Review

Functionalization of biomaterials with small osteoinductive moieties

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ABSTRACT

Human mesenchymal stem cells (MSCs) are currently recognized as a powerful cell source for regenerative medicine, notably for their capacity to differentiate into multiple cell types. The combination of MSCs with biomaterials functionalized with instructive cues can be used as a strategy to direct specific lineage commitment, and can thus improve the therapeutic efficacy of these cells. In terms of biomaterial design, one common approach is the functionalization of materials with ligands capable of directly binding to cell receptors and trigger specific differentiation signaling pathways. Other strategies focus on the use of moieties that have an indirect effect, acting, for example, as sequesters of bioactive ligands present in the extracellular milieu that, in turn, will interact with cells. Compared with complex biomolecules, the use of simple compounds, such as chemical moieties and peptides, and other small molecules can be advantageous by leading to less expensive and easily tunable biomaterial formulations. This review describes different strategies that have been used to promote substrate-mediated guidance of osteogenic differentiation of immature osteoblasts, osteoprogenitors and MSCs, through chemically conjugated small moieties, both in two- and three-dimensional set-ups. In each case, the selected moiety, the coupling strategy and the main findings of the study were highlighted. The latest advances and future perspectives in the field are also discussed.

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1. Introduction

Stem cells, in particular adult mesenchymal stem cells (MSCs), are currently recognized as a promising cell source for tissue engineering (TE) applications and cell-based therapies, namely for bone repair and regeneration. Under appropriate in vitro conditions, in the presence of morphogens and specific chemicals, MSCs can be induced to differentiate along several mesodermal lineages, including osteoblastic [1]. Moreover, in vivo, the osteogenic potential of MSCs has been clearly demonstrated by subcutaneous implantation of MSCs within a ceramic or polymer carrier in immunocompromised mice, a common assay for evaluating ectopic MSC differentiation into functional osteoblasts [2,3]. In view of the current limitations associated with bone grafting procedures, the combination of MSCs with adequate vehicles or scaffolding materials has been pursued as a therapeutic strategy to promote bone repair in response to injury, which still represents a major challenge and is a global health problem. Over the past years, biomaterials have evolved from essentially “bioinert” materials into sophisticated substrates, with the capability to instruct cells and tune their behavior. In this context, being able to drive MSCs’ fate in a controlled manner, namely by activating their differentiation into osteoblasts, is clearly relevant [4].

The effect of a variety of biochemical and mechanical cues [5], as well as other cues, such as cell shape and size and cell–cell contacts [6–9], on osteogenic differentiation has been extensively investigated. With respect to biochemical cues, which are covered in this review, studies have focused not only on the use of prototypical signaling molecules such as growth factors (GFs), extracellular matrix (ECM) proteins and hormones, but also on a wide variety of small compounds of diverse chemical nature. In the vast majority of available studies, these cues have been presented to cells and tissues in a soluble form, but their effect in an immobilized form and, in particular, associated with biomaterials has also been extensively investigated. Different immobilization strategies can be used to combine bioactive molecules with a substrate, including covalent bonding, physical adsorption and entrapment (Fig. 1). This review only covers examples involving the establishment of chemical bonds between the moieties and the material, which generally provides better control over their presentation in terms of density and orientation, and improves stability [10]. Compared with more complex biomolecules, approaches involving the use of smaller and simpler compounds can be advantageous by leading to less expensive and easily tunable biomaterial formulations [11]. These approaches are reviewed herein, and compounds

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have been organized into three major groups, according to their nature: (i) chemical moieties; (ii) peptides; and (iii) other small molecules.

The modification of biomaterials with specific functional groups is probably the most straightforward strategy to induce material-driven control over cell behavior. In most of the described studies, chemical groups of different character, broadly categorized as hydrophobic, hydrophilic, charged or uncharged, have been selected to convey a wide range of properties to the material surface. In a way, these moieties capture chemical features of the native ECM, as all of them are inherently present in living systems [11,12]. The selection of the functional properties that are the most akin to specific native microenvironaments can be pursued. For example, carboxylic acid groups are a prevalent chemical functionality of cartilaginous matrices, which are rich in glycosaminoglycans; negatively charged phosphate groups are present in the mineral phase of hard tissues, such as bone; and hydrophobic moieties can be associated with adipose tissues, as adipocytes are rich in lipids and secrete them into their extracellular milieu [11,12]. Mechanistic studies using model surfaces, such as self-assembled monolayers (SAMs), provide a valuable tool for investigating how the presentation of functional groups can be used to control MSC differentiation. Although SAMs cannot be used as implant materials, the knowledge gathered from such studies can be translated into the surface modification of scaffolds for tissue engineering, leading to more interactive and efficient materials that are able to guide the differentiation pattern of attached stem cells. As described in Section 2, several in vitro studies have demonstrated that surface chemistry can not only modulate short-term cell functions, such as adhesion and morphology (Fig. 2), but also affect long-term cellular functions such as differentiation, which is remarkable, given that most cells rapidly remodel their underlying matrix [13,14].

The molecular design of cell-instructive biomaterials decorated with peptide cues has also been fostered over the last decade or so [16–30]. The rationale for this strategy relies on the inherent bioactivity of such compounds. In fact, molecular recognition is highly dependent on specific amino acid sequences present in peptides and proteins, which regulate key biological and physiological processes, thus modulating cellular function and coordinating intercellular communication. The use of synthetic analogues of peptide sequences from biologically relevant proteins presents obvious advantages over the use of the full-length protein. The latter exhibit intrinsic bioactivity, but may also concomitantly present other binding sites for non-targeted biological ligands that may trigger unwanted, and often interfering, cellular responses. Moreover, whole proteins are much more sensitive and complex to use, are more expensive, and present greater risks of immunogenicity [28]. Peptides are commonly non-toxic, as they are composed of naturally occurring or metabolically acceptable amino acids. However, although generally they do not cause severe immune responses, this possibility must be taken into account, particularly for peptides with longer sequences.

One of the first peptidic sequences to be used for biomedical functionalization, which is still among the most widely used, is the prototypical cell adhesion sequence Arg-Gly-Asp (RGD). This motif was identified almost two decades ago by Pierschbacher and Rousslahti [31] as the minimal essential cell adhesion sequence in fibronectin (Fn), and shortly after was tested in a covalently immobilized form [32]. Different studies have addressed the effect of RGD surface density and accessibility (frequently controlled with the use of spacers), as these parameters are essential to guarantee that the peptide bioactivity is preserved [33,34]. Although not discussed in detail herein, the density and distance are clearly important issues when considering covalent bounding of any type of bioactive molecule. For example, the RGD nanospacing has been found to regulate specific cell adhesion [35,36]. Based upon a unique technical platform to fabricate RGD micropatterns and nanopatterns on PEG hydrogels [37–39] in their group, Wang et al. [40] recently examined MSC differentiation on RGD nanopatterns with varied nanospacings. Their stimulating finding is that RGD nanospacing regulates stem cell differentiation, including osteogenesis,
beyond cell adhesion. In fact, while it has been primarily used as an adhesive ligand, many studies have demonstrated that RGD is in itself a mild promoter of osteogenic differentiation [25,41–45]. However, RGD is not selective for a specific integrin, but triggers non-biased cell attachment. Moreover, in vivo data on the ability of RGD-modified materials to promote osteointegration and bone formation are contradictory and, in general, rather disappointing [46]. Therefore, for bone-related applications, other cell adhesion peptides have been proposed that selectively target integrin signaling cascades implicated in osteogenesis, which demonstrated improved performance. The most highly expressed integrins in osteoblastic-like cells belong to the β1 sub-family of integrins [46]. Although data reporting the expression of the specific alpha subunit by osteoblasts has been less consistent, it is well established that the α2β1 integrin is implicated in pro-osteogenic pathways and specifically binds to regions of collagen type I, the most abundant matrix component in bone [46]. Nevertheless, the modification of biomaterials with RGD-like peptides to promote cell adhesion has been often recurrent, as cell anchorage is a prerequisite for the survival of several cell types [47,48]. Advancements in terms of the grafting strategies and improved ligands have been reported. For example, techniques to covalently bind RGD into both hydrophobic and hydrophilic blocks have been recently set up, and the binding sites have been found to influence the cell adhesion efficacy [49]. A new and very potent peptide ligand combining both cyclic RGD and linear oligolysine has been designed to promote both specific and nonspecific cell adhesion [50]. Notably, many studies have shown that, when RGD and other osteoinductive peptides are co-grafted onto a substrate, they might actually operate synergistically to enhance osteogenic differentiation and mineralization of osteoprogenitor cells [21,51]. Presumably, as some authors have suggested, the increased adhesion and spreading of cells onto substrate promoted through binding to RGD motifs will in turn favor the interaction of the other peptides with specific cell surface receptors, potentiating their action. Other F-derivative peptide fragments have shown higher specificities for key integrins, such as α5β1, with important roles in the control of MSCs osteogenic differentiation [46,52]. In fact, independently of the activation state of α5β1 integrins, RGD alone seems to be insufficient to promote binding, even if it serves to activate and align the α5β1–Fn interface, and the simultaneous presence of the PHSRN synergy site is required to provide the mechanical strength of the bond [53].

To specifically promote osteoblastic differentiation and matrix mineralization, different peptide sequences derived from the active domains of typical bone ECM proteins, such as collagen type I (COL-I), osteopontin (OPN), osteocalcin (OCN) and bone sialoprotein (BSP), have been suggested. Peptides derived from bone-related GFs (such as bone morphogenetic proteins, BMPs) and peptide/protein hormones with recognized anabolic effects in bone (such as the parathyroid hormone, PTH) have also been suggested. Most of these peptides are expected to directly interact with cell receptors, activating particular signaling pathways similarly to their parental proteins. Alternatively, the immobilized peptides can have an indirect action, by promoting the non-covalent sequester of key biomolecules present in the extracellular milieu that, in turn, will exert their bioactivity [54–56]. In a way, these “indirect” strategies set up a new paradigm in biomaterials design, in the sense that these sequestered signaling compounds are not directly presented by the biomaterial. In addition, peptides themselves are paving the way as new biomaterials. This is the case of a new class of “smart” peptides, which self-assemble into nanostructures and create self-supporting hydrogels that can be used to culture cells under three-dimensional (3-D) conditions. These self-assembling (SA) peptides have been broadly proposed for the therapeutic regeneration of different tissues, including bone [57,58]. The bioactivity of these peptides can be molecularly tailored by changing their amino acid composition. In a common approach, the original SA sequence is extended, generally at one of its termini, with specific bioactive domains. Finally, there are a vast number of other classes of small compounds, including dexamethasone and statins, which have also been used as osteoinductive compounds, and these are described briefly here. Collectively, the examples provided in this review are intended to be illustrative rather than inclusive.

Importantly, for these compounds to have impact on the field, they must be suitable for use in a wide variety of biomaterials for bone regeneration. As described throughout the text, by selecting appropriate chemical routes, all these small molecules can in theory be used in the functionalization of different classes of materials, including polymers, ceramics, metals and composites, and processed in a variety of ways, such as films, membranes, micro-particles, porous TE scaffolds and hydrogel-based cell-encapsulation systems (Fig. 3). Polymers are the most common material class as they are quite versatile, offering a huge diversity in terms of nature, properties and composition. Additionally, they are generally easy to functionalize, as they intrinsically present adequate reactive groups to directly engage in different chemical modification schemes. Depending on the original material chemistry, pre modification steps to introduce adequate reactive groups might be needed, prior to functionalization with bioactive moieties. On the other hand, the bioactive moieties themselves might be directly reacted to the material or be previously modified. For example, in the case of peptides, the simplest grafting procedures involve a direct coupling via peptide N-terminal and C-terminal groups, or thiol groups present in terminal cystein residues. More complex procedures require the pre-derivatization of peptides, during or after their own synthesis, with different coupling functionalities, such as azides and acryloyl groups. In special cases, such as SA peptides or recombiant proteins, bioactive amino acid sequences are directly incorporated during biomaterial synthesis. Table 1 presents some examples of functionalization strategies employed in the covalent modification of different biomaterials with peptides. Throughout this review, the different chemical routes used for the functionalized of biomaterials with different small moieties are described in detail. Within each category of materials, examples describing both surface modifications (when performed after substrate/scaffold formation) and bulk modifications (when performed before scaffold formation) are provided.

2. Osteoinductive chemical groups

2.1. Surface-immobilized chemical groups

SAMs have emerged as an important tool to investigate the effect of specific surface chemistries on stem cell differentiation due to their controllable surface properties [72]. SAMs represent a class of well-ordered organic substrates formed by the adsorption of an active surfactant on a solid surface [73,74]. SAMs formed via chemisorption of alkanethiols onto gold-coated substrates are currently considered to be the best available class of model organic surfaces, allowing significant control over chemical properties of the underlying substrate [14,72,75,76]. A key advantage of this system is the simple creation of well-defined and reproducible surfaces presenting a wide range of chemical moieties, which allow the control of protein–surface and cell–surface interactions [14,77]. Although SAMs can only be used as model surfaces, results from these studies have been laying the groundwork for more complex studies with “real” implant biomaterials towards the identification of surface chemistries that are optimal to achieve the fine control over MSC differentiation in clinical applications.
Most of the published studies have demonstrated that cell interactions with surfaces are indirectly mediated by the characteristics of a pre-adsorbed protein layer, and the pattern of protein adsorption has been correlated with the underlying surface chemistry [15,73,78–94]. In fact, when a surface comes into contact with a protein-rich physiological medium, it instantaneously becomes coated with layers of adsorbed proteins, which will in turn direct the binding of cell adhesion receptors. The type, amount, conformation and surface distribution of adsorbed proteins modulate focal adhesion formation and intracellular signaling cascades, eventually leading to changes in initial adhesion and the long-term differentiation of cells [15,85]. Here, a selection of mechanistic studies performed with SAMs and other model surfaces is provided, where it was possible to identify chemical functionalities that were claimed to effectively promote or enhance osteogenic differentiation of MSCs, either in basal or pro-osteogenic conditions. Table 2 summarizes the main findings of the described studies.

Phillips and co-authors analyzed [14] SAMs functionalized with four different functional groups, namely methyl (–CH₃), hydroxyl (–OH), carboxyl (–COOH) and amino (–NH₂), and were able to demonstrate that the surface chemistry has an effect on the pattern of Fn adsorption, which in turn modulates the osteogenic differentiation of human MSCs (hMSCs). Differences in Fn conformation promoted different integrin–ligand interactions and the consequent activation of different intracellular signaling pathways [85]. In particular, NH₂-SAMs pre-coated with Fn promoted the strongest induction of hMSC differentiation along the osteoblastic lineage under osteoinductive medium. On this surface, mineralized nodules were primarily observed, alizarin red staining for calcium showed the greatest enhancement, and Runx2, BSP and OCN expression were significantly up-regulated compared to control surfaces (gold-coated tissue culture polystyrene, TCPS). It is noteworthy that, although the other functionalities also showed some evidence of osteoinductivity, they only affected the global magnitude of one or two phenotypic markers. In fact, only in NH₂-SAMs was it possible to demonstrate how a single surface variable predicts the most profound effect on lineage commitment.

Using silane-modified glass surfaces as models, Curran et al. [13] investigated the effect of the same chemical groups and also thiols (–SH) on hMSCs behavior. Again, hMSCs cultured on positively charged NH₂ surfaces were shown to be more likely to differentiate along the osteogenic lineage, rather than maintaining an undifferentiated phenotype or differentiating into other cell types. After 7 days, hMSCs cultured on these surfaces showed higher levels of viable cells adhering and also an increase in mRNA expression of Cbfa-1 (bone transcription factor) compared to day 1, and decreases in COL-I, COL-II, transforming growth factor β (TGF-β) and ornithine decarboxylase (a proliferation marker). Significantly, in this particular study, cells were cultured in basal growth medium, without soluble osteoinductors, allowing a direct correlation between surface chemistry and cell behavior. Although not studied in detail, the pattern of protein adsorption to NH₂ surfaces, and particularly the presence of adsorbed vitronectin, might have contributed to early osteogenic signaling, as suggested by the authors. Unfortunately, the unexpected decrease in COL-I expression were not discussed. The same authors subsequently performed a similar study in which hMSC behavior was monitored for a longer period of time (28 days) in the presence of basal and osteoinductive media...
Examples of functionalization strategies to covalently modify different biomaterials with peptides.

<table>
<thead>
<tr>
<th>Material</th>
<th>Material reactive group</th>
<th>Reactive group modification</th>
<th>Reactive group modification</th>
<th>Peptide reactive group</th>
<th>Reactive group modification</th>
<th>Material-peptide coupling reaction</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cermics</td>
<td>HA/[β-TCP]</td>
<td>Silanization to create NH2– followed by reaction with PEG diisuccinimidyl succinate</td>
<td>NH2–</td>
<td>–</td>
<td>–</td>
<td>NHS ester–NH2 coupling</td>
<td>[59]</td>
</tr>
<tr>
<td></td>
<td>HBP's</td>
<td>–</td>
<td>OH– (N- terminal Ser)</td>
<td>NH2–</td>
<td>–</td>
<td>Oxidation to create CHO–</td>
<td>[60]</td>
</tr>
<tr>
<td></td>
<td>Skeletrend</td>
<td>Silanization to create NH2– followed by succinylation to create a NHS ester</td>
<td>NH2–</td>
<td>–</td>
<td>–</td>
<td>NHS ester–NH2 coupling</td>
<td>[61]</td>
</tr>
<tr>
<td>Synthetic polymers</td>
<td>PEG</td>
<td>Acrylation to create H2C=CH–(C=O)–</td>
<td>S– (N-terminal Cys)</td>
<td>NH2–</td>
<td>–</td>
<td>Thiol-Acrylate coupling</td>
<td>[62]</td>
</tr>
<tr>
<td></td>
<td>pACAA</td>
<td>Oxidation to create COOH–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Carboximide chemistry (EDC/NHS)</td>
<td>[63]</td>
</tr>
<tr>
<td></td>
<td>PLEOF</td>
<td>Incorporation of propargyl acrylate during hydrogel formation</td>
<td>NH2–</td>
<td>–</td>
<td>–</td>
<td>Click chemistry</td>
<td>[21,65]</td>
</tr>
<tr>
<td></td>
<td>PLGA-(PEG-ASP)n</td>
<td>Derivatized with hydrazide by carbodiimide chemistry</td>
<td>COOH–</td>
<td>–</td>
<td>–</td>
<td>Derivatization with PEG–N3 during synthesis</td>
<td>[66]</td>
</tr>
<tr>
<td></td>
<td>PLGA</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Oxidation to create CHO–</td>
<td>[67]</td>
</tr>
<tr>
<td>Natural polymers</td>
<td>Silk fibroin</td>
<td>Carboxyl</td>
<td>–</td>
<td>NH2–</td>
<td>–</td>
<td>Carboximide chemistry (EDC/NHS)</td>
<td>[30]</td>
</tr>
<tr>
<td></td>
<td>Alginate</td>
<td>Carboxyl</td>
<td>–</td>
<td>NH2–</td>
<td>–</td>
<td>Carboximide chemistry (EDC/NHS or EDC)</td>
<td>[17,69,70]</td>
</tr>
<tr>
<td></td>
<td>Agarose</td>
<td>–</td>
<td>NH2–</td>
<td>–</td>
<td>–</td>
<td>Linked to the NHS-ester of heterobifunctional sulfo-SANPAH</td>
<td>[71]</td>
</tr>
</tbody>
</table>


The results from this study are somewhat confusing, as all the surfaces up-regulated at least one osteogenic gene at certain time points. In accordance with the previous results, however, NH2 surfaces were the ones that promoted and maintained cell osteogenic differentiation better overall, both at the gene and protein levels, in the presence and absence of biological stimuli. Interestingly, SH surfaces also had a positive effect on osteogenesis, with the up-regulation of OCN and Cbfa1 compared to control samples being observed at different time points, both in the presence and absence of biological stimuli. This was surprising, since in the previous study [13] –SH surfaces resulted in the decreased expression of proliferation and osteogenic markers after 1 week of culture in basal conditions, though this issue was not discussed.

A number of other chemical functionalities have been investigated with regard to their ability to promote osseointegration. For instance, using the H1PO4/P2O5/ET3PO4/hexanol method, Granja et al. [95] grafted phosphate functionalities onto regenerated cellulosic surfaces to enhance its bioactivity, inspired by the role of phosphoproteins in biomineralization. They demonstrated in vitro that the calcium salt of cellulose phosphate induces the mineralization of the surface, but the highly negatively charged surfaces promoted poor attachment, proliferation and differentiation of human bone marrow stromal cells [96]. This influence of chemical functionality and surface charge seems to be in agreement with the previously discussed effect of positively charged SAM surfaces. In vivo, phosphorylated surfaces promoted slightly better osseointegration than non-modified surfaces [96].

2.2. Bulk-immobilized chemical groups

Murphy et al. [97] proposed a chemical method for the preparation of silk fibroin derivatives with tailored structure and hydrophilicity, carrying different chemical groups, both hydrophilic (carboxyl, amino and sulfonic acid) and hydrophobic (ketone and heptyloxy). The diazonium coupling chemistry was chosen to target the tyrosine residues, as these are homogeneously distributed along the protein molecules and are present in sufficient amounts [97]. hMSC were cultured at the surface of modified-silk (azo-silk) films and, after 1 week of culture under osteoinductive conditions, higher expression of alkaline phosphatase (ALP) and COL-I was observed in all silk derivatives compared to non-stimulated cultures. Surprisingly, the variation in the functional groups attached to the silk did not differentially affect the pattern of hMSC differentiation. The authors did not provide an explanation for this, but proposed future studies to investigate whether azo-silk derivatives with higher surface charge (carboxylic and sulfonic acid) could enhance mineralization.
The effect of phosphoesters on hMSC differentiation was evaluated in a poly(ethylene glycol) (PEG) dimethacrylate-based cell encapsulation system [98]. Phosphate-containing hydrogels were obtained via photopolymerization of the macromer precursor PEG-di[ethylphosphatidyl(ethylene glycol)methacrylate], which incorporates PO₄-containing degradable polyester linkages [98,99]. Co-gels were prepared by combining PhosPEG with PEG. Compared to PEG gels, and in the absence of growth factors or other inducers, PhosPEG−PEG co-gels increased the gene expression of bone-specific markers (COL-I, ALP and osteonectin (ON)), secretion of bone-related matrix (ON and COL-I) and mineralization (both in a cellular and cellularized gels). In this case, the phosphate moieties in the hydrogel backbone played multiple key roles, as they not only acted as osteoinductive moieties, but also provided a site for ALP-responsive scaffold degradation, which, in turn, produced functional groups that promoted autocalcification.

Benoit et al. [11] tested the effect of amino, t-butyl, phosphate (PO₄), fluoro and carboxylic acid groups on hMSC differentiation, also with the aim of developing PEG-based cell-encapsulating hydrogel systems. In a first stage, hMSC were cultured on arrays of functionalized−PEG spots, as a 2-D screening platform. Some selected groups, and in particular PO₄ groups, shown to induce osteogenesis, were then tethered to 3-D PEG hydrogels, and their effect on the differentiation of encapsulated hMSCs was evaluated. These, PO₄−PEG hydrogels were prepared by photopolymerization of PEG monomers with ethylene glycol methacrylate phosphate [11,100,101]. Importantly, hydrogels were designed to have different chemistries, and hence different charges and hydrophilicities, but comparable matrix properties, such as stiffness and swelling [11]. In addition, 3-D cultured cells were trapped in a rounded morphology, irrespective of the material chemistry, in an attempt to eliminate confounding factors and show how a synthetic matrix could modulate hMSC commitment solely through interactions with small chemical groups. As predicted from the usual standards, from the N-terminus to the C-terminus. A compilation of covalently immobilized peptide sequences present in enamel matrix proteins [108], BMP-7 [105], neuropeptides [106,107] and amelogenin-derived peptide sequences present in enamel matrix proteins [108], no studies using such peptides in a covalently immobilized form have been found. Here, an overview of studies exploring the effect of ECM protein adsorption, which in turn directed hMSC differentiation down the osteogenic lineage without using induction medium.

### 3. Osteoinductive peptides

The general use of osteoinductive peptides in bone regeneration has been recently reviewed by Jabbari [103]. The use of ECM-derived and GF-derived peptides for implant functionalization to promote osteointegration and enhance bone healing within large defects has also been reviewed by Shekaran and García [46]. Although several recent reports exist uncovering the potentialities of new osteoinductive peptides, including collagen-binding peptides from BSP [104], peptides based on the prodomain region of BMP-7 [105], neuropeptides [106,107] and amelogenin-derived peptide sequences present in enamel matrix proteins [108], no studies using such peptides in a covalently immobilized form have been found. Here, an overview of studies exploring the effect of scaffold-grafted peptides on osteogenic differentiation and bone healing is provided. All peptide sequences are written, according to the usual standards, from the N-terminus to the C-terminus. A compilation of covalently immobilized peptide sequences tested as osteoinductors is presented in Table 3.

#### 3.1. BMP-derived peptides

BMPs are a group of 18 proteins belonging to the TGFβ family. Among them, BMP-2, -7 and -9 are the ones involved in the development of the skeleton and bone formation and remodeling.
[109–111]. They interact with the BMPR-type I and BMPR-type II cell receptors, which activate different signaling pathways. One of them involves the activation of LIM kinase-1 and has implications on cytoskeleton dynamics [112]; the other involves activation of Smad1/5/8 and its translocation into the nucleus, where it regulates the expression of some osteogenic genes [113–116]. In the case of BMP-2, the majority of its peptide derivatives was obtained from the “wrist” and “knuckle” epitopes of the parental protein, and preferentially interact with type I and type II cell surface receptors, respectively [110,117–119]. BMPs are potent osteoinductive agents, but their clinical use is often limited by their short biological half-life, rapid local clearance, propensity for side effects and high cost [120]. Some of these limitations can be partially overcome by the use of BMP-derived peptides, particularly when used in association with biomaterials.

3.1.1. Surface immobilization of BMP-derived peptides

Using SAMs, Moore and colleagues [51] investigated the synergistic and concentration-dependent effects of the peptides RGD and KIPKASSVPTELSAISTLYL (73–92 residues of the knuckle epitope of BMP-2) [19] on bone marrow stromal cells (BMSCs). Both peptides were derivatized with azide (N3–GRGDS and N3–KIPKASSVPTELSAISTLYL) and grafted onto alkyne-SAMs by click chemistry, alone or combined at a 1:1 M ratio, in gradients of 0–140 pmol cm⁻². Human BMSCs were seeded onto peptide-modified surfaces and cultured in the absence of osteoinductive supplements for 21 days. Runx2 expression increased in the presence of the BMP-2 peptide, when grafted at 80–120 pmol cm⁻², but not in the presence of RGD alone. When both peptides were present (1:1, total of 130 pmol cm⁻²) there was a synergistic enhancement of BSP expression and some signs of mineralization. This study reinforced the idea that, by providing cell-attachment sites, RGD peptides improve the interaction of BMP-2 peptide with its receptors. Unexpectedly, cell proliferation and BSP expression also increased in the presence of COOH groups alone (200 pmol cm⁻²), which seems to contradict previous reports [11]. It was hypothesized that COOH groups might interact electrostatically with exogenously expressed proteins, including BMP-2, and increase their accessibility.

Zouani and colleagues [63] tested a very similar BMP-2 derived sequence (RKIPKASSVPTELSAISTLYL) and also two other sequences, derived from BMP-7 (RTVPKASSVPTELSAISTLYF) residues

### Table 3

Examples of covalently immobilized peptide sequences tested as osteoinductors.

<table>
<thead>
<tr>
<th>Parental molecule</th>
<th>Peptide sequence</th>
<th>Material</th>
<th>Cells</th>
<th>Principal effects</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP-2</td>
<td>KIPKASSVPTELSAISTLYL</td>
<td>SAMs</td>
<td>hBMSC</td>
<td>Of osteogenic markers in OM improved by the synergistic effect of coupled RGD and BMP-2 peptide</td>
<td>[51]</td>
</tr>
<tr>
<td></td>
<td>pACAA</td>
<td>hBMSC</td>
<td></td>
<td>Of osteogenic markers in BM, in stiffer matrices and in the presence of BMP-2 peptide</td>
<td>[64]</td>
</tr>
<tr>
<td></td>
<td>PET</td>
<td>MC3T3-E1</td>
<td></td>
<td>Of osteogenic markers in OM (&gt;BMP-7 and BMP-9)</td>
<td>[63]</td>
</tr>
<tr>
<td></td>
<td>PEOF</td>
<td>rBMSC</td>
<td></td>
<td>Mineralization extent and ALP expression improved by the synergistic effect of coupled RGD and BMP-2 peptide in OM</td>
<td>[21]</td>
</tr>
<tr>
<td></td>
<td>PLEOF</td>
<td>rBMSC</td>
<td></td>
<td>Of osteogenic markers and vasculoegenic markers in OM improved by the synergistic effect of coupled RGD, BMP-2 and OPD peptides</td>
<td>[65]</td>
</tr>
<tr>
<td></td>
<td>PLGA</td>
<td>rBMSC</td>
<td></td>
<td>Of ALP expression in OM and promotion of ectopic bone formation in vivo</td>
<td>[66]</td>
</tr>
<tr>
<td></td>
<td>DWIVA</td>
<td>SAPs</td>
<td>hMSC</td>
<td>Of osteogenic markers in OM through the activation of Smad signal transduction</td>
<td>[58]</td>
</tr>
<tr>
<td>BMP-7</td>
<td>RTVPKASSVPTELSAISTLYF</td>
<td>PET</td>
<td>MC3T3-E1</td>
<td>Of osteogenic markers in OM (&lt;BMP-2 and &lt;BMP-9)</td>
<td>[63]</td>
</tr>
<tr>
<td>BMP-9</td>
<td>RKVGAASSVPTELPSILYK</td>
<td>NGLP</td>
<td>GICGP</td>
<td>GFOGER</td>
<td>Skeleté™</td>
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<td></td>
<td>COL-1</td>
<td>Agarose</td>
<td>cBMSC</td>
<td>Of osteogenic markers and of chondrogenic markers in CM (similar to RGD)</td>
<td>[71]</td>
</tr>
<tr>
<td></td>
<td>BSP</td>
<td>Quartz</td>
<td>PCL</td>
<td>Of mineralization in OM for peptide densities &gt;0.6 pmol/cm²</td>
<td>[29,134]</td>
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<tr>
<td></td>
<td>OPN</td>
<td>DVIDPDGRDGSLAY</td>
<td>OPF</td>
<td>rMSC</td>
<td>Of osteogenic markers, cell attachment and proliferation in OM in a concentration-dependent manner (similar to RGD)</td>
</tr>
<tr>
<td>OCN</td>
<td>GLRKSRRFRFRDQYDFATDIDTIHSH</td>
<td>γEPR-R/F-EY-EL</td>
<td>Alginate</td>
<td>hMSC</td>
<td>Of osteogenic markers in OM and new bone formation in vitro</td>
</tr>
<tr>
<td>PTH</td>
<td>SVSRLQHHNHELCKHN</td>
<td>SVERWLLRRKLQDQVHNF</td>
<td>Silk</td>
<td>S20s-2</td>
<td>Of osteogenic markers in OM (&lt;than RGD)</td>
</tr>
<tr>
<td>OGP</td>
<td>YFGCG</td>
<td>SAMs</td>
<td>PEU</td>
<td>Higher osteoblastic cells proliferation in BM</td>
<td>[158]</td>
</tr>
<tr>
<td></td>
<td>Heparin-binding</td>
<td>ALRQRGRTLYGF</td>
<td>FHKRIKA</td>
<td>GGGRGRTQGYK</td>
<td>SAPs</td>
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<td></td>
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<td></td>
<td>Of mineralization in OM (&lt; than RGD)</td>
<td>[133]</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Of osteogenic markers in OM enhanced by the sequester of heparin and improved by the synergistic effect with coupled RGD</td>
<td>[56]</td>
</tr>
</tbody>
</table>

ALP – alkaline phosphatase; BM – Basal medium; BMP-2 – bone morphogenetic protein-2; BMP-7 – bone morphogenetic protein-7; BMP-9 – bone morphogenetic protein-9; RGD – RGD motif; cBMSC – calf bone marrow stem cells; CM – chondroinductive medium; Col1 – collagen type 1; HA-PLG – hydroxyapatite-poly(lactide-co-glycolide); HBPs – hyaluronic acid-binding proteins; hBMSC – human mesenchymal stem cells; OCN – osteocalcin; OPG – osteogenic growth peptide; OM – osteoinductive medium; OPF – oligo[poly(ethylene glycol)] fumarate; OPN – osteopontin; pACAA – poly(acrylic acid-co-acrylamide); PCL – polycaprolactone; PET – polyethylene terephthalate; PLEOF – poly(lactide-co-ethylene oxide fumarate); PEU – polyetherurethane; PLGA – poly(lactic-co-glycolic acid); PTH – parathyroid hormone; rBMSC – rat bone marrow stem cells; rCC – rat calvaria cells; rMSC – rat mesenchymal stem cells; SAMs – self-assembly monolayers; SAPs – self-assembling peptides; | [133] |

[71] – increased.
acted with Az-mPEG–BMP and aminooxy-mPEG–OPD peptides by moieties and with 4-pentenal to create aldehyde moieties, then re-entration. In a more recent study [65], the OPN-derived sequence in BMP2–PLEOF and RGD–PLEOF hydrogels, but when both peptides effectively interacted with cell membrane receptors. Unsurprisingly, BMP-2 presenting surfaces were the more osteoinductive ones, which was further confirmed by an increased production of endogenous ECM. These differences were correlated with cell morphology. Moreover, BMP-presenting surfaces, and especially those with BMP-2, increased the mRNA expression of different GPs (BMP-2, TGF-β1 and VEGF), BMP receptors and OCN during 72 h of culture, as well as the extent of mineralization after only 5 days of culture. Overall, these results confirm the possible advantages that BMP-2 peptides may present over other peptides.

More recently, the interplay between biochemical (BMP-2 peptides) and mechanical cues on hMSC differentiation was addressed by the same group [64]. RGD and RIKPKASSVPTELSAISTLYL were grafted onto poly(acrylamide-co-acrylic acid) (pACAA) surfaces of different stiffnesses through carbodiimide chemistry. hMSCs sensed the different mechanical environments, committing into muscle-like and osteoblast-like cells on RGD–pACAA surfaces with 15 and 48 kPa stiffnesses, respectively, after 96 h of culture. The authors were able to show that the effect of biochemical stimuli may overlap those of mechanical ones (whenever stiffness >13 kPa), as hMSC only differentiated along the osteogenic lineage when BMP-2 peptides were present. However, osteogenic differentiation was inhibited on the softer BMP-2 surfaces (0.76–3.21 kPa), which presumably did not promote a favorable F-actin cytoskeleton reorganization, which is essential for BMP-induced Smad1/5/8 phosphorylation and nuclear translocation, as the authors also demonstrated. The results from this study contribute to uncovering the key role of matrix mechanical properties during osteogenesis and, importantly, their coordination with the biochemical environment.

He and colleagues [21] tested the same BMP-2 sequence, KIPKASSVPTELSAISTLYL, in the form of an azide-functionalized PEGylated peptide (Az-mPEG–BMP), which was grafted onto RGD-modified or unmodified hydrogels of poly(lactic-co-ethylene oxide fumarate) (PLEOF) by click chemistry. The final peptide concentrations used on cell studies were 1.6 pmol cm⁻² for RGD and 5.2 pmol cm⁻² for BMP-2, respectively. Under osteoinductive conditions, ALP activity and mineralization of rat BMSC were similar in BMP2–PLEOF and RGD–PLEOF hydrogels, but when both peptides were present they acted synergistically to enhance cell differentiation. In a more recent study [65], the OPN-derived sequence SVVYGLR (OPD peptide), which corresponds to residues 162–168 of OPN and is known to influence vasculosgenesis [121], was used as a third peptide. For orthogonal grafting, the RGD–PLEOF hydrogel was first conjugated with propargyl acrylate to create alkyne moieties and with 4-panetal to create aldehyde moieties, then reacted with Az-mPEG–BMP and aminooxy-mPEG–OPD peptides by click reaction and oxime ligation, respectively. The final peptide concentrations used on cell studies were 13.8 pmol cm⁻² for OPD and 5.4 pmol cm⁻² for BMP-2. Rat BMSCs were seeded on top of hydrogel disks and cultured in osteoinductive medium supplemented with vascularogenic factors for 28 days. The tested groups included hydrogels with RGD, RGD + BMP2, RGD + BMP2 + mOPD (a mutated OPD) and RGD + BMP2 + OPD peptides. The RGD hydrogels co-functionalized with OPD and BMP-2 yielded the best results in terms of ALP activity, mRNA expression of OPN and OC, and extent of mineralization. Moreover, both peptides were essential for the expression of vascular markers such as PECAM-1, α-SMA and VE-cadherin. In summary, the three peptides (RGD + BMP2 + OPD) acted cooperatively to provide a favorable microenvironment for concomitant BMSC osteogenesis and vasculogenesis.

Lin et al. [66] changed the terminal residues (underlined) of the previously described BMP-2 peptide, and showed that the sequence S₁₀⁴₈-KIPKASSVPTELSAISTLYLDD (designated by P24) was able to induce bone formation. P24 has a high content of D and (phosphorylated) S that might promote apatite nucleation and enhance mineralization. The peptide was coupled by carbodiimide chemistry through its terminal COOH groups to the amine groups of a biodegradable copolymer of poly(lactic-co-glycolic acid) (PLGA) with segments of PEG and aspartic acid units (PLGA–(PEG–Asp)) [66,122]. The incorporated PEG and Asp increased the polymer hydrophilicity and provided anionic functional groups that act as peptide-binding sites. The aim was to use the P24-modified scaffolds as a delivery system for the sustained release of P24. In vitro, in osteoinductive medium, the ALP activity and mineralization pattern of rat BMSC cultured on P24-modified scaffolds were increased compared to unmodified membranes. Moreover, these scaffolds promoted ectopic bone formation upon subcutaneous implantation in rats. Their performance was significantly better than that of the other groups (unmodified scaffolds or gelatin sponges), as demonstrated by radiographic and histological examination, Western blotting and reverse transcriptase-polymerase chain reaction (COL-I and OPN).

3.1.2. Bulk immobilization of BMP-derived peptides

One successful demonstration of ectopic bone formation using a covalently immobilized BMP-2-derived peptide was provided by Suzuki and colleagues in 2000 [17]. The authors prepared covalently crosslinked alginate hydrogel scaffolds by reacting 1-ethyl-(dimethylaminoenpropyl)-carboximide and sodium alginate in aqueous solution, followed by freeze-drying. The peptide, with the sequence NSVNSKIPKACCVPTELSAI, was grafted onto alginate COOH groups by carbodiimide chemistry. A control material was prepared by mixing the same amount of peptide with alginate but without the coupling agent, resulting in simple entrapment (10 mg of alginate with 180 μg of peptide). Both materials were implanted for 3 and 8 weeks in the calf muscle of Wistar rats. The formation of new bone tissue and vascular channels was only observed in the group with the covalently grafted peptide, highlighting the advantages of this type of immobilization. Surprisingly, given that alginate is non-cell adhesive, good results were obtained even in the absence of RGD peptides. The authors highlighted the importance of further characterizing the time course and dose–response dependence of the observed effects, but no subsequent studies using this exact peptide sequence have been found. In a subsequent study, Saito and co-workers [70] tested the slightly different sequence KIPKASSVPTELSAISTLYL (already described above). In its soluble form, this peptide was shown to increase ALP activity in the murine multipotent stromal cell line C3H10T1/2, and interact with both types of BMP receptor [19]. Peptide–alginate hydrogel scaffolds were prepared using the methodolody described above, and implanted in the same animal model (3 mg of alginate with 75 μg of peptide) [70]. Collagen gels impregnated with recombinant BMP-2 (3 mg of collagen with 3 μg of protein) were used as a control. While the peptide–alginate gel showed prolonged formation of a calcified bone-like tissue for up to 7 weeks, with large numbers of osteoblasts, the control exhibited maximal calcification after 3 weeks, which progressively
disappeared, probably being absorbed by osteoclasts, which were present in large amounts. Apparently, in contrast to the impregnated protein, the grafted peptide remained active at the implant site, exerting a continuous local effect. The differences between the materials (alginate vs. collagen) and the quite different molar amounts of peptide vs. protein implanted were not, however, taken into account when the results were discussed.

Using a different rationale, Lee and co-workers [58] tested the osteoinductive effect of several domains from human BMP-2, corresponding to residues 1–25, 26–50, 51–75 and 76–110. The segment 26–50, which provided the best results in terms of cell adhesion, was further segmented into short 4–5 amino acid sequences (VGWN, DWIVA, PPGYH, AFYCHG, and ECPPF), and out of these the sequence DWIVA was the one that presented the highest binding activity to BMP receptors type Ia and II. A hydrophobic aliphatic tail (C16) tail was attached to DWIVA, via an amide bond, to create a self-assembling peptide (SAP). In the presence of calcium, this amphiphile self-assembles into a nanofibrous hydrogel with surface-exposed DWIVA (or DFMGL, a control non-bioactive sequence). IMSCs were entrapped within this hydrogel and cultured under osteoinductive medium. Higher levels of phosphorylated Smad, ALP activity and mineralization were found in cells cultured within the DWIVA-SAP hydrogels, as compared to the control. Similar results were obtained when cells were treated with even higher concentrations of free peptide (1.67 vs. 0.7 mM), indicating that the bioactivity of DWIVA is retained upon immobilization.

3.2. Collagen type I-derived peptides

The most highly expressed integrins in osteoprogenitors and osteoblasts belong to the β1 sub-family, being predominant mediators of cell adhesion in these cells [46]. The α2β1 integrin is highly expressed on osteoblasts, being one of the major adhesion receptors for COL-I [123]. The interactions between this integrin and its ligand activate focal adhesion kinases, and initiate key pathways for the induction of osteoblastic differentiation and matrix mineralization [28, 124]. Based on this, COL-I-derived peptides have been used as cell adhesion mediators to ultimately induce osteogenic differentiation, as previously pointed out.

3.2.1. Surface immobilization of COL I-derived peptides

Wang and co-workers [61] selected the bone and cartilage synthetic peptide (BCSPTM1), derived from human COL-I, with the sequence NLGPLPQGP. BCSPTM1 was chemically bound to a commercial ceramic surface (SkeliteTM) in three steps. First, amine moieties were created at the ceramic surface by grafting [3-amino-propyl]triethoxysilane (APTES) to free hydroxyl groups through silanization. Then, through succinylation, amine functionalities were converted into esters, to which peptides were grafted via their terminal amines. Rat calvaria cells were first cultured for 3 days with 0.28 mM of ascorbic acid and then seeded on peptide-bound groups were activated by ultraviolet radiation for conjugation to the commercial ceramic surface (SkeliteTM), which was then incubated in PBS for 1 week. BMSC osteogenic differentiation was assessed under osteoinductive conditions by looking to the mRNA expression of COL-I and OCN. It was demonstrated that RGD peptides, but not GFOGER or FnIII7-10, increased COL-I. Moreover, the GFOGER and RGD peptides enhanced OCN at similar levels, whereas FnIII7-10 inhibited its expression. In general, the overall effect of GFOGER was not superior to that of RGD. However, the differential effects of the various ligands in osteogenesis were not studied in detail, as that was beyond the scope of the study.

3.3. Small integrin-binding ligand N-linked glycoproteins (SIBLING)-derived peptides

The ECM of bone and dentine is rich not only in collagenous but also in non-collagenous proteins, including those belonging to the SIBLING family [127]. This family of proteins is involved in the process of bone mineralization, and includes matrix extracellular phosphoglycoprotein (MEPE), OPN, BSP, dentin protein 1 and dentin sialophosphoprotein [128]. Although these phosphoproteins are distinct in their structures, they also share some similarities, such as presenting RGD-like motifs, glutamic acid-rich sites known to induce hydroxyapatite nucleation, and collagen-binding domains [127-129].

3.3.1. BSP-derived peptides

BSP is the major non-collagenous protein in bone, and it shows a high degree of specificity in osteoblastic-like cell attachment [130, 131]. It is considered as a major nucleator of hydroxyapatite crystal formation, and its expression correlates with the onset of matrix mineralization [132]. BSP is present in the bone matrix at late stages of osteoblast development, being primarily localized around mature osteoblasts and osteoblasts trapped in newly formed matrix [133].

3.3.1.1. Surface immobilization of BSP-derived peptides. Rezania and colleagues [29, 134] tested the effect of the BSP-derived peptide CCGNPEPRGDTSRY (pRGD) on rat calvaria cells. Peptides were grafted onto maleimide-functionalized quartz surfaces through thiol groups present in terminal C residues. Different peptide densities, ranging from 0.01 to 3.6 pmol cm−2, were tested. After 3 weeks of culture in mineralization medium (basal medium supplemented with ascorbic acid and β-glycerophosphate), enhanced mineralization was observed in cells exposed to ligand concentrations of 0.62 pmol cm−2 or higher, as compared with lower concentrations or control surfaces (with RGE in place of RGD). However, the mechanisms by which the initial engagement of adhesive ligands mediated the long-term effect were not elucidated. These mechanisms were more recently investigated by Drevelle and colleagues [135] using the same peptide, but this time immobilized on polyacrylatone (PCL). The peptides, pRGD or control pRGE, were linked through their C-terminus to PCL films previously functionalized with ethanalamine hydrochloride. In serum-free
medium, MC3T3-E1 osteoblasts were only able to spread on pRGD–PCL. Additionally, only on those surfaces cells presented an organized cytoskeleton, activated intracellular FAK signaling and responded to BMP-2 by activating their canonical Smad pathway. More recently, the same group tried to elucidate how MC3T3-E1 cells cultured in the presence of these peptides respond to BMPs, in terms of signal transduction and differentiation. Cells were cultured on PCL substrates in serum-free medium, and were treated with 0.38 nM of BMP-2 and/or BMP-9 [136]. Treatment with both BMPs similarly affected the kinetics of MAPK activation, but their effects on Smad activation and β-catenin stabilization were different. In terms of cells osteogenic differentiation, both BMPs increased Dlx5, osterix and OCN transcripts, and also activity, compared to unstimulated samples. This study shed some light on the mechanisms that underlie the effect of biomimetic materials on cells response to GFs.

3.3.2. MEPE-derived peptides

Similarly to other proteins from the SIBLING family, MEPEs also play a key role in phosphate regulation, bone mineralization and osteogenesis. MEPEs contain not only RGD-like motifs, but also the amino acid sequence SCGD known to promote glycosaminoglycan (GAG) attachment [137]. MEPE expression has been predominantly found in osteoblasts. Most of the studies to date have used this peptide in a soluble form [137–139].

3.3.2.1. Surface immobilization of MEPE-derived peptides. Only recently, Acharya and co-workers [59] immobilized a MEPE-derived peptide, with the sequence TDQERGDNDISPFSGDGQP, at the surface of hydroxyapatite/β-tricalcium phosphate (HA/β-TCP) particles to induce bone regeneration in local defects. The immobilization strategy comprised two steps. First, OH groups on the ceramic surface were silanized to create amine moieties, which were PEGylated with PEG disuccinimidyl succinate. Peptides were then coupled through the N-terminal to the PEG linker. It is noteworthy that, although the peptide immobilization was demonstrated, the effectively coated amount was not quantified. Peptide-functionalized ceramic particles were combined with a fibrin vehicle and implanted into calvarial defects created in mice. Eight weeks later, the composite was recovered and increased new bone formation was observed in peptide-modified HA/β-TCP particles compared to unmodified ones, as indicated by microcomputed tomography and hematoxilin & eosin staining. The newly formed bone was actively remodeled by osteoclasts. Overall, this multifunctional peptide combining RGD with a motif that promotes GAG attachment showed great potential for in vivo bone regeneration strategies. Nevertheless, future studies to elucidate the underlying mechanisms and its effect on MSC differentiation should be addressed.

3.3.3. OPN-derived peptides

OPN is a glycosylated phosphoprotein expressed by different cell types, but prominently localized in the ECM of mineralized tissues, such as bone, being involved in the regulation of their formation and remodeling [140,141]. OPN is able to bind not only to cells via a RGD sequence that recognizes the αvβ3 integrin, but also to other ECM components via less characterized peptide sequences [142]. As previously pointed out, similarly to other phosphoproteins, OPN has the capacity to complex to collagen fibrils and induce bone mineralization. This interaction involves a specific region, called collagen-binding motif [143].

3.3.3.1. Surface immobilization of OPN-derived peptides. Shin et al. [18,20,144] have been studying the effect of OPN-derived peptides on MSCs behavior using oligo(poly(ethylene glycol) fumarate) (OPF) macromers with alternating PEG chains and fumarate groups. Two cell adhesion peptides have been covalently grafted onto OPF, namely RGD and the DVPDGRGDSLAYG (ODP) sequence derived from rat OPN. To prepare peptide-modified hydrogels, each peptide was acrylated and reacted with acryloyl-PEG–NHS at different concentrations (0.1, 1.0 or 2.0 μmol g−1 of hydrogel). In an initial study, osteoblasts were shown to be able to attach and proliferate on ODP-modified and RGD-modified hydrogels at similar rates [20]. For differentiation studies, rat MSCs were seeded on top of both peptide-modified hydrogels and cultured under osteoinductive conditions [144]. When compared to unmodified hydrogels and TCPS surfaces, OPD hydrogels enhanced the differentiation and mineralization of MSCs, as verified by higher ALP activity, OPN secretion and calcium deposition, in a concentration-dependent manner. However, the effect ODP in enhancing osteogenic differentiation was not different from the effect of RGD.

Lee et al. [69] tested different fragments from human OPN, and identified the sequence GLRKSRRFRRFQYFDATDITEDTFSHM (CBM, residues 150–177) as the region presenting the highest binding affinity to collagen. Within this sequence, the amino acids D and E are the ones responsible for calcium binding, which in turn promotes mineralization [130]. CBM was grafted onto alginate by carbodiimide chemistry (0.007–35 mg of CBM per g of alginate) and the collagen-binding ability of CBM was apparently maintained in peptide–alginate hydrogels. hMSC were cultured on top of CBM-modified hydrogels, control peptide-modified hydrogels (residues 53–80, with the lowest collagen affinity) and non-modified gels for 28 days, and the cell response was analyzed. Cells adhesion, spreading and proliferation were improved on CBM–alginate. Differentiation assays were performed using hydrogels with 700 μg of peptide per mg of gel, under standard induction medium, for 28 days. The best results were obtained with CBM–alginate, as shown by the enhancement of ALP activity compared with control peptide-modified hydrogels, and by the expression of activated Smad. Importantly, when implanted in rabbit calvarial defects for 4 weeks, the CBM–alginate hydrogel induced significantly more new bone formation than unmodified alginate. Although the role and biologic activity of the OPN collagen-binding motif have not yet been well established, this study suggests its applicability as an active component for clinical bone regeneration procedures.

3.4. PTH-derived peptides

PTH is an 84-amino-acid hormone that acts as a regulator of calcium homeostasis and plays important roles in bone remodeling. PTH1–34 is a truncated peptide with 34 amino acids derived from the N-terminus of PTH that retains most of its bioactivity. Currently, PTH1–34 (commonly referred to as teriparatide) is used as an anabolic agent in the systemic treatment of osteoporosis. However, depending on the dose and administration regimen, it can also have catabolic effects. Moreover, multiple mechanisms of action and target cell types are involved in PTH1–34-stimulated bone repair, as recently reviewed by Takahata et al. [145]. A few studies have addressed the effect of immobilized PTH1–34 in local therapies and bone TE strategies.

3.4.1. Surface immobilization of PTH-derived peptides

In one of the first available studies, PTH1–34 was covalently coupled to silk films [30] by carbodiimide chemistry, at an estimated final density of 20 pmol cm−2. However, no positive effects in terms of differentiation of osteoblast-like cells (Saos-2) were demonstrated when compared to RGD-modified silk. Sharon and Puleo [67] reported a strategy for the controlled immobilization of bioactive PTH1–34 through attachment to a biodegradable polymer (PLGA) via its N-terminus. The N-terminal serine residue of the peptide was first oxidized to yield a single aldehyde moiety, which was then specifically bound to hydrazide–PLGA, forming a
hydrazide bond. Dihydrazide spacers with different lengths were tested, and the accessibility of the tethered peptide was shown to increase with spacer length, as probed with antibodies directed to both the N- and C-terminus of the peptide. The longest spacers also increased the bioactivity of immobilized PTH₁₋₃₄, compared to randomly bound or adsorbed peptide, showing a higher stimulation of intracellular synthesis of cAMP by pre-osteoblastic MC3T3-E1 cells. In a more recent study by Yewle et al. [60], oxidized PTH₁₋₃₄ was conjugated via the N-terminal aldehyde to single molecules of hydrazine bisphosphonates (HBP’s), which present a high bone-binding affinity. The PTH–HBP conjugates were immobilized on bone wafers, to simulate the bone surface, and the bioactivity of PTH₁₋₃₄ was demonstrated as described in the previous study. The selective conjugation of PTH₁₋₃₄ with HBP’s increased its affinity to bone and improved its interaction with cells once delivered. Although these results are quite interesting, the effects of conjugated PTH₁₋₃₄ are yet to be investigated further in more specific in vitro osteogenesis assays, or in terms of in vivo bone formation.

### 3.4.2. Bulk immobilization of PTH-derived peptides

The beneficial effects of PTH locally delivered into bone defects were recently demonstrated in an elegant work by Arrighi et al. [146]. The strategy consisted on derivatizing fibrin matrices with an engineered active fragment of PTH₁₋₃₄, using a plasmin-sensitive substrate sequence as a linker (pl). Once in vivo, the enzymatic cleavage of the linker by endogenous plasmin results in cell-driven release of PTH₁₋₃₄ in situ. The fusion peptide (TGpPTH₁₋₃₄) was obtained by linking the N-terminus of the PTH₁₋₃₄ to the coagulation transglutaminase (TG) factor Xllla, which covalently crosslinks the peptide into the fibrin network during polymerization. The TGpPTH₁₋₃₄ pro-drug is inactive, preventing the eventual activation of osteoclasts at the time of injection when the local concentration of PTH₁₋₃₄ is high [147], but it converts into fully functional PTH₁₋₃₄ upon plasmin-induced proteolytic cleavage. In vivo studies were performed using epiphysial drill defects created in sheep, which were filled with TGpPTH₁₋₃₄-derivated (50–1000 μg ml⁻¹) or non-derivatized fibrin gels. Bone formation was observed in the presence of PTH in a dose-dependent fashion, demonstrating a strong healing potential, with evidence of both osteoconductive and osteoinductive mechanisms. The same group started clinical trials in humans to investigate the therapeutic efficacy of this PTH-fibrin matrix in bone healing.

In another study, Jung et al. [62] used an RGD-modified PEG-based matrix containing covalently bound PTH₁₋₃₄ peptides. To prepare functionalized PEG hydrogels, cys-PTH₁₋₃₄ and cys-RGD were both added to a four-arm PEG-acrylate solution, and cysteine thiol groups were allowed to react with the PEG-acrylate. Afterwards, this solution was combined with another solution of linear PEG-dithiol and the gelling mixture was loaded into a syringe for surgical application. The final concentrations of each peptide in the PEG hydrogels were 20 μg ml⁻¹ (PTH₁₋₃₄) and 350 μg ml⁻¹ (RGD). The RGD–PTH₁₋₃₄–PEG matrix and different controls were implanted in canine alveolar bone defects. Histomorphometric analysis after 4 and 12 weeks revealed that the PTH₁₋₃₄ group presented enhanced bone formation when compared with PEG alone or empty defects, with similar results being obtained with autogenous bone. However, the effect of RGD–PEG without PTH₁₋₃₄, which would be an important control, was not evaluated.

### 3.5. Osteogenic growth peptide (OGP) and OGP-derived peptides

OGP is a naturally occurring tetradecapeptide identical to the C-terminal amino acid sequence (residues 89–102, ALKRQGRTL YGF GG) of histone H4 (H4), which is naturally present in human plasma at micromolar concentrations [148–151]. Chen and co-workers [152–154] demonstrated that the C-terminal sequence of OGP (residues 10–14, YGFGG, hereafter designated as OGP₁₀–₁₄) corresponds to the bioactive portion of OGP that directly interacts with the cell surface. Upon intravenous administration in animals, both peptides were shown to promote increased bone mass and promote fracture healing [148,150,151]. In vitro, soluble OGP peptides were shown to increase osteoblasts and MSCs proliferation and to promote osteogenic differentiation in a biphasic concentration-dependent manner [155]. The use of physically [156,157] and chemically immobilized OGP has been explored in a few works.

#### 3.5.1. Surface immobilization of OGP and OGP-derived peptides

To clarify the effect of concentration and bioavailability of surface-immobilized OGP, Moore and colleagues [158] performed a systematic study using SAMs. OGP and OGP₁₀–₁₄ derivatized with azide at the N-terminal were grafted by click chemistry onto SAMs functionalized with alkyn gradients [158]. Along the gradients, the density of grafted peptides ranged from 0 to 140 pmol cm⁻². MC3T3-E1 cells were cultured on these substrates for up to 7 days in serum-free medium. From day 0 to day 3, immobilized OGP increased cells proliferation independently of concentration. It is possible that OGP activity was dependent on the cleavage of the bioactive (10–14) sequence that, once free, subjected all cells to similar OGP₁₀–₁₄ concentrations. Also, there was probably a concomitant effect of cell-secreted OGP. A positive effect of OGP₁₀–₁₄ was only observed at a lower density (40 pmol cm⁻²). Globally, the outcome of immobilized OGP was considered to be small compared to other immobilized GFs described in the literature. In another study, the effect of OGP on gene expression of ostegenic markers was only investigated in cells treated with soluble peptides (at 10⁻⁷ M). From day 3 to day 7, both peptides increased the expression of COL-I while decreasing the expression of Runx2, apparently indicating a transition of MC3T3-E1 cells from a proliferative to a maturation phase [159].

#### 3.5.2. Bulk immobilization of OGP and OGP-derived peptides

OGP was also used to functionalize the SAP AcN–RADARADARADARA–CONH₂ (RAD16), through direct solid-phase synthesis extension at the C-termini (Ac(RADA)₄GGLRKQRGTLYGF–CONH₂) [22]. Its effect was compared to that of unmodified RAD16, and of OGP₁₀–₁₄ functionalized with either an OPN-derived motif (Ac(RADA)₄GGLDKRGDSVAYG–CONH₂) or a double RGD-containing sequence (Ac(RADA)₄GPRDGSYGRDG–CONH₂). Gel-precursor solutions were prepared (1 wt.%) by mixing unmodified with modified RAD16 at a ratio of 1:1, and MC3T3-E1 cells were cultured on top of the hydrogels in OM. After 2 weeks, the cell proliferation rate in all modified peptides was higher than that of pure RAD16. ALP activity and OCN expression were also increased, especially when PRG was used. Although this study demonstrated the effectiveness of immobilized OGP in enhancing cellular proliferation and differentiation, the biofunctional region of the peptide was apparently used in a truncated form (YGF) that, according to previous studies, is not fully active [154].

In a recent study, OGP₁₀–₁₄ was used as a crosslinker in amino acid-based poly(ester urea) (PEU) scaffolds [68]. OGP₁₀–₁₄ was symmetrically functionalized with K residues at both termini (KYFGGGK), with reactive vinyl groups grafted onto K side chains. It was then used (at 0.5% and 1%) to photochemically crosslink phenylalanine or leucine-based PEU scaffolds, enhancing their mechanical properties in a concentration-dependent manner. The proliferation of MC3T3-E1 cells cultured on PEU scaffolds was slightly increased in the presence of OGP₁₀–₁₄ at both concentrations. As the peptide was immobilized through both ends, the release of bioactive OGP₁₀–₁₄ into the medium upon enzymatic cleavage is improbable, suggesting that it retains bioactivity even in an immobilized form. Upon subcutaneous implantation in rats...
covalently sequestering specific biomolecules from the medium, in this case heparin and heparin-binding GFs such as BMPs; (ii) presenting those GFs at the cell-material interface at a high local concentration; and (iii) amplifying key signaling pathways involved in proliferation and differentiation.

3.6.2. Bulk immobilization of heparin-binding peptides

No studies using bulk-immobilized HBP for promoting osteogenesis were found, even if heparin-functionalized PEG hydrogels have been shown to induce the osteogenic differentiation of 3-D cultured hMSC [162]. Alginate scaffolds have been functionalized with HBP and RGD, but have only been tested for cardiac applications, showing good potential to improve cardiac muscle tissue formation in vitro [163]. In another, very recent, example, HBP have been used to modify a hydrogel obtained by the copolymerization of N-vinyl pyrrolidone, diethylene glycol bis allyl carbonate and acrylic acid. In this case, the HBP consisted on positively charged tri-lysine (KKK) or triarginine (RRR) sequences, but the objective was the development of a pro-angiogenic biomaterial [164].

3.7. Calcium-binding peptides

Phosphorylated amino acid (serine) residues have a high capacity for binding calcium ions. For this reason, they play a key role in the regulation of the early stages of mineralization in vivo, being involved in the process of crystal formation. For this reason, these amino acids have been exploited as functionalization molecules to improve the osseointegration of implant materials [165, 166].

Recently, peptide amphiphile (PA) materials capable of self-assembling into well-defined nanofibrous hydrogels were designed using peptide sequences that contained phosphoserine residues (S-PA) and RGDS motifs (RGD-PA). The combination of these two modifications, when implanted in a critical-size rat femoral defect, showed greater bone formation compared with serine and RGD alone [57]. The authors hypothesized that the observed effect was related to the capacity of these nanofibers to stimulate hydroxyapatite (HA) nucleation and enhance mineral deposition. Later, in an in vitro study, in which calcium-supplemented medium was used, demonstrated that these PA nanofibers containing serine and phosphoserine were effectively able to nucleate spheroidal aggregates of carbonated hydroxyapatite [167].

A number of strategies have been proposed to localize GF activity to mineralized ECMs and mineral-based biomaterials [54]. With this aim, a class of modular peptides conjugating BMP-2-derived peptides with a natural HA binding unit present in OCN (γEPRR-γEVCyEL, γ-carboxylated glutamic acid) has been proposed by Lee et al. [168–170]. The Gla residues coordinate with calcium ions in the HA crystal lattice to promote high levels of binding. The modular peptide KIPKASSVPTELSAISTLYL-AAAA-γEPRRγEVCyEL, where the (Ala)4 sequence acts as a spacer, has been prepared by solid-phase peptide synthesis. In a first set of studies, the sequence that more effectively binds to a HA-coated poly(lactide-co-glycolide) surface was identified, and then its biological activity on hMSC osteogenic differentiation was evaluated. ALP activity, BMP-2 secretion and OCN, OPN and Cbfa1 production were enhanced when cells were cultured in HA surfaces modified with the modular peptide under induction standard medium, as compared with cells treated with the soluble modular peptide.

Recently, another interesting type of material with HA-binding ability has been proposed, consisting on recombinamers (recombinant protein materials obtained from a synthetic gene) containing peptide motifs derived from elastin, a structural protein, and statherin, a salivary protein that has a high affinity for calcium phosphate [171]. Multifunctional elastin-like recombinamers containing the SN3,15 domain of statherin (an analogue of the amino-terminal 15-residue fragment) were prepared, and their
potential as mineralization templates were tested by incubation in simulated body fluid at 37 °C. In materials with a triblock structure, it was possible to observe calcium phosphate deposition after 1 week. However, there have been no studies exploiting the effect of such materials on MSC differentiation in vitro nor on bone formation in vivo.

4. Other osteoinductive small molecules

There are several other classes of small compounds, with different mechanisms of action, that have also been covalently coupled to biomaterials and used as osteoinductors. In many cases, the beneficial effect of those compounds in a soluble form had been previously demonstrated, providing the rationale for investigating their use as bioactive molecules in drug-releasing scaffolds, to be released in situ upon material and/or linker degradation.

For example, Nuttelman et al. [101] covalently grafted dexamethasone, typically used as supplement in osteoinductive medium, to PEG through a degradable lactide acid link using diisopropyl carbodiimide chemistry. Dexamethasone was released from this system upon degradation of the lactic acid bonds, and proved to be biologically active as it enhanced the osteogenic differentiation of encapsulated hMSCs in vitro, as suggested by a significant increase in Cbfa1 gene expression.

A similar strategy has been followed using statins as bioactive molecules [172]. These small naturally occurring compounds are composed of a dihydroxyheptanoic acid unit and a ring system with different substituents. Structural/compositional variations give rise to different types of statins with distinct pharmacological properties [173]. Statins are widely recognized for their effect in lowering blood cholesterol levels, as inhibitors of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase. However, back in 1999 [174,175], statins were included in a study in which showed a positive result [175]. Later, this effect was corroborated in an in vivo study, where statins were shown to stimulate bone formation when administered to rats, either systemically or locally in bone. In more recent studies, these compounds were combined with biomaterials for bone regeneration therapies. Be. noit et al. [172] synthesized a flavastatin-releasing monomer that was covalently incorporated into PEG through a lactide acid link. In the absence of other osteoinductive factor, the released flavastatin not only promoted hMSCs osteogenic differentiation, as suggested by the increase in Cbfa1, ALP and COL-I gene expression, but also stimulated BMP-2 production. Whang et al. [176] grafted simvastatin to hydrolytically degradable poly(lactide-co-glycolide) before scaffold formation, and tested its effect on rat BMCs in vitro.

The immobilized simvastatin was able to significantly enhance ALP activity and mineralization.

Some of these novel molecules have been shown to act cooperatively with BMPs [177], but in many cases the molecular basis for their activity has not yet been identified. Most of these compounds were not included in this review because no studies were found describing their use in an immobilized form. Nevertheless, by way of example, some recently described compounds are listed in Table 4.

With the advent of high-throughput screening (HTS), many other small molecules with osteogenesis-inducing activity have been identified recently [177–180]. In fact, HTS not only allows for the rapid checking of thousands of molecules involved in a specific biological process, but may also lead to the discovery of unpredicted signaling pathways and molecular mechanisms. For example, Alves and co-workers [179] recently identified some promising compounds from a library of 1280 pharmacologically active small molecules using an in-house developed HTS assay, even without the need of expensive robotic techniques. From a tissue engineering perspective, these small molecule discoveries could be valuable for the design of novel biofunctional materials.

Finally, there are additional types of small molecules that target other regulatory processes, namely by modulating the expression of specific genes, which also hold great potential in directing stem cell fate. This is the case, for example, of small interfering RNA [186], and other forms of gene delivery [187–191], which are not covered in the present review.

5. Conclusions and future perspectives

It has become clear that biomimetic approaches to modulate cell-material interactions are vital not only to implement advanced TE strategies involving stem cells transplantation, but also to improve osseointegration of current orthopedic biomaterials. In fact, by inducing peri-implant bone formation, these modifications might contribute to decreasing the failure rate of implant devices, which still represents a large socioeconomic burden [192]. As reviewed herein, several small compounds with different mechanisms of action have been described to enhance osteogenesis and bone formation. On the one hand, small molecules are advantageous comparing to more complex ones. On the other hand, immobilized molecules can be more effective than their soluble counterparts. In some cases, as with peptides, the combined use of different bioactive motifs also seems to be an attractive strategy, as they can act synergistically and potentiate each other’s effects. However, while many of these compounds have shown promise as osteoinductors in vitro, more studies need to be done to demonstrate their bioactivity in vivo as well. Moreover, the beneficial ef-

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Table 4

Examples of small molecules recently shown to have osteogenic-inducing activity. a

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Cell type</th>
<th>Mechanism/target(s)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP</td>
<td>hMSC</td>
<td>Activation of PKA pathway</td>
<td>[181]</td>
</tr>
<tr>
<td>Forskolin</td>
<td>hMSC</td>
<td>Activation of PKA pathway</td>
<td>[182]</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>hMSC</td>
<td>BMP and Wnt/beta-catenin signaling pathways</td>
<td>[183]</td>
</tr>
<tr>
<td>Pumorphamine</td>
<td>Mouse embryonic mesodermal fibroblasts C3H10T1/2</td>
<td>Unknown</td>
<td>[178]</td>
</tr>
<tr>
<td>Phenamil</td>
<td>M2-10B4 (M2) mouse marrow stromal cells, mouse ES cells, C3H10T1/2 MSC line, Mouse calvarial organ culture</td>
<td>Trb3-independent promotion of BMP action</td>
<td>[177]</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>Human ES-derived MSC, canine AD-MSC, mouse MSC, mouse MC3T3-E1</td>
<td>Uregulation of RUNX2 gene expression via the SIRT1/FOXO3A axis</td>
<td>[184,185]</td>
</tr>
</tbody>
</table>

a Examples depicted in this table are illustrative rather than inclusive. BMC – bone morphogenetic proteins; cAMP – cyclic adenosine monophosphate; canine AD-MSC – canine adipose mesenchymal stem cells; C3H10T1/2 MSC – C3H10T1/2 mesenchymal stem cells; ES cells – mouse embryonic stem; hMSC – human mesenchymal stromal cells; Human ES-derived MSC – human embryonic stem-cell derived mesenchymal progenitors; mouse MSC – mouse mesenchymal stem cells; PKA – protein kinase A; Trb3 – tribbles homolog 3.
fects of many of them are still limited or controversial, making the search for novel molecules and immobilization strategies essential. As pointed out, this challenging task is being facilitated by the emergence of new technologies and methodologies for HTS. Yet, while HTS methods will indeed deliver many new molecules, these can only make an impact when they can be applied to a large range of 3D materials for bone regeneration. HTS approaches have already been applied to the study of cell-material interactions by using miniaturized material-based array platforms obtained by various microfabrication technologies, such as micropatterning, bioprinting and microfluidics [193]. Anderson et al. [194] performed one of the first studies demonstrating the usefulness of material-based HTS systems. This work examined the effects of different biomaterial compositions in human embryonic stem cell attachment, growth and differentiation using an array of synthetic polymers, where 1700 different conditions were screened to evaluate cell-material interactions. Since then, many different other cell-material HTS platforms have been developed, both in two dimensions, e.g., microarrays of SAMs [195], ECM proteins [196] and biomaterials [197], and in three, such as spots of cell-laden hydrogels [198]. Although young, this field has been progressing rapidly and more complex HTS platforms are being produced. Greater investment in existing and new HTS techniques will certainly contribute to our understanding of the role of several cues in MSC osteogenic differentiation, and help to more rapidly identify novel and unique biomaterial–molecule combinations with a view to optimizing therapeutic approaches. In a certain way, materials become “cell-instructive”, a property that was previously reserved for growth factors and other signaling molecules [199]. This paves the way to the improvement of the biomaterials’ biological performance for clinical applications, in a more cost-effective way.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 1–3, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at 10.1016/j.actbio.2013.08.004.

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and COOH functionalized surfaces.

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