SPECIAL ISSUE REVIEW

New trends in the development of multifunctional peptides to functionalize biomaterials

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Improving cell-material interactions is a major goal in tissue engineering. In this regard, functionalization of biomaterials with cell instructive molecules from the extracellular matrix stands out as a powerful strategy to enhance their bioactivity and achieve optimal tissue integration. However, current functionalization strategies, like the use of native full-length proteins, are associated with drawbacks, thus urging the need of developing new methodologies. In this regard, the use of synthetic peptides encompassing specific bioactive regions of proteins represents a promising alternative. In particular, the combination of peptide sequences with complementary or synergistic effects makes it possible to address more than one biological target at the biomaterial surface. In this review, an overview of the main strategies using peptides to install multifunctionality on biomaterials is presented, mostly focusing on the combination of the RGD motif with other peptides sequences. The evolution of these approaches, starting from simple methods, like using peptide mixtures, to more advanced systems of peptide presentation, with very well defined chemical properties, are explained. For each system of peptide's presentation, three main aspects of multifunctionality—improving receptor selectivity, mimicking the extracellular matrix and preventing bacterial colonization while improving cell adhesion—are highlighted.

KEYWORDS
biomaterials, cell-material interactions, multifunctionality, oligopeptides, branched peptides, peptide mixtures, surface functionalization

1 | INTRODUCTION

Tissues in the human body are characterized by their potential to self-regenerate. However, when there is an injury, disease or due to aging, the body's capacity to regenerate by itself may be compromised. In such cases, the use of biomaterials that guide tissues to restore their original function and structure is required. During this regeneration process, the biomaterial should serve as a structural frame to favor host cells attachment and ideally to promote the migration of stem cells and their proper differentiation into tissue-specific cell types.[1,2]

The current gold standard to address tissue regeneration is still the use of autografts, mainly due to their optimal support to the host cells to colonize the graft and the absence of immunological reactions. However, the drawbacks associated to autologous grafting, like the limitation in the obtainable quantity, the patient morbidity and the need of additional surgery, makes it necessary to find other approaches. A feasible alternative is the use of allografts or xenografts, but although they solve most of the problems related to autografts, in both cases the risk of infection, disease and rejection of the grafts by the immune system exists.[3,4]

To overcome the drawbacks of natural grafts, synthetic biomaterials can be used. Synthetic biomaterials are largely available and may be tailored to meet the desired properties for a specific application. However, although most of current synthetic biomaterials fulfill the requirements from a structural point of view, many of them are bioinert, meaning they lack regulatory signals as well as bioactivity, both essential to control cell-material interactions. This may trigger low promotion of cell adhesion and growth as well as limited vascularization, causing eventually the failure of the biomaterial.[5,6] Consequently, it is of paramount importance to enhance bioactivity of synthetic scaffolds by combining biomaterials with biological cues to drive a particular tissue function.[7] In this regard, functionalization with biologically active peptides is a powerful strategy to engineer tailored cell-material interactions and to mimic the signaling microenvironment to enhance tissue regeneration.[8–10]

The aim of this review is thus to cover the existing possibilities to increase bioactivity of biomaterials with peptides by mimicking the healing microenvironment of body tissues. More in detail, the combination of peptides—mainly the RGD cell adhesive motif—together with other sequences with different biological functions will be presented. This report will first focus on the use of mixtures of biologically active cues to equip inert biomaterials with multifunctional bioactivity. Next, we will further present how functionalization strategies have been evolved to obtain more advanced systems of presentation of peptides with very well defined properties, such as peptide orientation, distribution and spacing.

2 | SURFACE BIOFUNCTIONALIZATION WITH PEPTIDES

Surface functionalization consists on incorporating specific biological functions on the surface of a biomaterial, providing bioinert materials with bioactive molecules without modifying their bulk properties. To obtain a successful functionalization, mimicking the cellular microenvironment is of special importance. In this regard, it is possible to reproduce the biochemical signals involved in the regeneration of a tissue by incorporating biological cues that recapitulate the extracellular matrix (ECM) of the target tissue.[8,11] Nonetheless, recreating cell-matrix interactions is challenging due to the complex array of biochemical processes taking place in native tissues. For instance, cells are sensitive to the intrinsic mechanical properties of the matrix, which besides providing a three dimensional network for tissue growth, also influence cell fate due to the capacity of cells to sense biomechanical forces.[12,13] Furthermore, ECM provides cells with soluble molecules, like growth factors (GFs) or cytokines, which are tissue-specific and orchestrate cell functions. Similarly, ECM contains a great variety of proteins that directly interact with cells through their receptors (e.g., integrins), mainly driving cell adhesion. Cells also communicate with nearby cells, driving tissue homeostasis and cell development.[14,15]

The incorporation of these processes into a biomaterial is a complex task and depending on the target tissue, the approach followed to engineer and functionalize the biomaterial will differ. For instance, the use of ECM proteins, like fibronectin, vitronectin or collagen type I, which are involved in cell adhesion, are paramount to functionalize biomaterials that present low affinity for cells. On the other hand, installing GFs on tissue grafts will provide crucial signals to stimulate the differentiation of stem cells into a particular lineage. This is the case of bone morphogenetic 2 (BMP-2) for osteogenesis or the vascular endothelial growth factor (VEGF) for vasculogenesis.[16–18]

Due to the potential of proteins and GFs to regulate cell behavior, they have been extensively used to functionalize biomaterials for tissue regeneration.[15,19] In this regard, fibronectin has been combined with scaffolds, as it plays a key role not only in cell adhesion but also in cell migration, differentiation and wound healing, enhancing the scaffold bioactivity.[20,21] The interaction of fibronectin with cells takes places through integrins, transmembrane receptors that mediate cell-matrix interactions. In detail, integrins recognize the tripeptide sequence arginine-glycine-aspartic acid (RGD), which is present in the tenth type III domain of fibronectin.[10,22,23] Such interaction triggers specific functions of the cells by activating particular signaling pathways, responsible of cell fate. In the field of bone regeneration, BMP-2 also plays a crucial rule, as it orchestrates bone formation and remodeling and fracture repair.[24,25] Thus, BMP-2 has been also combined with biomaterials to enhance their bioactivity.[26–29]

Despite the potential of using different ECM proteins as well as GFs for tissue regeneration, the use of native, full-length proteins in biomaterials entails some limitations. Due to their fast degradability, proteins have a short-term biofunctionality. They also are very sensitive to temperature and pH changes, compromising their stability. In addition, although their synthesis has been significantly improved over the last years, there are still problems related to the presence of contaminants or bacterial endotoxins after purification. Finally, their production is generally expensive, and it is hard to obtain them pure in large quantities due to their low solubility.[8,30]

To overcome these shortcomings, the use of synthetic peptides that include specific regions of ECM proteins has been proposed. Peptides are normally cheaper to produce, structurally simpler and more stable than proteins. Moreover, they are easily modifiable for functionalization of surfaces, reaching, in some cases, similar biological activity than the entire protein for a specific target.[31,32]

However, no single peptide has yet come close to mimic the specificity and complexity of ECM proteins. This is mainly because linear peptides are flexible and can adopt different conformations, decreasing the specificity towards a particular receptor.[33] Furthermore, proteins are multifunctional by nature, meaning that they contain more than one functional site, which may trigger synergistic or complementary biological effects. Single synthetic peptides, however, cannot promote such multiple interactions, and consequently, alternative approaches have been developed.[8,34,35]

2.1 | Installing multifunctionality on biomaterials with peptides

As previously mentioned, the main limitation of using peptides to enhance the bioactivity of implantable materials is that they can only

address one biological target. A possibility to improve the performance of peptidic molecules is to combine peptide sequences with synergistic or complementary effects, enabling to address two or more biological effects. In this way, multifunctionality may be installed on biomaterials without the need of using proteins. This strategy thus exploits the advantages of native ECM proteins while avoiding their documented drawbacks. As a result, functionalization of biomaterials combining peptides has gained increasing relevance in the field of tissue engineering over the last years.[36,37]

Interestingly, by combining peptides from diverse nature, different biological effects may be targeted. Besides mimicking the complexity of the ECM, there are other issues that biofunctionalization needs to address. For example, in the case of vascular implants, preventing inflammation at the implanted site, as well as protein and platelet adhesion, is important, as aggregation may lead to thrombosis on vascular grafts. Such problem may be minimized by modifying the grafts with peptides to reduce non-specific protein adsorption.[38,39] In bone regeneration, the use of BMP-2-derived peptides is also of crucial importance to promote osteodifferentiation of mesenchymal stem cells (MSCs) and guide bone formation.[40,41] Another important aspect when implanting a biomaterial is to avoid bacterial adhesion, which ultimately may lead to implant infection. In this regard, antimicrobial peptides (AMPs) can be incorporated too on the material surface.[42–45]

Alternatively, chimeric bifunctional peptides have been designed combining sequences that have the potential to selectively adsorb on a particular type of material with bioactive motifs, allowing the incorporation of signaling cues on biomaterials in a substrate-specific manner.[46–50] However, this dual-function peptides will not be covered in this review, as we will only focus on peptides targeting biological entities, for example, cells or bacteria.

Thus, the combination of peptides to functionalize materials is a powerful tool to improve their bioactivity. In particular, multifunctionality aims at addressing three main aspects (Figure 1):

- Improving receptor selectivity: through this strategy, the use of complementary peptides may increase affinity for a particular

receptor. A clear example is the combination of RGD with the PHSRN sequence. When RGD is used alone, cell adhesion is enhanced by allowing interaction of this sequence with integrins. However, such interaction is unspecific, as the RGD motif interacts with different types of integrins. To increase receptor selectivity, the PHSRN peptide may be combined together with RGD, thereby mimicking the 10th and 9th type III repeats of fibronectin (i.e., its cell attachment site), respectively. The combination of both sequences synergistically increases the selectivity of RGD towards α5β1 integrin.[51] Another example is the combination of RGD with the REDV peptide, which selectively binds to α4β1 integrin,[52] highly expressed in endothelial cells. Such combination may then increase the specificity of a biomaterials towards endothelial cells compared with other type cells, like fibroblasts.[53]

- Mimicking the ECM microenvironment: the aim of this strategy is to combine peptides derived from different ECM proteins. In this regard, GFs play a crucial role as signaling molecules, orchestrating cell behavior in terms of migration, proliferation and differentiation. Although GFs are soluble biomolecules involved in paracrine signaling, they also exert their biological effects bound to the ECM, which acts as a GF reservoir and regulates their functions. Also, many GF receptors, like BMP-2 receptors, cooperate with integrins to foster healing of tissue.[54,55] Consequently, the combination of integrin binding ligands, such as the RGD motif, with BMP-2-derived peptides may be a powerful strategy to better recreate the ECM microenvironment, simultaneously addressing cell adhesion and osteogenic cell differentiation. Most interestingly, integrins and BMP receptors have shown synergistic effects, opening the way to engineer novel surface functionalization techniques to enhance bioactivity of biomaterials.[56–58]

- Preventing bacterial adhesion without loosening cell adhesive properties: in addition to mimicking ECM characteristics, bacterial colonization is another challenge. This is especially important in orthopedics and dental applications, in which metals, which are prone to bacterial infection, are commonly used. In such cases, bacterial adhesion and the further biofilm formation may
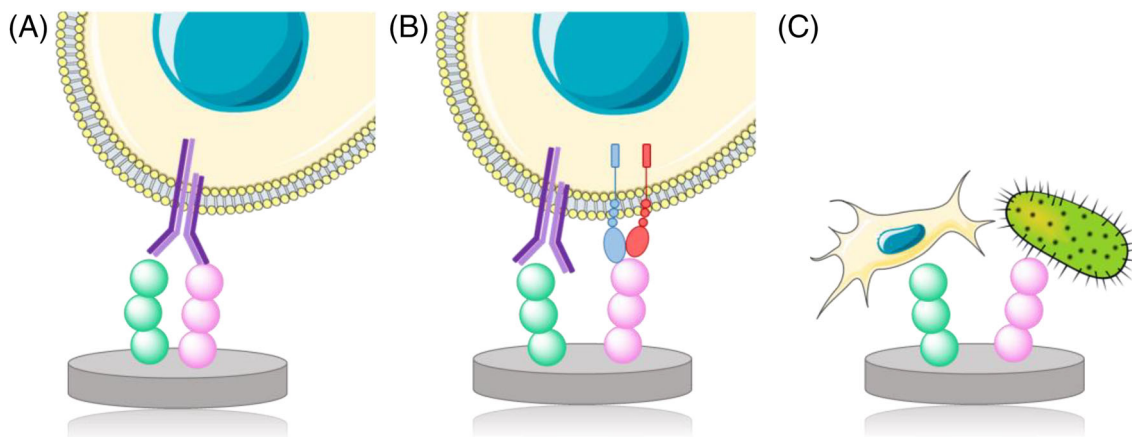

FIGURE 1 Installing multifunctionality on biomaterials by (A) enhancing receptor selectivity, (B) mimicking the ECM microenvironment, and (C) preventing bacterial colonization while improving cell adhesion

compromise the successful integration of the scaffold, and ultimately lead to implant failure. To overcome this serious risk, combining integrin binding ligands with antibacterial sequences holds promise to suppress bacterial adhesion while simultaneously enhancing cell adhesion.[42] This may be achieved, for example, by combining the RGD sequence with a lactoferrin-derived peptide (LF1-11).[59,60]

These examples illustrate the potential of peptides to enhance the bioactivity of materials and to provide them with multi-functionality. Nonetheless, peptides can be incorporated on biomaterial surfaces following different approaches. Fine-tuning key parameters, such as the orientation, spacing, conformation and distribution of the surface-bound peptides is essential to optimally target a particular biological effect. The following section describes representative systems of peptide presentation and how such strategies have evolved from simplistic to more complex and engineered approaches, in which tailor-made peptides have allowed significantly improving the bioactivity of materials.

2.2 | Systems of presentation of peptides

The integration of multiple bioactive peptides on a biomaterial surface has been achieved through different synthetic approaches. Reasonably, the most straightforward strategy to obtain a bifunctional coating is to use mixtures of peptides. This strategy relies on the combination of two (or more) peptides in solution at a defined ratio (normally equimolar) and subsequent coating of the desired surface. Albeit simple, the major limitation of this approach is that binding of the individual peptides to the surfaces strongly depends on the chemistry of each peptide (e.g., peptide charge, hydrophilicity/hydrophobicity, size, and conformation) and hence ensuring a defined ratio of the two peptides on the biomaterial is difficult. This is coupled to the fact that the characterization at the surface level is not trivial, that is, discriminating the efficiency of grafting per each individual sequence. To address this, oligopeptides or fusion peptides, which contain the two sequences within the same peptide backbone, have been developed. The evident advantage of this method is that the two bioactive

peptides are integrated with the same chemical structure and thus the ratio is chemically controlled and does not depend on the properties of the individual peptides. Such strategy contemplates several approaches. Although a clear distinction is not always possible, in this review, we will differentiate between linear constructs and branched architectures. In the first case, the peptide contains the two sequences linked in a linear fashion (separated or not via a linker) and are grafted on a surfaces by either nonspecific physical adsorption or by surface specific anchoring units present at the *N*- or *C*-termini. In the second case, the peptide is designed directly as a branched molecule (e.g., using a lysine residue as branching point) or contains a suitable anchoring unit in its central backbone, thereby allowing binding to the surface in a configuration that optimally exposes the two motifs for interaction with biological entities (cell receptors and/or bacteria). For this reason, branched conformations allow for a higher accessibility of the peptides and often improved biological effects. These different configurations are schematically summarized in Figure 2.

The following section presents representative examples of each strategy, focusing on the biological targets described in Section 2.2 and in Figure 1. A comprehensive list of reported peptide combinations used to functionalize biomaterials is shown in Tables 1, 2, and 3.

2.2.1 | Peptide mixtures

As previously introduced, the use of peptide mixtures represents the simplest approach to incorporate multiple biological functions onto biomaterials. In particular, this strategy has been commonly pursued to improve the lack of specificity of the RGD motif towards a particular integrin subtype (Table 1). A canonical example is the use of the PHSRN sequence, which synergistically enhances the affinity of RGD towards $\alpha5\beta1$ integrin.[51] In this regard, Chen et al. covalently bound RGD and PHSRN motifs to titanium substrates. The combination of both sequences significantly improved MC3T3 adhesion in comparison with the presentation of the peptides alone. However, no significant enhancement was observed on cell proliferation and ALP activity.[64] Alternatively, the RGD peptide has also been co-immobilized with either the $\alpha4\beta1$-binding sequence REDV or with the
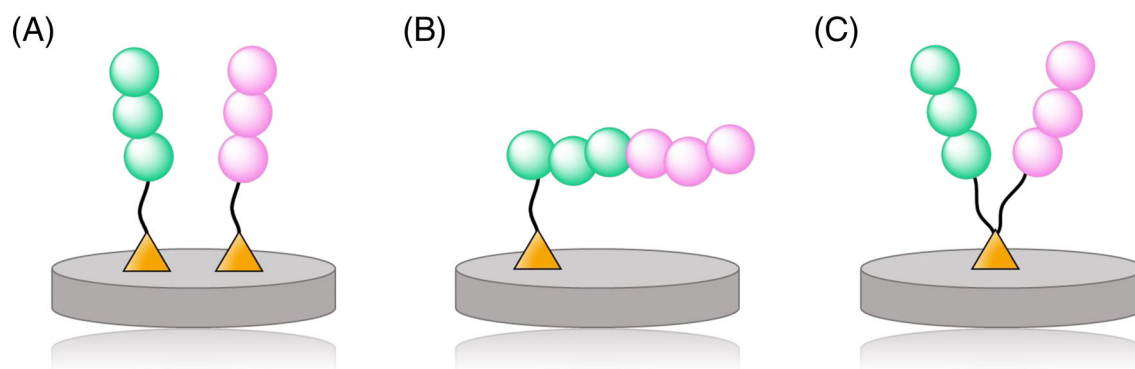

FIGURE 2   Main systems of presentation of peptides: (A) peptide mixtures, (B) linear oligopeptides, and (C) branched peptides

TABLE 1 Combination of peptides to enhance receptor selectivity

Biofunctional motifs	System of presentation	Substrate/immobilization	Main biological effect	References
RGD + PHSRN or YIGSR	Mixture of peptides	PEG hydrogel/covalent	↑ hMVEC migration (only YIGSR)	Fittkau et al.[61]
RGD + PHSRN	Mixture of peptides	Langmuir–Blodgett films on mica/physical adsorption	↑ HUVEC spreading	Ochsenhirt et al.[62]
RGD + PHSRN	Mixture of peptides	Alginate hydrogel/covalent	↑ NHOsts adhesion, ALP activity, OCN production and mineralization	Nakaoka et al.[63]
RGD + PHSRN	Mixture of peptides	Ti/covalent	↑ MC3T3-E1 adhesion, proliferation and ALP activity	Chen et al.[64]
RGD + PHSRN [G_3PHSRNG_6RGDG]	Linear fusion peptide	TCPS/physical adsorption	↑ MG63 adhesion, spreading and MAPK activity	Il et al.[65]
RGD + PHSRN [RGDG$_{13}$PHSRN]	Linear fusion peptide	PEG hydrogel/covalent	↑ RCO adhesion, spreading, FA, proliferation, metabolic activity, ALP activity	Benoit and Anseth[66]
RGD + PHSRN	Branched peptide	Ti/physical adsorption	↑ Saos-2 adhesion, spreading and proliferation	Mas-Moruno et al.[67]
RGD + PHSRN	Branched peptide	Ti/covalent	↑ hMSCs adhesion, spreading, mineralization and ITGA5 and Runx2 gene expression; ↑ bone formation in vivo	Fraioli et al.[68]
Cyclic RGD + PHSRN	Mixture of peptides	Gold nanodots on glass substrates/covalent	↑ REF WT adhesion and spreading	Schenk et al.[69]
RGD + FHRRIKA	Mixture of peptides	Quartz/covalent	↑ RCO adhesion and mineralization	Rezania and Healy[70]
RGD + FHRRIKA	Mixture of peptides	P (NIPAAm-co-AAc) hydrogels/covalent	↑ RCO adhesion, spreading and proliferation	Stile and Healy[71]
Cyclic RGD + FHRRIKA	Branched peptide	Ti/chemisorption	↑ Saos-2 adhesion, spreading and viability	Pagel et al.[72]
Cyclic RGD + FHRRIKA	Branched peptide	PCLLC scaffolds/chemisorption	↑ HUVEC adhesion, survival, migration and differentiation	Clauder et al.[73]
RGD + FHRRIKA or KRSR	Mixture of peptides	Ti/physical adsorption	↑ RCO migration	Schuler et al.[74]
Cyclic RGD + FHRRIKA or KRSR	Mixture of peptides	HA/physical adsorption	↑ hMSCs spreading	Sawyer et al.[75]
RGD + KRSR	Mixture of peptides	Silk/covalent	= NHOsts adhesion and proliferation	Kim et al.[76]
RGD + KRSR	Mixture of peptides	Ti/physical adsorption	= BIC, bone fill, interfacial shear strength in vivo	Broggini et al.[77]
RGD + KRSR	Mixture of peptides	Ti/physical adsorption	↑ MG63 adhesion; ↓ ALP activity and OCN, TGF-β1 and PGE2 expression of MG63	Bell et al.[78]
RGD + KRSR	Mixture of peptides	$C_2S^H_{48}C_2$/yeast production	↑ MG63 survival and morphology	Włodarczyk-Biegun et al.[79]
RGD + KRSR	Linear fusion peptide	TCPS/physical adsorption	= RCO adhesion	Dettin et al.[80]
RGD + KRSR	Branched peptide	Ti/covalent	↑ Saos-2 adhesion and mineralization	Hoyos-Nogués et al.[81]
RGD + REDV or YIGSR	Mixture of peptides	CoCr/covalent	↑ HUVEC adhesion and proliferation (only YIGSR)	Castellanos et al.[82]
RGD + REDV or YIGSR	Mixture of peptides	dPVCs in vitro; dAoGs in vivo/physical adsorption	↑ HUVEC adhesion (only REDV)	Aubin et al.[53]
RGD + YIGSR	Mixture of peptides	CoCr/covalent	↑ Expression of adhesion, vascularization and anti-thrombogenic genes on HUVEC	Castellanos et al.[83]

Abbreviations: ALP, alkaline phosphatase; BIC, bone-to-implant contact; CoCr, cobalt-chrome alloy; dAoGs, decellularized aortic grafts; dPVCs, decellularized ovine pulmonary heart valve cusps; FA, focal adhesions; hMSCs, human mesenchymal stem cells; hMVEC, human microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells; ITGA5, integrin subunit alpha 5 integrin gene; MAPK, mitogen-activated protein kinase; MC3T3-E1, murine preosteoblast cell line; MG63, hypotriploid human cell line; NHOsts, normal human osteoblasts; NIPAAm-co-AAc, N-isopropylacrylamide (NIPAAm) and acrylic acid (AAc); OCN, osteocalcin; PCLLC, polycaprolactone-co-lactide; PEG, poly(ethylene glycol); PGE2, prostaglandin E2; RCO, rat calvaria osteoblasts; REF WT, rat embryonic fibroblasts, wild type; Runx2, Runt-related transcription factor 2; Saos-2, human osteosarcoma cell line; TCPS, tissue culture polystyrene; TGF-β1, transforming growth factor beta 1; Ti, titanium.

TABLE 2 Combination of peptides to mimic ECM microenvironment

Biofunctional motifs	System of presentation	Substrate/ immobilization	Main biological effect	References
RGD + BMP-2 (KIPKASSVPTELSAISTLYL)[a]	Mixture of peptides	PLEOF hydrogel/ covalent	↑ rBMSC growth, ALP activity, mineralization	He et al.[84]
RGD + BMP-2 (KIPKASSVPTELSAISTLYL)	Mixture of peptides	Glass/covalent	↑ hBMSC density and Runx2 and BSP gene expression	Moore et al.[85]
RGD + BMP-2 (KIPKASSVPTELSAISTLYL)	Mixture of peptides	TCPS/physical adsorption	↓ hMSC ALP activity, mineralization; = hMSC Runx2, ALP and Coll-IA gene expression	Kim et al.[86]
RGD ((K)$_{16}$GRGDSPC) + BMP-2 (KIPKASSVPTELSAISTLYL)	Mixture of peptides	PLGA-[Asp-PEG]$_n$ scaffold + HA/ chemisorption	↑ rBMSC adhesion, proliferation, ALP activity, osteogenic expression	Pan et al.[87]
RGD (GRGDS) + BMP-2 (KIPKASSVPTELSAISTLYL)	Mixture of peptides	Glass/covalent	↑ hMSC proliferation and Runx2 and BSP gene expression	Ma et al.[88]
RGD + BMP-2 (KIPKASSVPTELSAISTLYL) + OPN (SVVYGLR)	Mixture of peptides	PLEOF hydrogel/ covalent	↑ rBMSC growth, ALP activity, mineralization and OPN, OCN, ON gene expression	He et al.[89]
RGD + BMP-2 (KIPKASSVPTELSAISMLYL)	Mixture of peptides	Glass/covalent	↑ hBMSC Runx2 expression; ↓ hBMSC Stro1 expression	Bilem et al.[90]
RGD + BMP-2 (KIPKASSVPTELSAISMLYL)	Mixture of peptides (micropatterning)	Glass/covalent	↑ hMSC ALP activity and Runx2 and OPN expression; ↓ hMSC Stro1 expression	Bilem et al.[91]
RGD + BMP-2 (KIPKASSVPTELSAISMLYL) or OGP (YGFGG)	Mixture of peptides	PET/covalent	↑ hMSC Coll-IA and ALP gene expression	Padiolleau et al.[92]
RGD + BMP-2 (KIPKASSVPTELSAISMLYL) or OGP (YGFGG)	Mixture of peptides (micropatterning)	PET/covalent	↑ hMSC osteogenic gene expression	Padiolleau et al.[93]
RGD + OGP (YGFGG)	Mixture of peptides	Ti/chemisorption	↑ hMSC ALP activity, mineralization and osteogenic gene expression; ↑ bone formation in vivo	Pan et al.[94]
RGD (GGRGDSP) + FGF-2 (GGGKRTGQYKL)	Mixture of peptides	Gold/covalent	↑ hMSC adhesion, ALP activity and OPN gene expression	Hudalla et al.[95]
RGD (RGDSP) + FGF-2 (TYRSRKY)	Mixture of peptides	Gold/covalent	↑ hMSC spreading	Hudalla et al.[96]
RGD (KGGPQVTRGDVFTMP) + BMP-7 (KGGQGFSYPYKAVFSTQ)	Mixture of peptides	TCPS/chemisorption	↑ ALP activity, protein expression, osteogenic gene expression, mineralization of hESCs and hiPSCs	Wang et al.[97] and Deng et al.[98]
Laminin (PPFLMLLKGSTRFC) + ameloblastin (VPIMDFADPQFPT)	Mixture of peptides	Ti/covalent	↑ TERT-2/OKF-6 proliferation and hemidesmosome formation	Koidou et al.[99]
BMP-2 + OCN [KIPKASSVPTELSAISTLYLAAAAγEPRRγEVAγEL]	Linear fusion peptide	HA/ionic	↑ hMSC ALP activity, mineralization and BMP-2 and OCN gene expression	Lee et al.[100]
RGD + Collagen [H$_2$(POG)$_4$PK(Byp) G (POG)$_4$GGRGDS]	Linear fusion peptide	CMP scaffold/ionic	↑ spheroid formation of MCF10A	Hernandez-Gordillo et al.[101]

TABLE 2 (Continued)

Biofunctional motifs	System of presentation	Substrate/ immobilization	Main biological effect	References
RGD (CGGGRGDS) + AG73 (CGGGRKRLQVQLSIRT)	Branched peptide	MPP nanoclusters/ covalent	↑ HUVEC adhesion and spreading, FA and endothelialization	Karimi et al.[102]
RGD + BMP-2 (DWIVA)	Branched peptide	Glass/chemisorption	↑ C2C12 adhesion and spreading, osteogenic transdifferentiation and p38 protein expression	Oliver-Cervelló et al.[58]
Cyclic RGD + Laminin (SIKVAV)	Branched peptide	Ti/chemisorption	↑ HUVEC adhesion, proliferation and angiogenesis	Clauder et al.[103]

Abbreviations: ALP, alkaline phosphatase; BMP-2, bone morphogenetic protein 2; BSP, bone sialoprotein; C2C12, mouse myoblast cell line; CMP, collagen mimetic peptide; Coll-IA, collagen type I alpha 1; FA, focal adhesions; HA, hydroxyapatite; hESCs, human embryonic stem cells; hiPSCs, human induced pluripotent stem cells; hMSC, human mesenchymal stem cells; HUVEC, human umbilical vein endothelial cells; MCF10A, human non-tumorigenic epithelial cells; MPP, terpolymer of methyl methacrylate/PEG-methacrylate/PEG-methacrylate-norbornene; OCN, osteocalcin; ON, osteonectin; OPN, osteopontin; PET, poly(ethylene terephthalate); PLEOF, poly(lactide-co-ethylene oxide-co-fumarate); PLGA-[Asp-PEG]n, poly(lactide-co-glycolide)-aspartic acid-poly (ethylene glycol); rBMSC, bone marrow stromal cells; Runx2, Runt-related transcription factor 2; Stro1, stem cell marker; TCPS, tissue culture polystyrene; TERT-2/OKF-6, immortalized human oral keratinocyte cells; Ti, titanium.

aThe underlined amino acids represent mutations from the BMP-2 original sequence KIPKACCVPTELSAISMLYL.

laminin-derived YIGSR peptides (Table 1),[53,82] aiming in both cases at improving the adhesion and proliferation of endothelial cells over other cell types (e.g., fibroblasts, platelets, or smooth muscle cells), a prerequisite for ensuring re-endothelialization of stents and vascular grafts. Similarly, heparin-binding peptides, such as KRSR and FHRRIKA, which mimic the interactions between cells and heparan sulfate proteoglycans (HSPGs), have been also used together with RGD to improve osteoblast adhesion and osteogenic differentiation (Table 1).[112,113] For example, nanofibrillar hydrogel scaffolds presenting the combination of KRSR and RGD sequences improved the spreading and proliferation of osteoblastic cells compared with the use of the individual peptides as cotronls.[79] Nonetheless, the co-presentation of RGD with either KRSR or FHRRIKA has shown conflicting results in other studies. For instance, this strategy proved inefficient to significantly enhance MSC adhesion on hydroxyapatite surfaces relative to RGD alone.[75] In addition, the functionalization of titanium with RGD and KRSR mixtures inhibited osteoblast differentiation,[78] and failed to improve bone formation in vivo.[77] The presence of these two peptides on silk nanofibrous mats did not show either synergistic effects on the adhesion and proliferation of osteoblasts.[76]

The discrepancies observed in the previous examples highlight that the biomaterial's nature, and the ratio and spatial disposition of the peptides play crucial roles in the biological performance of the functionalized surfaces. In this regard, the non-homogeneous distribution of RGD and heparin binding peptides on the surfaces, as achieved by using peptide mixtures, is likely responsible for the often varying and even contradictory biological outcomes reported in the literature.

Recreating the ECM microenvironment has also been a major goal to improve cell-material interactions. In particular, the combination of integrin-binding ligands (mainly RGD) together with GF-derived peptides is a promising approach to induce integrin/GF signaling, orchestrating cell fate without the need of using GFs.[85,90] For instance, in the field of bone tissue engineering, the use of RGD in combination with BMP-2-derived sequences has been widely investigated (Table 2).[90,114,115] BMP-2 comprises two binding epitopes, namely, the knuckle epitope, which mainly interacts with BMP receptor type II (BMPR-II), and the wrist epitope that binds to BMPR-I with high affinity.[116,117] Both epitopes have been used to functionalize biomaterials.

In this regard, Durrieu's group has extensively studied the biological effects of combining RGD and a peptide derived from the knuckle epitope of BMP-2 (RKIPKASSVPTELSAISMLYL). Both sequences were covalently attached on glass substrates in 1:1 molar ratio. Biological results revealed a decrease in the expression of the stemness marker STRO-1 on the substrates functionalized with RGD and the BMP-2 mimetic peptide, together with a higher expression of the osteogenic RUNX-2 marker, which indicated the differentiation of human MSCs into the osteogenic lineage (Figure 3).[90] Similarly, by coating poly (ethylene terephthalate) (PET) surfaces with these peptides, it was demonstrated the ability of the motifs to synergistically enhance human MSCs differentiation, as highlighted by the overexpression of ALP and Coll-IA genes.[92]

Based on the same rationale, Madl et al. compared the osteogenic capacity of the knuckle- and wrist-BMP-2-derived peptides (RKIPKASSVPTELSAISTLYL and DWIVA, respectively) in combination with RGD on alginate hydrogels. When functionalizing hydrogels with the knuckle-derived peptide and RGD, Smad signaling, upregulation of ALP and osteopontin production as well as an enhancement of mineral deposition were observed. However, such improvements were not observed when combining RGD with the wrist-derived peptide,

TABLE 3 Combination of peptides to prevent bacterial adhesion without loosening cell adhesive properties

Biofunctional motifs	System of presentation	Substrate/immobilization	Main biological effect	References
RGD + HHC36 (KRWWKWWRR)	Mixture of peptides	Ti/covalent	↓ S. aureus and E. coli adhesion; ↑ BMSC adhesion	Lin et al.[104]
RGD (GCGYGRGDSPG) + AMP (ILPWRWPWWPWRR)	Mixture of peptides	Silicon/physical adsorption	↓ S. aureus, S. epidermidis and P. aeruginosa adhesion; ↑ FB adhesion	Muszanska et al.[105]
P15 (GTPGPQGIAGQRGVV) + CSP (SGSLSTFFRLFNRSFTQALGK)	Linear fusion peptide	Hydrophilic and hydrophobic model substrates/physical adsorption	↓ S. mutans biofilm formation; ↑ MSC adhesion and mineralization	Li et al.[106]
RGD + Phe(4-F)	Linear fusion peptide	Ti/chemisorption	↓ E. coli adhesion; ↑ CHO-K1 and Saos-2 adhesion	Yuran et al.[107]
RGD + LF1-11 (GRRRRSVQWCA)	Branched peptide	Ti/covalent	↓ S. aureus and S. sanguinis adhesion; ↑ Saos-2 adhesion, proliferation and mineralization	Hoyos-Nogués et al.[60]
Cyclic RGD + LF1-11 (GRRRRSVQWCA)	Branched peptide	Ti/chemisorption	↓ S. aureus adhesion; ↑ MSC adhesion	Martin-Gómez et al.[108]
QK (IGKYKLQYLEQWTLK) + AMP (KRWWKWWRR)	Branched peptide	Ti/covalent	↓ S. aureus, E. coli, P. aeruginosa and MRSA adhesion; ↑ proliferation and gene expression of HUVEC and BMSC; In vivo: ↓S. aureus and ↑ vascularization and osseointegration	Chen et al.[109]
GL13K (GKIIKLKASLKLL) + LamLG3 (KKGGGPPFLMLLKGSTRFC)	Mixture of peptides	Ti/covalent	↓ Streptococcus grodonii biofilm activity; ↑ proliferation and hemidesmosome formation of TERT-2/OKF-6	Fischer et al.[110]
GL13K + ELR [GKIIKLKASLKLLVLG$_{10}$LVG (VPGVGVPGKG (VGPVG)$_4$)$_8$VCC]	Linear fusion peptide	Gold/covalent	↓ S. epidermidis and S. aureus biofilm formation	Acosta et al.[111]

Abbreviations: BMSC, bone marrow stromal cells; CHO-K1, Chinese hamster ovary cells; E. coli, Escherichia coli; FB, fibroblast; HUVEC, human umbilical vein endothelial cells; MRSA, methicillin-resistant Staphylococcus aureus; MSC, mesenchymal stem cell; P., Pseudomonas; S., Staphylococcus; Saos-2, human osteosarcoma cell line; TERT-2/OKF-6, immortalized human oral keratinocyte cells; Ti, titanium.

which led the authors to conclude the non-osteogenic capacity of the DWIVA sequence.[118]

As discussed in Section 2.2, in addition to recreating the complexity of the ECM, biomaterials face other challenges. For example, the susceptibility of medical implants, especially metallic materials, for bacterial attachment and subsequent biofilm progression represents a major concern. In this regard, biomaterial-associated infections have been described as one of the primary causes leading to implant failure.[42] Hence, biomaterials capable of inhibiting bacterial colonization warrant special attention. AMPs stand out as promising candidates for such purpose.[119,120] These peptides are normally cationic and amphipathic in nature, and interact with bacterial membranes causing their disruption. This mechanism neither affects eukaryotic cell membranes nor promotes bacterial resistance, offering clear advantages compared to other antibacterial approaches such as the use of inorganic compounds or antibiotics.[121–124]

The combination of cell adhesive peptides with AMPs thus offers excellent opportunities to develop multifunctional biomaterials

(Table 3). Such approach resulted in the inhibition of S. aureus and E. coli attachment on titanium surfaces coated with a 1:1 mixture of RGD and the AMP HHC36. Of note, in addition to remarkable antibacterial effects, the presence of RGD supported the adhesion of bone marrow stromal cells.[104] In an alternative approach, the anti-adhesive polymer Pluronic F127 (PF127) was modified with either RGD (PF127-RGD) or a 13-mer AMP (PF127-AMP). Subsequently, silicone rubber sheets were functionalized with different mixtures of the polymer-peptide conjugates yielding antibacterial and cell adhesive properties.[105] AMPs have been also combined with laminin-derived peptides to avoid peri-implant infections of dental implants. For example, Fischer et al. engineered a multifunctional biomaterial by co-immobilizing the antimicrobial peptide GL13K—derived from the parotid secretory protein—and the laminin-derived peptide LamLG3 on titanium surfaces. Such functionalized surfaces decreased biofilm activity of Streptococcus gordonii and improved proliferation and hemidesmosome formation of keratinocytes, without affecting gingival fibroblasts.[110]
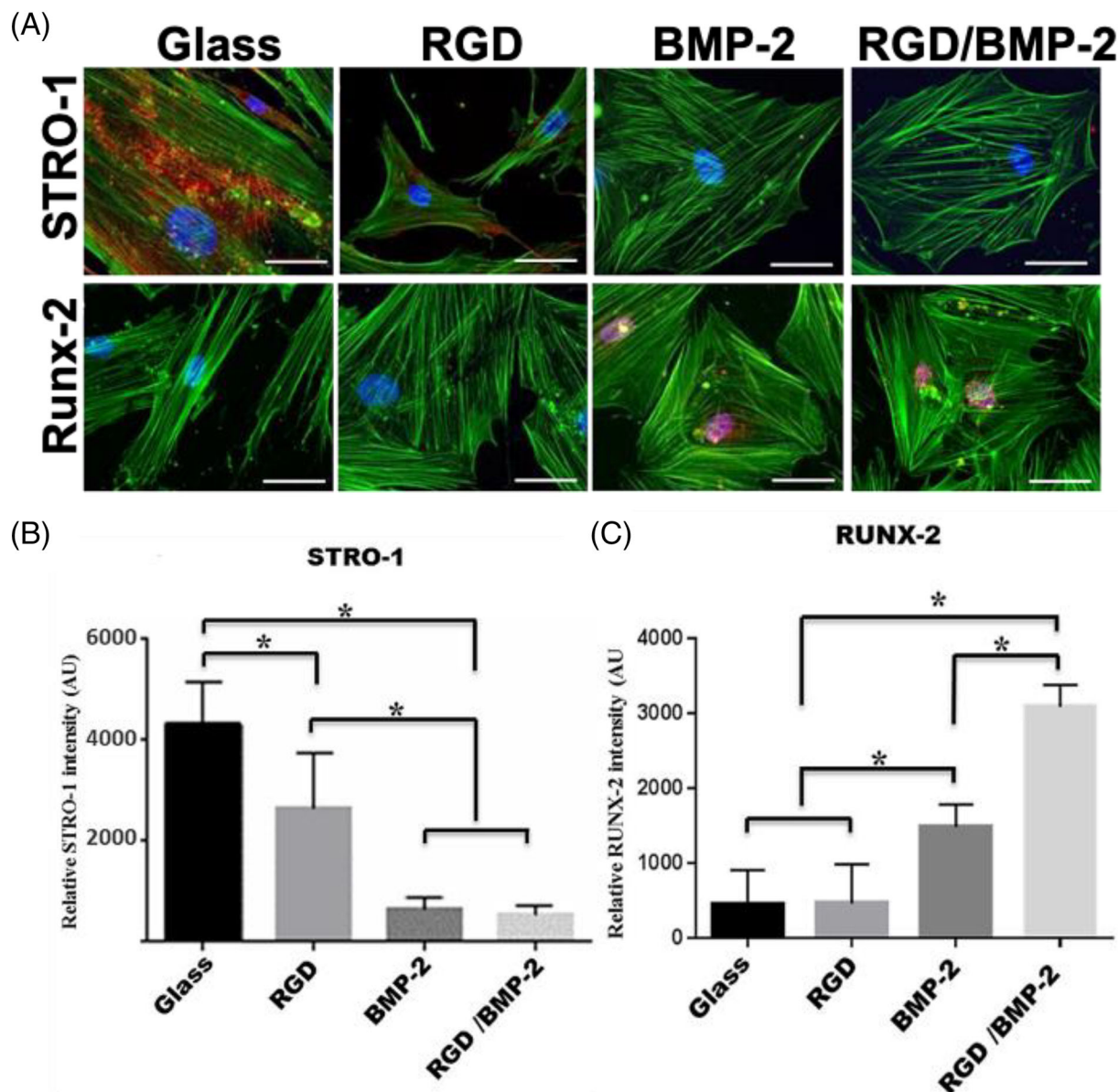
FIGURE 3 Example of peptide mixture to mimic ECM. (A) Immunostaining of STRO-1 and Runx-2 markers on human MSCs (scale bars = 50 μm). (B) STRO-1 gene expression. (C) Runx-2 gene expression. Adapted from Bilem et al.[90]

2.2.2 | Linear and fusion peptides

Although the use of peptide mixtures to functionalize biomaterials is a straightforward and simple approach, the aforementioned limitations associated to this procedure make it necessary to find alternatives. A possible solution is the use of oligopeptides, in which two or more peptide sequences are found in the same peptidic backbone, allowing to chemically control the presentation of the peptides in well-defined ratios. This strategy thus ensures a homogenous distribution of the bioactive peptides on a biomaterial surface. Such degree of geometrical control is normally not attainable if mixtures of individual peptides are used instead.

This strategy has been widely employed to improve the selectivity of peptides for cell expressed receptors at the surface level (Table 1).

For example, Kim et al. developed oligopeptides containing RGD and PHSRN sequences separated with different glycine residues to promote synergistic $\alpha5\beta1$-signaling. By physically adsorbing the active sequences on tissue culture polystyrene (TCPS) substrates, an enhancement in cell adhesion, spreading and MAPK signaling was observed. Of note, these effects were highly dependent on both the concentration of the coatings and the spacing between the two peptides, being the 6-glycine linker the one that performed the best.[65] Following a similar approach, Benoit et al. engineered PEG hydrogels incorporating oligopeptides made of RGD and PHSRN with a 13-glycine spacer aiming at mimicking the 30–40 Å distance at which the two peptides are separated in fibronectin (Figure 4A). Such configuration enhanced osteoblast adhesion, spreading and focal adhesion in comparison with RGD alone, as well as proliferation and ALP
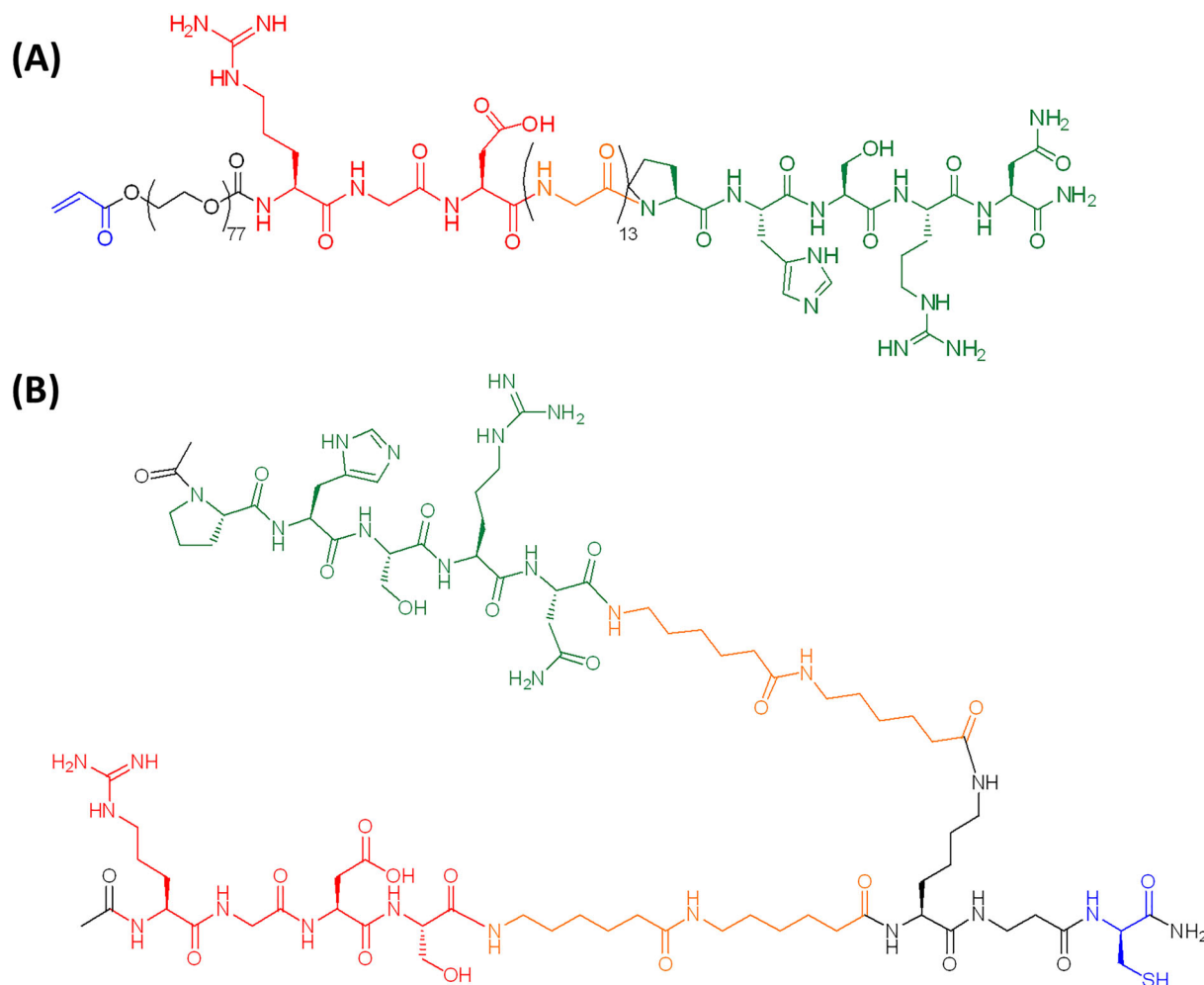
FIGURE 4 Representative multifunctional peptides to improve receptor selectivity. (A) Linear oligopeptide combining the RGD and PHSRN sequences.[66] (B) Branched peptide combining the RGD and PHSRN sequences.[67] Active sequences represented in red and green colors, spacers in yellow and anchorage units in blue

production.[66] Nonetheless, these results are in disagreement with the previous study, in which a 12-glycine spacing did not promote synergistic effects. These differences may arise from the distinct substrates used and also suggest that besides the relative distance between the bioactive sequences, their spatial disposition (i.e., their presentation and accessibility) are important to achieve synergistic signaling. Similar behaviors are observed when two different receptors are targeted, such as integrins and HSPGs. In this case, for instance, using linear peptides of RGD and KRSR separated by two glycine units did not induce any improvement of cellular behavior in comparison with control RGD.[80]

Mimicking the ECM has also been addressed by using linear fusion peptides (Table 2). To this end, different regions of proteins from the ECM are incorporated into a linear peptide to target more than one biological function. For example, the combination of the knuckle BMP-2-derived peptide with an osteocalcin-inspired peptide has been use to functionalize hydroxyapatite substrates.[100] In this way, the osteocalcin peptide was able to mimic the native hydroxyapatite-binding affinity of the full protein, while the BMP-2

peptide provided the biomaterial with higher osteodifferentiation potential, as demonstrated by an increase in ALP activity, mineralization and overexpression of BMP-2 and osteocalcin genes, both osteogenic markers. Following a different approach, Hernandez-Gordillo et al. engineered three collagen mimetic peptides containing the RGD cell adhesive motif covalently bond to the collagen sequences (Figure 5A). Such peptides assembled into 3D scaffolds under the influence of metal ions and had the ability to be easily modified with GFs. Indeed, the addition of epidermal growth factor (EGF) in the presence of the RGD sequence triggered the formation of spheroids of epithelial cells, thereby indicating the potential of these peptidic scaffolds to support growth of tissues for regenerative medicine as well as to build in vitro models to study tissue development (Figure 6).[101]

Oligomeric peptides combining cell instructive sequences and AMPs have also been explored (Table 3). In this regard, Li et al. described a fusion peptide combining P15, an osteogenic peptide derived from collagen type I, and the AMP competence-stimulating peptide (CSP), a quorum sensing peptide produced by *S. mutans*. This
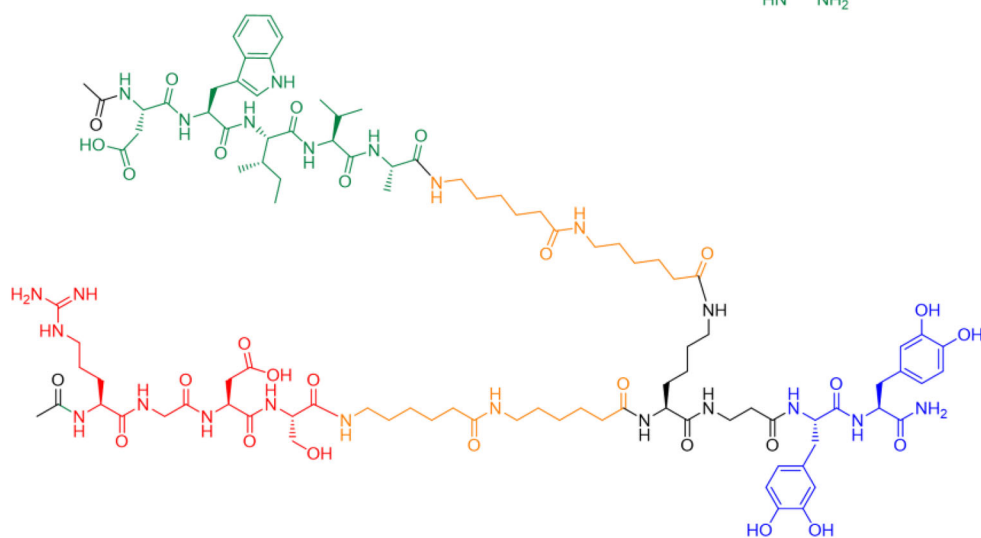
(A)
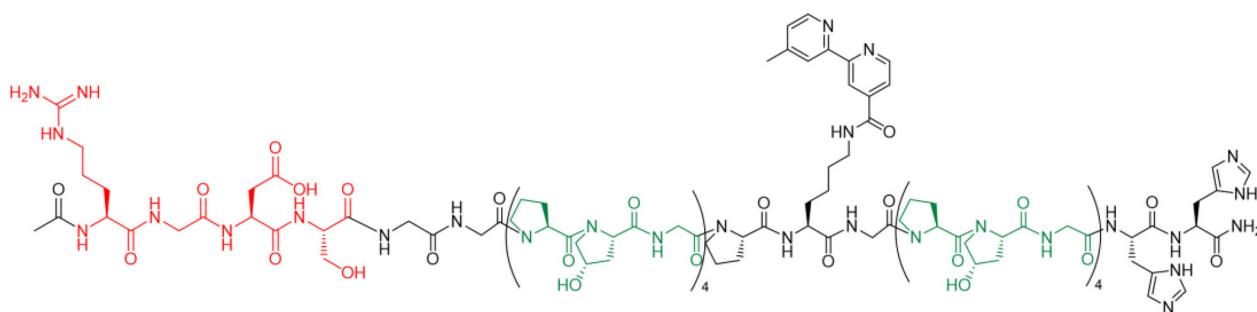
(B)
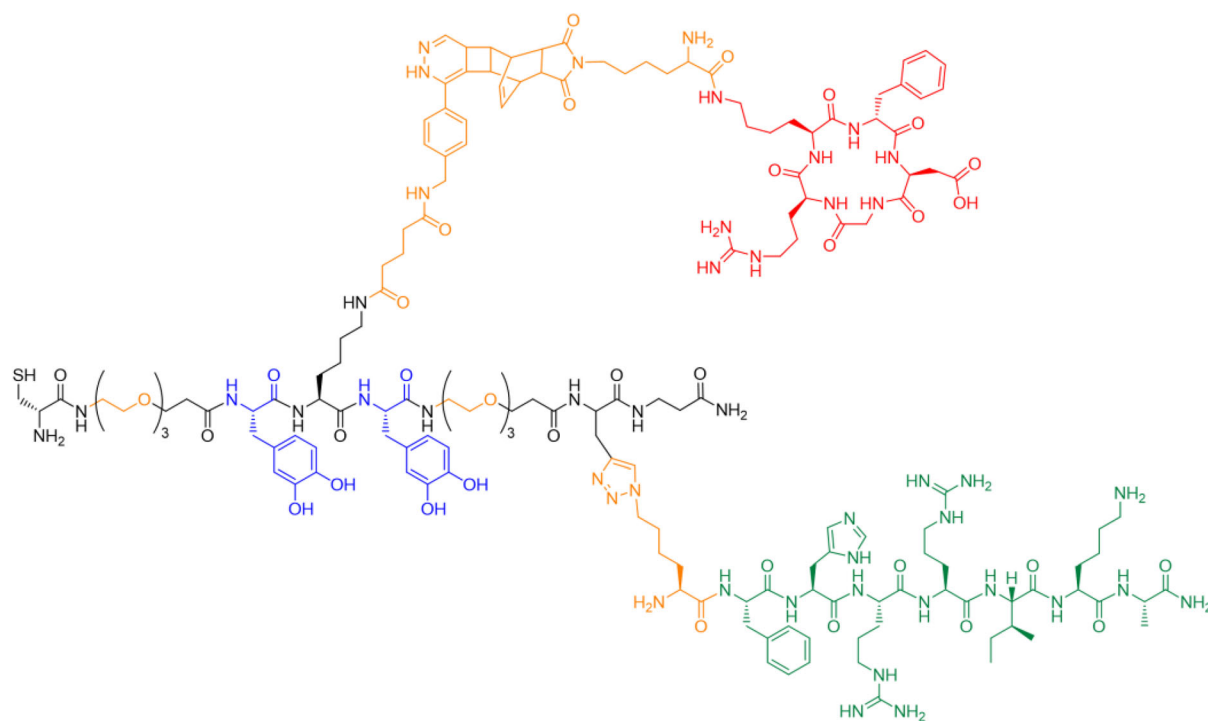
(C)

FIGURE 5 Representative multifunctional peptides to mimic the ECM. (A) Linear oligopeptide combining a type I collagen mimetic with the RGD sequence.[101] (B) Branched peptide incorporating the cyclic RGD with the FHRRIKA motifs.[72] (C) Branched peptide combining the RGD and DWIVA sequences.[58] Active sequences represented in red and green colors, spacers in yellow and anchorage units in blue
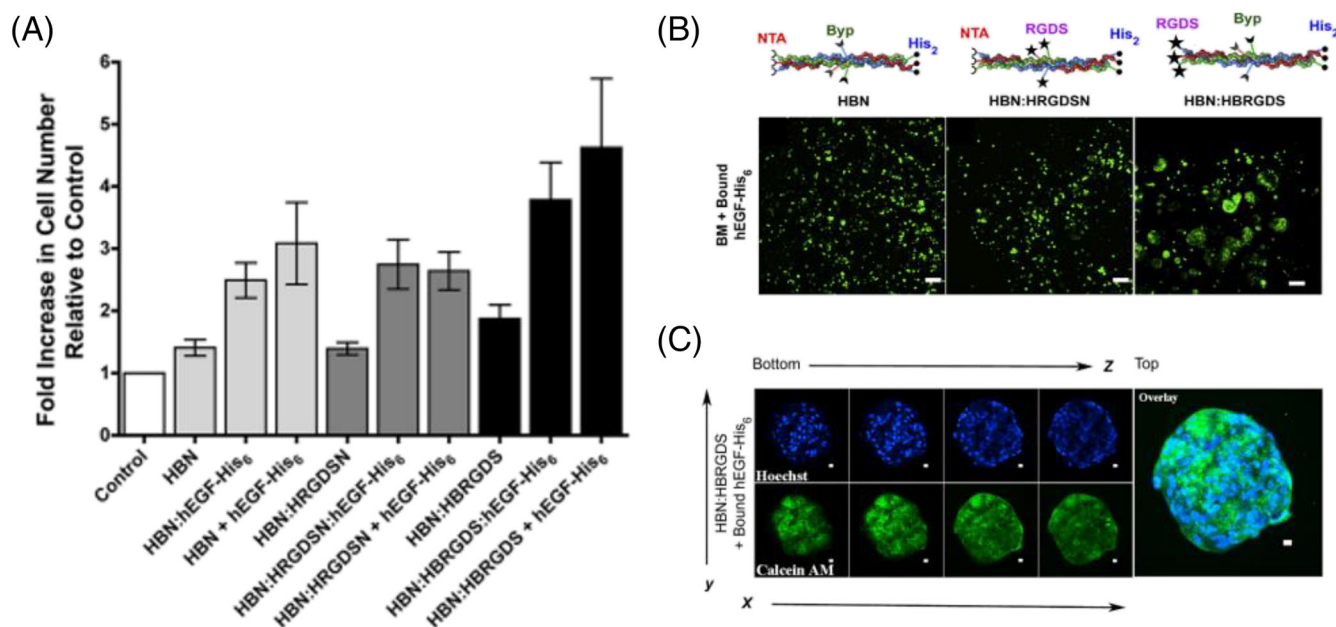
FIGURE 6 Example of oligopeptides to mimic ECM. (A) Number of human non-tumorigenic epithelial MCF10A cells on different scaffolds composed of collagen mimetic peptides in combination with RGD and/or EGF. (B) MCF10A cells encapsulated in three different scaffolds. (C) Organization of MCF10A cells in spheroids in HBN:HGRGDS hydrogels. Adapted from Hernandez-Gordillo and Chmielewski[101]

peptide showed positive effects in reducing *S. mutans* biofilm formation and promoting MSC adhesion and mineralization.[106] However, the peptide was coated on the surfaces by simple physisorption and the biological effects were highly dependent on the chemistry of the surface used. An alternative strategy has been recently reported by the group of Meital Reches. She designed a modular multifunctional peptide containing the cell adhesive RGD peptide, two self-assembling and bacterial resistant units of fluorinated phenylalanine (Phe(4-F)), and L-3,4-dihydroxyphenylalanine (DOPA) to assist anchoring of the peptide to titanium[125] (Figure 7A).[107] This minimalistic approach proved useful to reduce the attachment of *E. coli* and to enhance the adhesion of CHO and Saos-2 cells. This peptide has been used in further studies to functionalize other types of materials.[126,127] Following a different approach, Acosta et al. designed a multifunctional fusion peptide by combining the GL13K peptide, with high antibiofilm activity, together with an elastin-like recombinamer (ELR), with low-fouling activity. The capacity of the GL13K peptide to reduce biofilm formation was synergistically enhanced when combined with the antifouling potential of the ELR.[111]

2.2.3 | Branched peptides

As we have discussed in the previous sections, controlling the spatial distancing between different bioactive sequences is paramount to achieve enhanced biological profiles. Such spacing may be controlled, to a certain extent, by using linkers of different length; however, using linear peptides it is often difficult to present the active sequences with an optimal conformation that is fully accessible for the cells.

Alternatively, branched peptides might offer a better geometrical control and orientation of the bioactive motifs, consequently improving the biological performance of the biomaterial.

Fraioli et al. functionalized titanium disks by covalently grafting a branched peptide containing the two synergistic sequences RGD and PHSRN. Each motif was incorporated on a different arm of a peptidic platform, which also contained aminohexanoic acid residues as spacers, and a lysine as a branching unit. A cysteine at the *C*-terminus served as anchoring point (Figure 4B).[67] Peptide-modified titanium surfaces supported human MSCs adhesion with formation of focal contacts. Furthermore, the branched molecule was able to trigger overexpression of integrin $\alpha 5\beta 1$ and Runx2, which was not achieved when both sequences were randomly distributed on the surface (Figure 8). An increase of calcium deposits was also observed and in vivo results revealed new bone formation in a rat calvarial defect (Table 1).[68]

Heparin-binding peptides have been also combined with RGD using a branched strategy (Table 1). For instance, Beck-Sickinger's group studied the synergistic effects exerted on osteoblasts by combining cyclic RGD and the FHRRIKA sequence. To this end, the two sequences were incorporated on a mussel derived peptide, containing DOPA, which binds with high affinity to metallic oxides[125] (Figure 5B). The conjugation of both peptides in one branched molecule enhanced the adhesion, spreading and proliferation of osteoblast-like cells, as well as the formation of focal adhesions.[72] More recently, they installed the same kind of branched peptides on polycaprolactone-co-lactide (PCLLC) scaffolds. The heparin binding peptide induced the immobilization of heparin, which in turn recruited the wound-healing C-X-C motif chemokine ligand 12 (CXCL12),
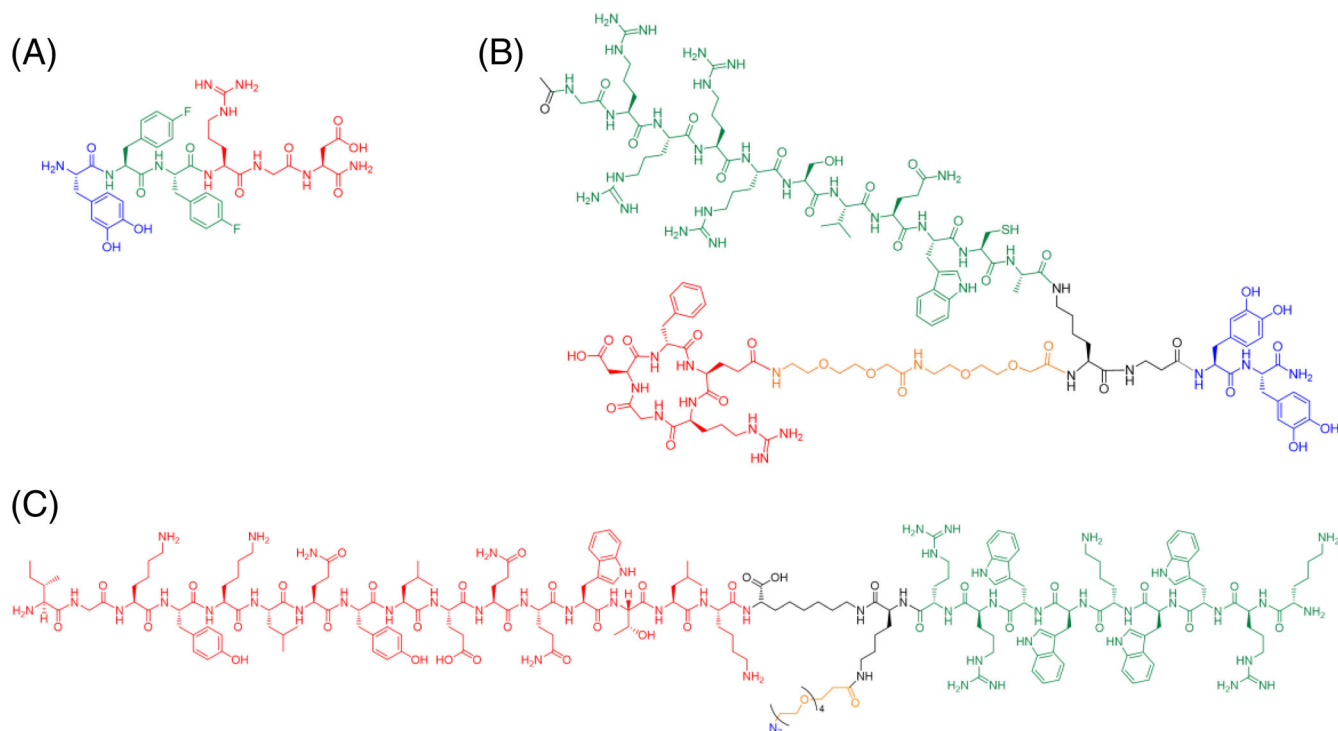
FIGURE 7 Representative multifunctional peptides combining cell adhesive and antibacterial properties. (A) Linear peptide incorporating the RGD sequence, two units of Phe(4-F) and 1 DOPA residue.[107] (B) Branched peptide combining the cyclic RGD and LF1–11 sequences.[108] (C) Branched peptide incorporating the HHC36 and QK motifs.[109] Active sequences represented in red and green colors, spacers in yellow and anchorage units in blue
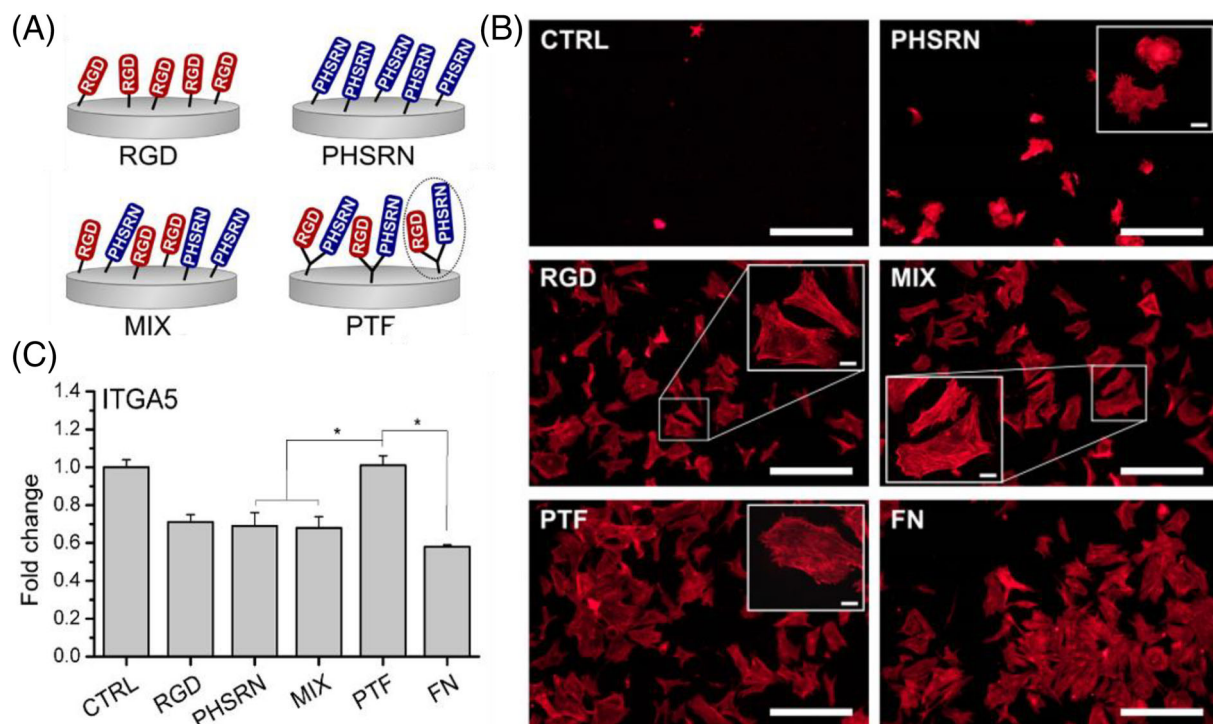

FIGURE 8 Example of branched peptide to improve receptor selectivity. (A) Schematic representation of the different peptide conditions. (B) F-actin immunostaining (scale bar = 500 μm, scale bar = 50 μm in the insets). (C) ITGA5 gene expression. Adapted from[68]

fibroblast growth factor (FGF-2) and VEGF. Such loaded systems with cytokines improved human umbilical vein endothelial cell (HUVEC) adhesion and stimulated cell survival and differentiation, which was boosted by the delivery of CXCL12 and VEGF. These results led the authors to suggest a cooperative effect between integrins and cytokines, highlighting the crosstalk between adhesion ligands, proteoglycans and signaling molecules of the ECM (Figure 9).[73] In addition, integrin and proteoglycan binding has also been mimicked by combining the RGD and KRSR sequences within a branched platform, which was covalently bound to titanium substrates. Although such system did not show a synergistic effect on osteoblast-like cell adhesion or proliferation, it improved mineralization, an important late marker of osteodifferentiation.[81]

The ECM microenvironment has also been recreated with branched peptides to improve surface endothelialization (Table 2). In this regard, Karimi et al. developed polymeric nanoclusters containing RGD and a syndecan-4-binding peptide (AG73), which synergistically promoted HUVEC adhesion and spreading. In addition, both ligands were required to further improve focal adhesion formation, endothelialization and to drive cell morphological changes under laminar shear flow, which demonstrated the importance of controlling the distance between integrin and syndecan receptors to optimally tune cell-material interactions as well as mechanotrasduction.[102] In another study, Clauder et al. co-installed the laminin-derived peptide SIKVAV and cyclic RGD on a branched structure to functionalize titanium

plates through DOPA interactions. The geometrically defined presentation of both peptides, synergistically enhanced adhesion, proliferation and angiogenesis of endothelial cells. Importantly, the peptidic coatings were hemocompatible and neither induced hemolysis nor platelet adhesion.[103]

In bone tissue engineering, the replacement of BMP-2 by peptidic analogues has been a focus of intensive research. Recently, we have engineered a biomimetic multifunctional peptide containing the RGD and the DWIVA motifs in a spatially defined geometry and two DOPA molecules to anchor the molecule to model glass substrates (Figure 5C).[58] The biomimetic interface significantly increased C2C12 adhesion and spreading, and inhibited myotube formation, a well-known indicator of BMP-2 activity.[128] Moreover, activation of BMP-dependent signaling via p38 was observed. Interestingly, these effects were not observed on surfaces displaying only one bioactive motif, a mixture of both peptides or soluble DWIVA, demonstrating the potential of the branched molecule to transdifferentiate the C2C12 cells towards the osteogenic lineage (Table 2, Figure 10). Although this strategy was validated on glass substrates, it could be easily applied to more relevant biomaterials, like titanium, due to the well-defined affinity of DOPA to metallic oxides.[125]

Branched peptides have also been successfully used to combine cell adhesive and antibacterial effects (Table 3). Hoyos-Nogués et al. described a peptidic branched platform containing the RGD sequence and the AMP derived from lactoferrin LF1-11.[60]
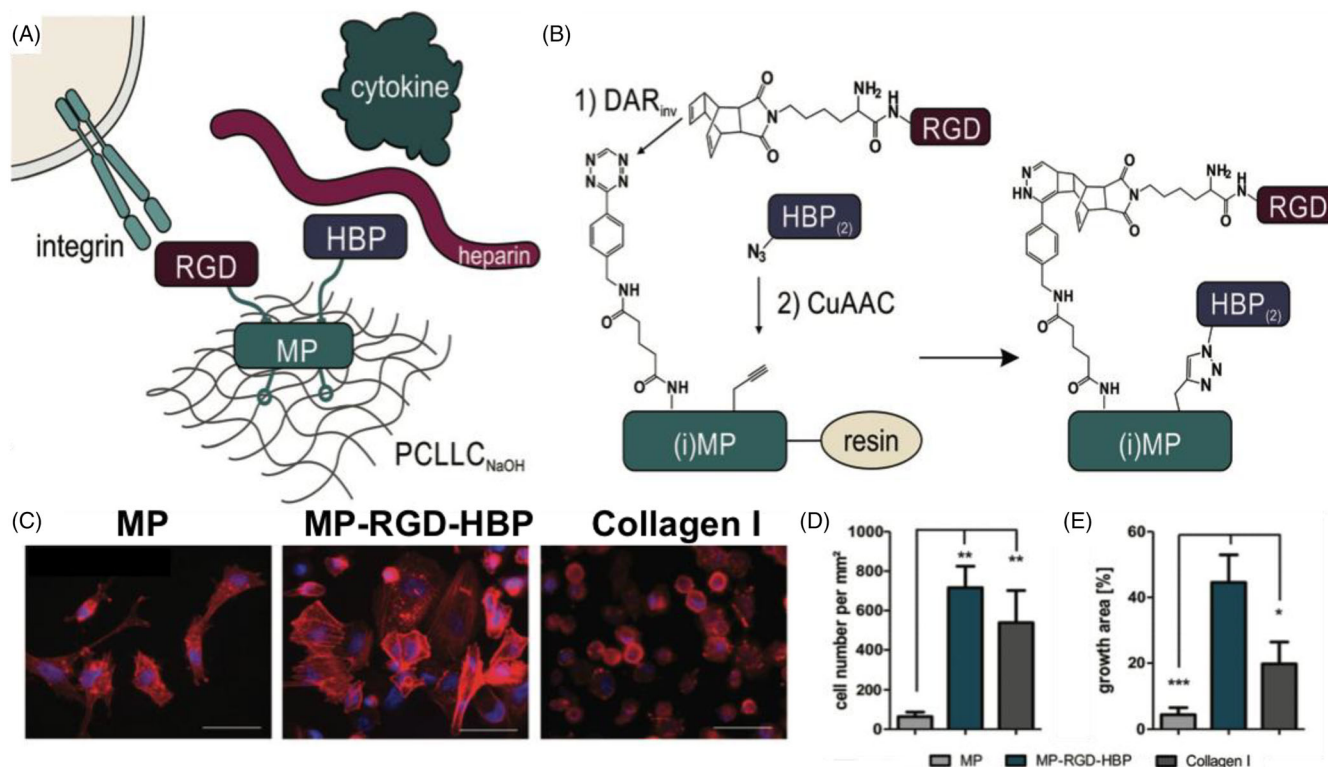

FIGURE 9 Example of branched peptide to mimic the ECM. (A) Schematic representation of the multifunctional peptide. (B) Schematic representation of the chemical synthesis of the peptide. (C) F-actin immunostaining of HUVECs in fluidic conditions (scale bar = 50 μm). (D) Cell number and (E) growth area of HUVECs in fluidic conditions. Adapted from Clauder et al.[73]
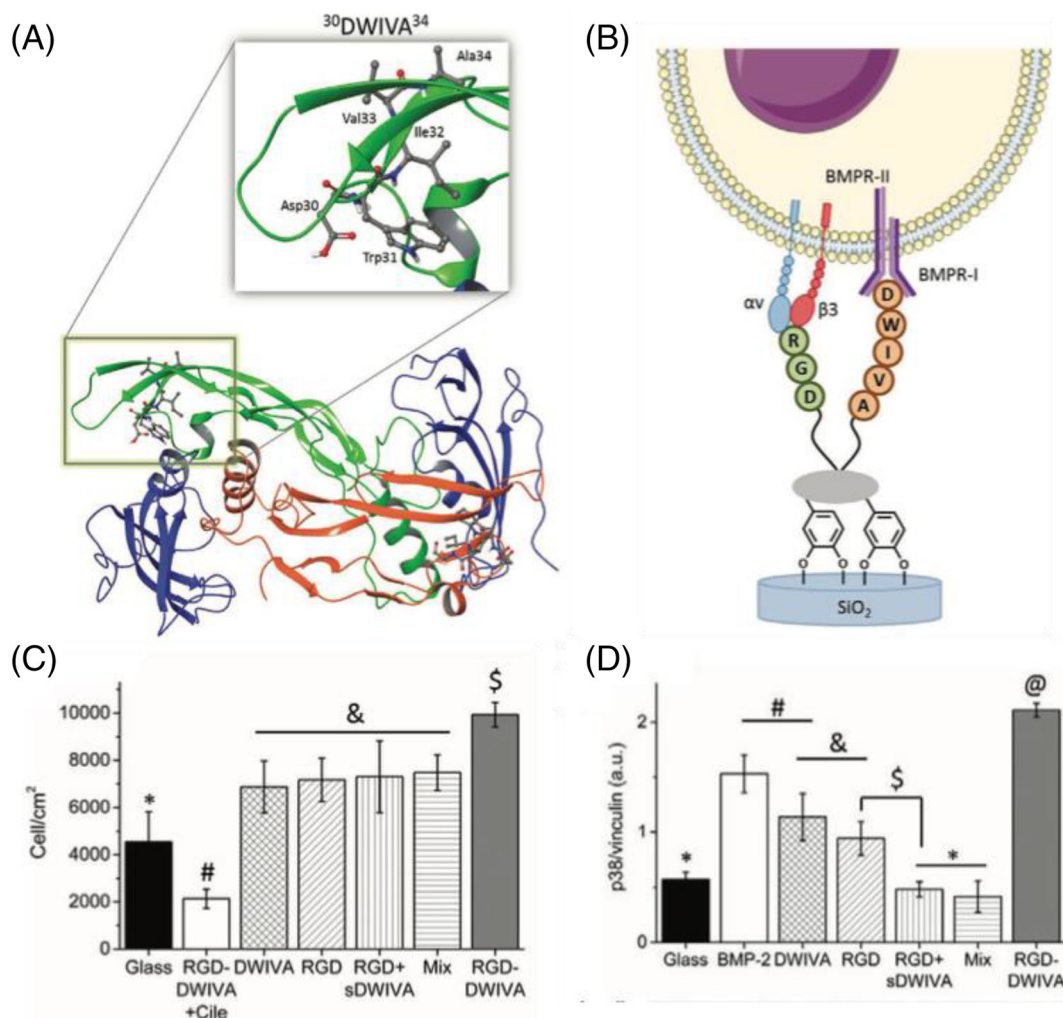
FIGURE 10 Example of a branched peptide to mimic the ECM. (A) BMP-2-BMPR-IA complex. The DWIVA sequence is highlighted. (B) Interaction of RGD-DWIVA with integrin and BMP-2 receptors. (C) Number of C2C12 cells adhered to the different substrates. (D) p38 protein expression obtained by western blot. Adapted from Oliver-Cervelló et al.[58]

Functionalization of titanium with this peptide enhanced the adhesion, proliferation, and differentiation of osteoblasts and efficiently reduced bacterial attachment and biofilm formation. Of note, this strategy was validated in co-culture studies in which titanium surfaces were pre-infected with bacteria. Indeed, infected surfaces abolished the adhesion of osteoblasts; in contrast, peptide-coated surfaces proved capable of killing bacteria and supported notable levels of cell adhesion and proliferation. In a subsequent study, the same peptide was grafted into polyethylene glycol (PEG), which is known for its anti-fouling potential. Such trifunctional coating (cell adhesive, bacterial repellent, and bactericidal) was applied to titanium and rendered surfaces highly antibacterial but with the capacity to support osteoblast adhesion.[59] To further optimize the biological potential of this peptide, a chemical peptide library of RGD-LF1-11 analogues was recently described.[108] In detail, the peptides were customized with two catechol groups (i.e., two DOPA units) to ensure their direct binding to titanium. Moreover, PEG linkers of different length were introduced to study the effect of

peptide accessibility on the cell adhesive and antibacterial activity (Figure 11). All designed analogues improved MSC adhesion and inhibited *S. aureus* adhesion, but, interestingly, the peptides displaying too long spacers showed reduced potency, thereby indicating the importance of properly exposing the peptide motifs and the fact that increased spacer length and/or flexibility above a certain threshold may be detrimental. In addition, replacing linear RGD by its cyclic counterpart (Figure 7B) further enhanced MSC adhesion while preserving excellent antimicrobial potential.[108]

Very recently, Chen et al. also engineered a multifunctional peptide to functionalize titanium, simultaneously achieving strong antibacterial effects and promoting vascularization and osseointegration in vivo.[109] In detail, the peptide contained the antimicrobial sequence HHC36 and the QK angiogenic peptide, derived from VEGF. The peptide incorporated an azide group in its backbone, which was used to assist the functionalization of titanium via sodium borohydride reduction promoted Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC-SB) (Figure 7C).
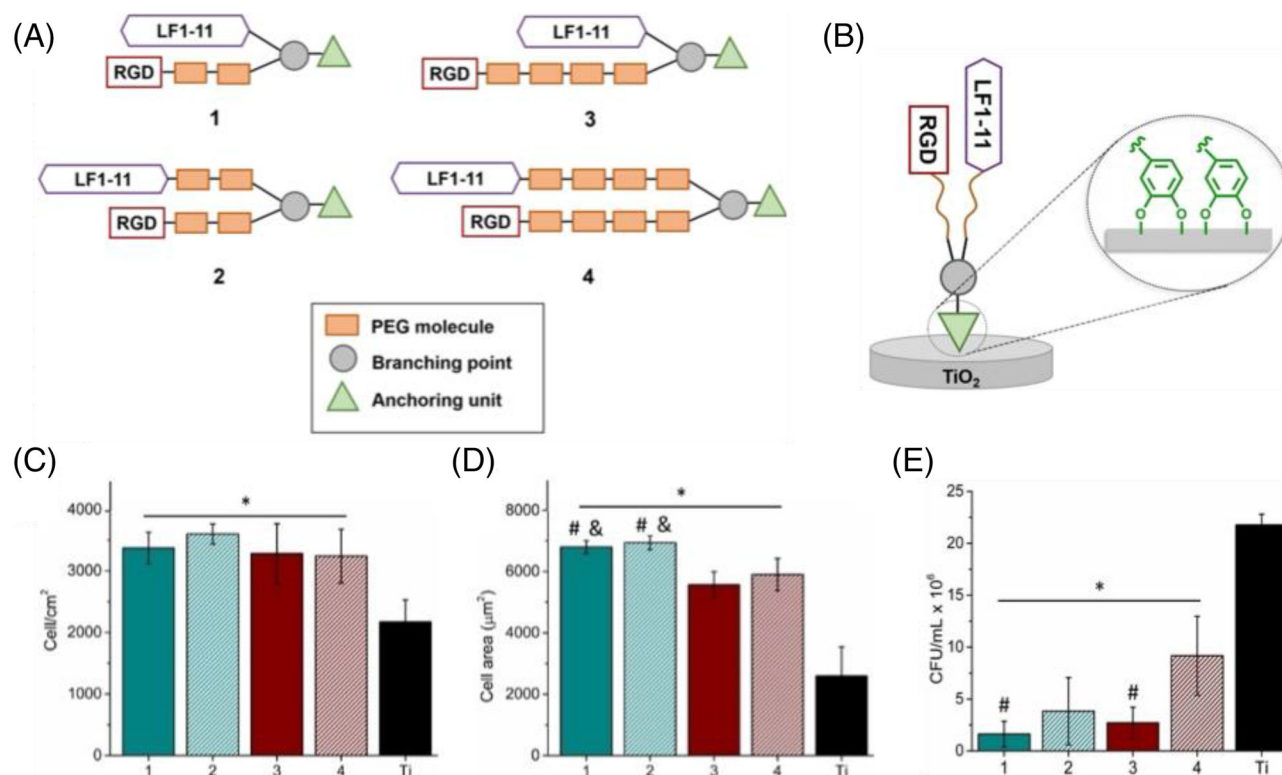
FIGURE 11 Example of branched peptide. (A) RGD-LF peptide analogues displaying PEG linkers of different length. (B) Catechol units are introduced to bind the molecules to titanium. Number (C) and spreading (D) of MSCs on the functionalized titanium surfaces. (E) Adhesion of *S. aureus* on the functionalized surfaces. Adapted from Martin-Gómez et al.[108]

2.3 | Conclusions and future perspectives

The use of peptides to increase the biological activity of biomaterials has gained increasing relevance over the last years. One important advantage of this strategy is that it overcomes the majority of drawbacks associated with the use of native proteins of the ECM. Nonetheless, no single peptide is known to be able to mimic the complex microenvironment and signaling of the ECM and the reported biological effects in vitro and in vivo are often reduced compared with full-length ECM proteins and GFs. Consequently, the combination of peptides of different nature is required to address more than one biological target, thus achieving multifunctional potential. In this regard, the functionalization of biomaterials with peptide mixtures has been studied. However, although such approach is simple and easy to apply, controlling the exact molar ratio and spatial distribution of the different bioactive molecules on the biomaterial surface may be complicated. These limitations may decrease the biological response at the surface level and compromise the feasibility of this strategy. Alternatively, linear oligopeptides or fusion peptides have allowed installing multiple peptides within the same peptidic backbone, thereby controlling the presence of the bioactive peptides at well-defined ratios and addressing the problems detected with peptide mixtures. However, in this case, the accessibility of the active sequences for interacting with cellular receptors may be not optimal due to the linear conformation of the scaffolds. This, in turn, may be translated into reduced biological outcomes. In recent years, the development of branched peptides with more sophisticated chemical structures represented an important step forward towards achieving multifunctional biomaterials. Branched configurations have demonstrated the necessity of co-presenting the bioactive sequences in a geometrically controlled manner in order to elicit favorable receptor signaling.

Besides the importance of regulating the spatial distribution and orientation of the peptides, the properties of the biomaterial surface, the peptide density and the method of immobilization used to functionalize the biomaterial are crucial factors that may also strongly influence the biological performance of peptide-material systems. Thus, the same peptidic sequences may elicit different (or even contradictory) biological results depending on the method of immobilization chosen. This has been highlighted throughout this review in several cases. For example, the combination of RGD and DWIVA sequences as a mixture failed to show a significant impact on osteo-differentiation, while the same peptides integrated in a branched system, effectively promoted synergistic biological effects. Such results stress the importance of controlling the spatial geometry and distance between bioactive peptides. Indeed, it is well reported that the co-localization of BMP-2 receptors and integrins is crucial to elicit an optimal integrin-GF signaling.

Another important aspect, which is often overlooked in the literature, is the nature and role of the linker units, which greatly influence the efficiency of the functionalization as well as the biological

performance of the anchored peptides. Thus, the chemical features of the linker, including its length, rigidity, conformation and hydrophilicity, may ultimately determine the biological outcome of the coatings. For instance, the linker length is known to be crucial for ensuring an adequate presentation and accessibility of the peptides for an optimal interaction with integrins, with too short or too long linkers failing to provide adequate values of cell adhesion.[10,23] The same principle has been shown to affect the antibacterial potential of AMPs.[108] In this regard, in a study by Liu et al. the use of a rigid linker, compared to a flexible one, significantly increased the adsorption of an AMP on titanium surfaces and improved its antibacterial activity.[129] Conversely, other studies have shown that more hydrophilic linker sequences enhanced the peptide solubility and their accessibility for cell receptors recognition.[94,130] Thus, the selection of an appropriate linker needs to be carefully considered when designing peptides to functionalize biomaterials.

Consequently, installing multifunctionality on biomaterials by using peptides in a chemically and geometrically well-defined manner holds promise to overcome the drawbacks associated with the use of full-length ECM proteins. Nonetheless, in many cases, such in integrin-GF signaling, it remains to be clearly elucidated the mechanisms underlying crosstalk between different receptors, and therefore more insight is required to better understand synergistic signaling, which finally will influence cell fate. In this way, it would be possible to engineer biomimetic peptides with even more potential to significantly improve the bioactivity of materials.

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