin interacting protein; DM, dimethacrylate; FGF-2bp, FGF-2 binding peptide poly(AAC), poly(acrylic acid); Kringle, fibrin binding peptide from human plasminogen; BDNF, brain-derived neurotrophic factor; CBD, collagen binding domain.

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6. Biomimetic hydrogels for growth factor delivery to regenerate bone

Here we summarize studies that implemented the above strategies for the regeneration of bone defects. The chosen examples should illustrate that sophisticated GF immobilization and release is an efficient way to foster safe and localized bone healing and that GF-functionalized hydrogels are on their way to becoming a potential replacement for bone autografts.

6.1. Implementation using covalent growth factor tethering

An early example of growth factor delivery from fibrin hydrogels to regenerate bone tissue was reported by Hubbell and co-workers [113]. The authors used recombinant technology to engineer a BMP-2 variant, such that it could be delivered in a timescale matching the local morphogenetic processes. A tri-functional peptide was designed consisting of a C-terminal BMP-2 domain and an N-terminal Gln acceptor substrate separated by a plasmin sensitive cleavage sequence (TG-pl-BMP). The peptide was expressed in E. coli, chromatographically purified and could then be covalently incorporated into the forming fibrin matrices via the Gln sequence at the N-terminus. Although much more specific than chemical cross-linking, the genetic addition of the Gln-domain and the plasmin cleavage sequence could be interfering with the biological activity of BMP. However, it was shown in in vitro assays that the TG-pl-BMP fusion protein was as active as the wild-type BMP. As expected, release studies showed that only the fusion protein was retracted in the fibrin implant, whereas the wild-type was quickly liberated. The constructed BMP-2 functionalized implants were applied in critical size defect models in rat, cranial defects in rabbit, and used for fusion of the carpus in dogs.

The fusion protein Gln-pl-PTH provides another impressive example for the design of a therapeutic biomolecule to foster local bone regeneration without the safety risks associated with systemic delivery [111]. A team of scientists at Kuros Biosurgery designed this bifunctional peptide which can be covalently incorporated into clinically approved fibrin matrices by the factor XIII specific substrate sequence at the N-terminus. Further, adjacent to the Gln sequence they cloned a plasmin sensitive cleavage site and amino acids 1 to 34 of the human PTH active fragment, which was known to stimulate bone growth [79]. Probably due to masking amino acids 1 and 2 crucial for the bioactivity, the addition of the plasmin site and the factor XIII substrate resulted in reduction of PTH bioactivity, which was fully restored after plasmin mediated cleavage. In order to show the medical potential of Gln-pl-PTH functionalized matrix, they created metaphyseal and epiphyseal defects in sheep and convincingly demonstrated that delivery of fibrin-tethered PTH led to dose-dependent bone formation and that the carrier was completely resorbed, making the presented implant a potential replacement for bone autograft treatment.

6.2. Implementation using non-covalent growth factor tethering

The use of bone grafts based on biological materials such as fibrin or collagen bears potential risks of disease transmission and initiation of a foreign body immune response in patients. For example, a clinical trial was reported on the use of type 1 bovine collagen as carrier for recombinant human BMP-7 (rhBMP-7) for treatment of tibial non-unions. Although the study was able to prove the efficacy of the treatment, it also showed development of antibodies against collagen and rhBMP-7 by 5% and 10% of patients, respectively [15]. The fabrication of engineered bone grafts that are based on biologically inert synthetic polymers offers a solution to the potential immunogenicity of biological materials. For example, Hubbell and co-workers designed an injectable, plasmin-sensitive PEG-co-peptide hydrogel crosslinked by Michael-type addition reaction [146]. To make the implant suitable for controlled recombinant BMP-2 delivery, a heparin-binding delivery system was employed. A strong improvement in cranial regeneration compared to control samples containing no BMP-2 or a plasmin-insensitive linker was shown. However, in the case where the heparin binding system was used, the authors found a significant larger area of the defect covered by novel bone (94%) compared to the soluble growth factor (73%). This data shows that tailor-made biomimetic hydrogel carriers allow for in situ control of bone formation and indicates, in a preclinical model, that bone healing can be enhanced by releasing BMP in a sustainable manner.

Recently, a fibrin scaffold possessing integrin-binding sites adjacent to growth factor-binding sites to obtain synergistic effects was used to regenerate bone [63]. For this purpose a trifunctional peptide was created consisting of an N-terminal Gln sequence for covalent incorporation into fibrin matrices, the major integrin binding domain of fibronectin (FN III9-10) and at the C-terminus another domain of fibronectin (FN III12-14) to bind various growth factors. Since FN III12-14 binds growth factors from different families it allows for straightforward delivery of multiple growth factors. By delivering BMP-2 and PDGF-BB, two growth factors known to induce bone formation and previously shown to bind to FN III12-14, the authors tested for improved growth factor efficiency in ectopic positions in nude mice and calvarial critical-size defects in rats. By using growth factor concentrations that didn’t show any in-vivo effects when delivered in empty fibrin matrices, they were able to demonstrate that the FN III9-10/12-14 functionalized matrices enhanced growth factor-induced bone formation and recruitment of bone forming progenitor cells. Due to the enhanced growth factor signalling, concentrations could be dramatically reduced and bone tissue deposition was observed with much lower doses than elsewhere reported, showing promise for the use of such implants in clinical applications.

7. Future directions

During bone healing and regeneration, a sequential cascade of cellular processes must occur, including chemotaxis, differentiation, and proliferation. Since these processes are tightly orchestrated by the wound microenvironment, we believe that there lies tremendous underexplored clinical potential in engineering the natural bone healing capacity. In order to do so, the microenvironmental key players, growth factors, must be presented in the appropriate combination, delivery mode and with appropriate time scales. In order to do so, more profound knowledge on all relevant cellular events and corresponding growth factors will be necessary.

We see room for advances in at least three areas of smart implant engineering: i) There exists a huge body of literature on pro-osteogenic growth factors and the bone formation step in the regeneration. We believe that there still lies great potential in targeting the other stages of natural fracture healing, for example by presenting angiogenesis and inflammation regulating biomolecules. ii) One of the bottlenecks to successful bone healing is the availability of responsive bone forming progenitor cells, which have to be recruited to the wound, proliferate, and eventually differentiate into bone cells (Fig. 3A). Therefore, we believe that research towards the creation of next-generation bone healing implants should also be directed towards enhancing these three processes by presenting thoroughly selected growth factors from screening approaches in a spatio-temporal fashion (Fig. 3B). iii) Only recently, studies to elaborate the role of ECM components in growth factor signalling and bone healing have been initiated and translated to new implant designs. We believe that more in-depth knowledge about integrin growth factor receptor co-signalling and its translation to biomaterials design will lead to more effective and thus safer implants.

So far, these issues had to be exclusively addressed using in vivo experiments, simply because physiologically relevant in vitro models were missing. More sophisticated in vitro technologies that allow for
mimicking the in vivo microenvironment and thereby for example enable the elucidation of cell-matrix and growth factor-cell interactions in high-throughput and in tissue-like 3D contexts are necessary. Exciting progress is being made in this direction. For example, microfluidics and robotic spotting/printing have emerged as means to miniaturize experiments allowing for experiment parallelization [147-149]. In order to create 3D in vitro models that mimic the spatio-temporal organisation of a healthy or damaged bone, flexible growth factor immobilization strategies will be essential to tailor the simultaneous immobilization and release of multiple candidate growth factors. Technologies applied thus far either rely on affinity interactions that vary among growth factors, need site-specific chemical engineering or labor-intensive genetic engineering of growth factors. Therefore, the development of promiscuous and reliable growth factors binding strategies would be desirable.

Since layer-by-layer deposition and robotic printing of hydrogels and cells now allows for precise positioning of growth factor-presenting matrices, microtissues, or single dispersed cells, the creation of spatially defined 3D microenvironments becomes possible (Fig. 3C left panel) [150-152]. Such approaches may permit the high-throughput screening of signal candidates that enhance progenitor cell recruitment, proliferation and differentiation in 3D contexts. By using bone progenitor cell invasion assays, the growth factor-induced recruitment can for example be studied and optimized (Fig. 3C right panel). Additionally, reproducibly printed bone tissue-like 3D constructs could bridge the gap between 2D cell culture and animal models, permitting the testing of drug and biomolecule candidates under more physiologically relevant conditions (Fig. 3D left panel) [150,153,154]. Fabrication of models that mimic bone fracture will show if a selected candidate triggers the same behavior in a more complex setup (Fig. 3D right panel).

Finally, the gathered basic information on bone regeneration mechanisms should, most likely in an iterative process, be translated to next-generation matrices that could, by temporally controlled presentation of growth factors from ECM mimicking matrices, act on the relevant stages and cellular processes involved in bone healing and regeneration.

8. Conclusion

We believe that great strides in the creation of more efficient and clinically safe bone healing implants will come from an increased understanding of the fundamental bone healing processes. In one promising strategy, smart cell-instructive bone healing implants of the future should aim at substituting or accelerating naturally occurring signals to augment the healing process in deficient tissues. Extrinsic signal presented by these implants should be tailored to sequentially, and in a spatiotemporally well-controlled fashion, induce the recruitment, proliferation, and differentiation of cell contributing to the healing cascade.

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