

Peptide-functionalized Biomaterials with Osteoinductive or Anti-biofilm Activity



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Abstract Peptides are short sequences of amino acids. Peptides with biological functionality can be derived from the active domain of proteins or determined from peptide screening experiments. Combined with modern chemical techniques to facilitate peptide synthesis, this leads to peptide modification as an interesting approach to render synthetic biomaterials bioactive. Peptides have been used to functionalize implant surfaces as well as bulk biomaterials, and they can be incorporated within controlled release systems. This chapter considers both osteoinductive peptides and anti-biofilm peptides with the goals to improve bone regeneration and reduce implant-associated infection, respectively.

Keywords Peptide synthesis · Phage display · Surface modification · Hydrogels · Nanofibers · Titanium · Controlled release · Osteoinductive peptides · Osteogenic differentiation · Bone morphogenetic protein · Osteogenic growth peptide · Hydroxyapatite · Antimicrobial peptides · Anti-biofilm · Bone tissue engineering · Dentistry

Introduction

Proteins are the functional building blocks of cells and tissues. They are made up of differing combinations of 20 natural amino acids (Table 1), which have an amino group on one end, a carboxylic acid group on the other end, and a variable side chain coming off the central carbon atom. Within a protein or polypeptide, the amino acid residues are joined together by the formation of an amide bond between the carboxylic acid of one amino acid and the amino group of the next. The sequences of natural proteins are determined from the genetic code whereby DNA is first tran-

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Table 1 The 20 standard amino acids, their abbreviations, and types of side chains

Amino acid	Three-letter abbreviation	One-letter abbreviation	Type of side chain
Alanine	Ala	A	Hydrophobic
Cysteine	Cys	C	Thiol
Aspartic acid	Asp	D	Negatively charged
Glutamic acid	Glu	E	Negatively charged
Phenylalanine	Phe	F	Hydrophobic
Glycine	Gly	G	None (hydrogen)
Histidine	His	H	Positively charged
Isoleucine	Ile	I	Hydrophobic
Lysine	Lys	K	Positively charged
Leucine	Leu	L	Hydrophobic
Methionine	Met	M	Hydrophobic
Asparagine	Asn	N	Polar uncharged
Proline	Pro	P	Special
Glutamine	Gln	Q	Polar uncharged
Arginine	Arg	R	Positively charged
Serine	Ser	S	Polar uncharged
Threonine	Thr	T	Polar uncharged
Valine	Val	V	Hydrophobic
Tryptophan	Trp	W	Hydrophobic
Tyrosine	Tyr	Y	Hydrophobic

scribed to messenger RNA and then translated to protein with each three-base-long codon specifying an amino acid. Similar to proteins, peptides are shorter chains of amino acids, also linked by amide bonds, that are typically less than 50 amino acids in length. With advances in chemical and molecular biology techniques, peptides can be chemically synthesized and modified, and de novo sequences can be designed. As a treatment strategy, peptides offer many of the advantages of protein therapeutics while addressing some of their limitations.

This chapter focuses on the use of peptides in biomaterial-based approaches for bone regeneration and anti-biofilm applications. First, a perspective is given on peptide design with two main approaches being the derivation of amino acid sequences from natural proteins and the screening of de novo libraries of peptides for specific functions. Then, relevant peptide synthesis techniques are presented along with chemical methods that allow functionalization of surfaces and bulk materials with peptides. The main focus of the chapter is the introduction of specific peptides from two main classes: osteoinductive peptides and anti-biofilm peptides. In both cases, in vitro assays to assess peptide activity as well as in vivo studies to demonstrate efficacy are presented. To date, however, few studies have combined osteoinductive peptides with antimicrobial peptides to stimulate bone formation while simultaneously reducing infection. The chapter concludes with some future perspectives on the topic.

Peptide Design

Peptides Derived from Proteins

The primary structure of a protein is its chemical composition determined by the specific sequence of amino acids. Protein secondary structure, which includes units such as α -helices and β -sheets, arises from the organization of portions of the protein by weak intramolecular bonds, such as hydrogen bonding and ionic interactions. Hydrogen bonding can occur between atoms on the protein backbone, but in other cases, these interactions are driven by the chemical composition of the side chains of the amino acids. With some exceptions, these side chains can be either positively or negatively charged, polar uncharged, or hydrophobic (Table 1). In de novo protein design, specifying the pattern of hydrophobic and hydrophilic amino acids in the protein sequence is in many cases sufficient to drive protein folding into α -helices [1] or β -sheets [2], as measured by circular dichroism. As with proteins, peptides with specific patterns of amino acids can self-assemble to form these structural units, such as RADA16 [RADARADARADARADA [3]; single-letter amino acid abbreviations used throughout this chapter, see Table 1] and Q11 [QQKFQFQFEQQ [4]] that form β -sheets or LD₆ [LIVAGD [5]] that forms α -helical intermediates. These self-assembling peptides have also been used to generate cyto-compatible hydrogels for biomedical applications, but further discussion of their use is outside the scope of the current chapter.

Likewise, these interactions can occur intermolecularly, that is, between two proteins, leading to receptor–ligand pairing or enzyme–substrate docking for example. As the amino acid sequences that are involved in these interactions are usually specific, they can serve as a starting point for the design of bioactive peptides that mimic these active domains of the protein. One of the most simple and commonly used peptide mimetics is the cell adhesive RGD sequence. RGD is found in several proteins including fibronectin, laminin, collagen, vitronectin, and others and mediates cell attachment through various integrins on the surface of cells [6]. Cell attachment to RGD is sequence specific, as evidenced by reduced binding to scrambled peptides such as RDG, and exposure to soluble RGD causes cells to detach from their substrate [6]. Functionalization of an otherwise nonadhesive biomaterial with RGD will allow cells to attach to and spread on or within the material. Patterning of RGD can also restrict cell attachment to specific areas, as has been shown by observing encapsulated cell spreading in inert poly(ethylene glycol) (PEG) hydrogels compared to PEG hydrogels with uniform functionalization with RGD and with spatially patterned RGD (Fig. 1) [7].

Besides RGD, there are a number of other integrin binding peptides that can be used as cell adhesion ligands, as summarized in Table 2. The use of integrin-binding peptides is a generic strategy in tissue engineering approaches, as most cell types are adherent and thus will attach, spread, proliferate, and migrate on the peptide-functionalized materials. Knowledge of the surface receptor profile of different cells can aid in the selection of which ligand or ligands to choose to be able to bind a

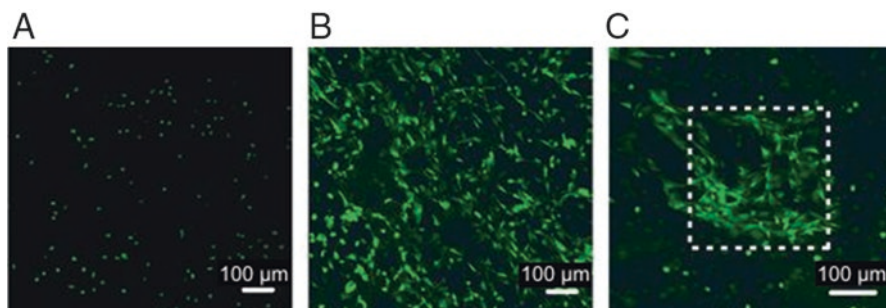


Fig. 1 Effect of the addition of RGD uniformly throughout compared to spatially patterned within PEG hydrogels that were formed by click chemistry on 3 T3 fibroblast spreading. (A) No RGD. (B) Uniform RGD. (C) Patterned RGD. The scale bars are 100 μm . (Reprinted by permission from Springer Nature, *Nature Materials*, ©2009 [7])

Table 2 Cell adhesion (integrin binding) peptides

Peptide sequence	Binding partners	Source and/or function	References
RGD	Several integrins	Fibronectin and others	[6]
YIGSR	Integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$	Laminin; binds endothelial cells	[8–10]
VAPG	Galactoside-binding protein	Elastin; binds smooth muscle cells	[8, 11]
SIKVAV	Integrins α_3 , α_6 , β_1 ; $\alpha_v\beta_3$	Laminin; binds endothelial cells	[12, 13]
RNIAEIIKDI	Integrins $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, $\alpha_7\beta_1$	Laminin	[9]
EILDVPST	Integrin $\alpha_4\beta_1$	Fibronectin; binds lymphoid and tumor cells	[14]
KLDAPT	Integrins α_4 , β_1 , β_7	Fibronectin; binds lymphoid cells	[15]
WQPPRARI	Heparin-binding	Fibronectin; binds endothelial cells and fibroblasts	[16]
PHSRN	Integrin $\alpha_5\beta_1$	Fibronectin	[17]
AEIDGIEL	Integrin $\alpha_9\beta_1$	Tenascin-C	[18, 19]
REDV	Integrin $\alpha_4\beta_1$	Fibronectin; binds endothelial cells	[20]

specific cell type. These peptides can then be used to functionalize biomaterials in both 2D and 3D. For example, nanofibrous matrices were formed from the self-assembly of peptide amphiphiles functionalized with either YIGSR or VAPG. The materials presenting YIGSR enhanced the attachment of endothelial cells, whereas the matrices containing VAPG promoted greater spreading of smooth muscle cells [8]. Hydrogels formed from photo-cross-linked PEG diacrylate combined with VAPG-functionalized PEG monoacrylate were also shown to preferentially support attachment of smooth muscle cells over fibroblasts, endothelial cells, and platelets [11]. Further, SIKVAV-conjugated chitosan hydrogels were demonstrated to promote angiogenesis and re-epithelialization in vivo [12]. These cell adhesive peptides

can potentially be combined with peptides targeting other receptors, and the design of osteoinductive and anti-biofilm peptides that are based on naturally occurring domains within proteins are further described in sections “Osteoinductive Peptides” and “Anti-biofilm Peptides” of this chapter.

Moving beyond the direct use of peptides derived from natural proteins, these mimetics can be varied, often in a systematic way, by changing individual amino acids within the peptide sequence to increase or decrease the activity of the peptide. One good example of this is the use of collagen-derived peptides to explore the sequence specificity of matrix metalloproteinases (MMPs), which are a family of enzymes that are active during normal tissue remodeling processes and also in disease states. Starting from an eight-amino-acid-long MMP substrate sequence found in the $\alpha 1$ chain of type I collagen, GPQGIAGQ, Nagase and Fields systematically varied the amino acids in each position and found that the substitution of A with W led to increases in the Michaelis-Menten parameter k_{cat}/K_M and the relative rate of hydrolysis by MMP-1, MMP-8, and MMP-3 [21]. These peptide sequences were then used in innovative enzymatically degradable synthetic hydrogels formed by the Michael-type addition reaction of end-functionalized multi-arm PEG macromers with cross-linker peptides containing the MMP substrate sequences [22, 23]. Indeed, the hydrogels containing the modified GPQGIWGQ substrate degraded faster than the hydrogels containing the collagen-derived GPQGIAGQ sequence, and this allowed for faster remodeling of the matrix and migration of embedded fibroblasts (Fig. 2) [22]. These degradable hydrogels were also found to support bone formation in combination with the osteoinductive molecule, bone morphogenetic protein (BMP)-2 [23]. In a rat calvarial defect model, hydrogels that were not degradable but loaded with BMP-2 led to similar low amounts of bone formation as degradable hydrogels without BMP-2 (approximately 20% bone coverage); however, the

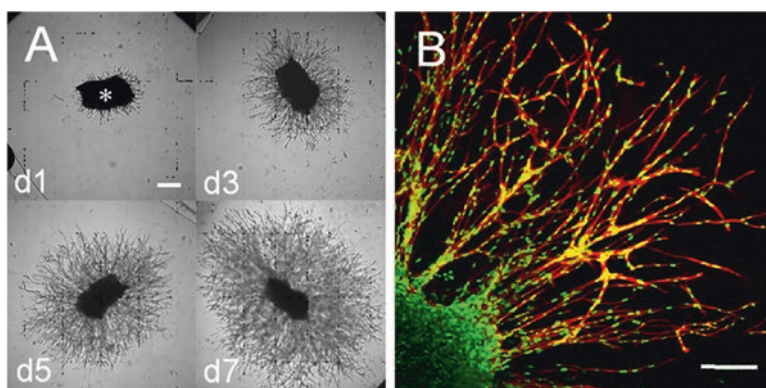


Fig. 2 Fibroblast migration into PEG hydrogels prepared through a Michael-type addition reaction with the MMP-sensitive GPQGIWGQ peptide cross-linker. **(A)** Phase contrast images after 1, 3, 5, and 7 days with scale bar = 250 μm . **(B)** Confocal image showing cell membranes and nuclei with scale bar = 150 μm . (Reprinted with permission from [22]. ©2003 National Academy of Sciences, USA)

hydrogels that were degradable and loaded with BMP-2 resulted in similar amounts of bone formation as the standardly used collagen sponge with BMP-2 (approximately 90% bone coverage) [23].

Library Screening Approaches

It is also possible to design peptides completely from scratch, and several methodologies have been developed to screen libraries of peptides for specific biological activity. In fact, half of the 2018 Nobel Prize in Chemistry was jointly awarded to George P. Smith and Sir Gregory P. Winter for developing the technique of phage display of peptides [24] and antibodies [25]. Phage display utilizes gene editing of the coat proteins of bacteriophage to incorporate peptide sequences that are displayed as part of the coat proteins. These phage can then be screened against target molecules for an interaction of interest and further amplified. The DNA within the selected phage can finally be sequenced to determine the relevant peptide sequence. Phage display can be useful in determining substrate specificity of enzymes. For example, to determine mutant substrates that are not cleaved by an enzyme, the coat proteins are modified with the library of peptide sequences, each with a tag on the terminus to allow for separation using antibody-coated beads (Fig. 3). After incubation with the enzyme, the phage that are still attached to the beads can be separated, washed, amplified, treated with enzyme, and sorted again. After several rounds of sorting and amplification, the individual phage can be isolated and sequenced. This approach has been used to identify substrates that are less susceptible to cleavage by the metalloproteinase ADAMTS13 [26], and by changing the method to select the phage of interest, one can identify enzyme substrates that are preferentially cleaved, peptides that bind to specific proteins or other molecules, etc. [24].

An alternative to phage display for peptide design is mixture-based oriented peptide libraries [27]. The mixture-based oriented peptide library approach screens the peptides in solution and relies on peptide sequencing methods (Edman degradation) to determine the sequence. It has been applied for determining enzyme substrates and protein interaction domains [28–31]. For example, to determine an enzyme substrate, in a first round, a totally degenerate peptide library with the amino (N-) termini acetylated is treated with the protease. The fragments that are generated have a free amine group on the N-terminus and are subjected to N-terminal sequencing. The amino acids that are most abundant in each position are then fixed in a second-generation library, which have the N-termini as free amines and the carboxy (C-) termini tagged with biotin. After cleavage with the protease, the C-terminal fragments and uncleaved peptides can be separated using immobilized avidin, and the N-terminal fragments can be sequenced. This approach has been used to generate substrates for a variety of MMPs [27]. Another approach to screen for enzyme substrates is a technique called cellular libraries of peptides substrates (CLiPS) [32]. A library of peptides containing potential substrates tagged with a ligand for a fluorescent probe are displayed on the surface of *E. coli*.

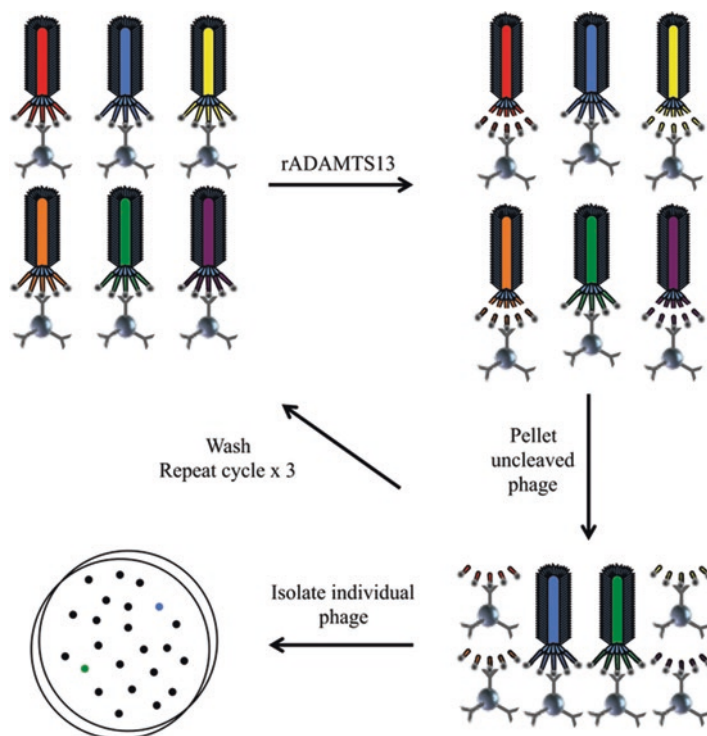


Fig. 3 Working principle of phage display. In this case, the substrates that are not cleaved by the enzyme ADAMTS13 are selected and amplified. (Reprinted under the terms of the Creative Commons Attribution License from Desch et al. ©2015 [26])

After treating the cells with the protease of interest, the intact peptides are able to be detected using fluorescence-activated cell sorting (FACS), and the clones with hydrolyzed substrates can be enriched by repeating this cycle several times. Compared to phage display and soluble peptide libraries, up to 10^4 copies of the substrate are displayed on a single cell, which allows quantitation of substrate conversion for an individual clone [32].

These techniques are commonly used to identify peptides that bind to target receptors or to natural ligands, for epitope mapping or mimicking, in drug discovery to find new enzyme inhibitors and receptor agonists and antagonists, for epitope discovery in vaccine development and diagnostics, and selection of DNA-binding motifs, among others [24]. Of relevance to biomaterials development, they have also been used to identify new substrates for various MMPs, such as MMP-2 [33], MMP-3 or -7 [34], MMP-11 [35], MMP-13 [36], and MT1-MMP [37]. These peptide substrates were evaluated with the PEG hydrogel system described above, and sequences resulting in hydrogels with increased susceptibility to MMP-1, MMP-2, or plasmin were identified [38, 39]. The faster degrading hydrogels supported increased proliferation of encapsulated fibroblasts as well as sprouting of endothelial cells in a chick aortic ring

outgrowth assay [38, 39]. Similar to the cell adhesion peptides already described, the functionalization of scaffolds with enzyme substrate peptides also provides a generic strategy to support the matrix remodeling, proliferation, and migration of cells within the matrix. More specific peptides that are osteoinductive or anti-biofilm, which have been found by library screening approaches, are detailed in sections “Osteoinductive Peptides” and “Anti-biofilm Peptides” of this chapter.

Chemical Methods

Peptide Synthesis

During protein translation from messenger RNA, the nascent protein chain is synthesized from N- to C- terminus in the ribosome via the action of transfer RNA, which recognizes the codon in the RNA strand. A new amide bond is formed to attach the amino acid to the growing polypeptide chain. Specific codons initiate and terminate the synthesis of the protein. On the other hand, peptides are typically synthesized chemically using a technique called solid-phase peptide synthesis, which allows excess reagents and by-products of the reactions to be easily removed by washing and filtration after each step. They can also be synthesized in solution, although this requires additional work to separate the peptide intermediate after each step. In the case of solid-phase peptide synthesis, the peptide is synthesized on a solid support from the C- to N-terminus. Cleavable protecting groups typically block reactive functionalities on the side chains and amino group of the amino acid. The latter protecting group is often an Fmoc (base-labile) or Boc (acid-labile) group, which can be removed before the addition of the next amino acid. Merrifield was the first to describe the solid-phase peptide synthesis technique in 1963 [40], and the Fmoc/*t*-Bu orthogonal protecting group strategy has become the most common approach applied today. Solid-phase peptide synthesis typically proceeds by coupling of the first amino acid to the supporting resin followed by a deprotection step to remove the Fmoc group. The next amino acid is added, followed by deprotection, and so on until the last amino acid or other N-terminal group is added. Then, the peptide is cleaved from the resin, and the protecting groups are also removed from the side chains. The peptide is typically purified by reversed-phase HPLC-MS, with the MS used to confirm the molecular mass of the synthesized peptide.

Important considerations when planning to synthesize a peptide include the general synthesis strategy, the choice of protecting groups for the side chains, the resin used as solid support as well as how it is linked to the C-terminus of the peptide, and the choice of coupling reagents [41]. The speed and quality of solid-phase peptide synthesis can be improved by the application of microwave irradiation, which raises the temperature and helps to break up chain aggregation of the peptide intermediates [42]. The microwave energy reduces the reaction time for both the coupling of amino acids and also the removal of the Fmoc protecting group [42, 43]. Nevertheless, synthesis of longer peptides tends to result in lower yields, and some amino acids are more difficult to couple than others. As a result, chemical ligation strategies can

be used to join two shorter peptides together in solution phase. These methods result in the formation of amide or other chemical bonds between the two peptide segments, and typically chemoselective functional groups are chosen to allow ligation in the presence of the unprotected side chains on the peptides. Commonly used reactions include thioester ligation, thioether ligation, and imine ligation [44]. Enzyme-catalyzed methods, which have been primarily developed for ligation of proteins, can also be applied to peptides. For example, sortase A derived from *Staphylococcus aureus* recognizes and cleaves the LPxTG motif, which can be tagged on one peptide. The resulting thioester is then ligated to the N-terminus of a second peptide that starts with a G residue [45, 46]. The enzyme subtiligase, an engineered mutant of subtilisin, can also catalyze the reaction between a peptide thioester or peptide ester and the N-terminus of a second peptide [47, 48].

An advantage of solid-phase peptide synthesis is that it is not limited to the 20 natural amino acids, and in fact any compounds with appropriate reactive groups can be added to the peptide chain. Some examples are provided in Table 3. The functional group on the C-terminus of the peptide is usually determined by the choice of starting resin, and the N-terminus is often acetylated to block the reactivity of the amine group. Fluorescent labels or dyes can be incorporated into the peptides, including them as modifications on side chains or directly within the peptide chain if proper reactive groups are available. Examples of molecules used in this approach are summarized in Table 4. Fluorescent dyes such as fluorescein isothiocyanate (FITC), 5-carboxyfluorescein (5-FAM), Dansyl, 5- (and 6-) carboxytetramethylrhodamine [5(6)-TAMRA],

Table 3 Potential modifications to peptides prepared by solid-phase peptide synthesis

Modification	Molecules
N-terminus	Free amine, acetylation, Fmoc
C-terminus	Free acid, amidation
D-form amino acids	D-Ala, D-Cys, D-Asp, D-Glu, D-Phe, D-His, D-Ile, D-Lys, D-Leu, D-Met, D-Asn, D-Pro, D-Gln, D-Arg, D-Ser, D-Thr, D-Val, D-Trp, D-Tyr
Phosphorylation	pSer, pTyr, pThr
PEGylation	PEG _x
Cyclization	Disulfide bridge

Table 4 Fluorescent tags for incorporation into peptides, their excitation (λ_{ex}) and emission (λ_{em}) wavelengths, and their function

Fluorophore	λ_{ex}	λ_{em}	Function
Biotin	NA	NA	Binding-tagged streptavidin
FITC	492 nm	515 nm	Fluorophore, donor for FRET
5-FAM	492 nm	518 nm	Fluorophore
Dansyl	333 nm	518 nm	Fluorophore, FRET
TAMRA	546 nm	575 nm	Fluorophore, acceptor for FRET
Dnp	350 nm	NA	Acceptor for FRET (quencher)
MCA	322 nm	390 nm	Fluorophore, FRET with Dnp
5(6)-CR6G	522 nm	550 nm	Fluorophore
DABCYL	428 nm	NA	Acceptor for FRET (quencher)

7-methoxycoumarin-4-acetic acid (MCA), and 5- (and 6-) carboxyrhodamine 6G [5(6)-CR6G] have different excitation and emission wavelengths, and they can be selected to avoid overlap with tissue autofluorescence or with other fluorescent molecules used in a biomaterial formulation. Biotin can be incorporated for later tagging with fluorophores conjugated to streptavidin, and other molecules such as dinitrophenol (Dnp) or DABCYL can be used as part of Forster energy resonance transfer (FRET) pairs. Some of these molecules can be used as an N-terminal modification by reaction of the molecule with the amine group at the end of the peptide as the last step during peptide synthesis. This reaction can be achieved through a carboxylic acid group already present in the molecule or by first modifying the molecule with a succinimidyl ester group, which can then react with the terminal amine group. Alternatively, the fluorescent molecule can be added to the side chain of a lysine residue, and then this modified lysine can be incorporated anywhere in the peptide using standard coupling and deprotection reactions.

Labeling of peptides with fluorescent dyes can be a useful strategy for examining the loading and release of the peptide from a biomaterial carrier. For example, FITC-labeled peptides were used to track release from electrospun nanofibrous membranes and aerogels of poly(lactide-co-glycolide) (PLGA)/collagen/gelatin [49, 50]. A fluorescence plate reader can directly measure the concentration of the released peptide over time without the need for more complicated assays, such as ELISA, for the detection of the peptide. FRET pairs and dye quenching can also be used in peptide design to track spatial information about the peptides or peptide fragments. Fluorescent dyes and molecules that quench their fluorescence when in close proximity, such as the combination of fluorescein with DABCYL, can be incorporated on the opposite ends of enzyme substrate peptides similar to the ones described above. When the peptide is intact, the dye and quencher are close enough that there is no fluorescence; however, once the substrate is cleaved, the two fragments can diffuse away from each other, and the fluorescence is restored. In addition to being useful for determining kinetic parameters of the enzyme substrate interaction in solution [21], this approach has been used to track the local degradation of a PEG hydrogel incorporating such peptides (Fig. 4) [51].

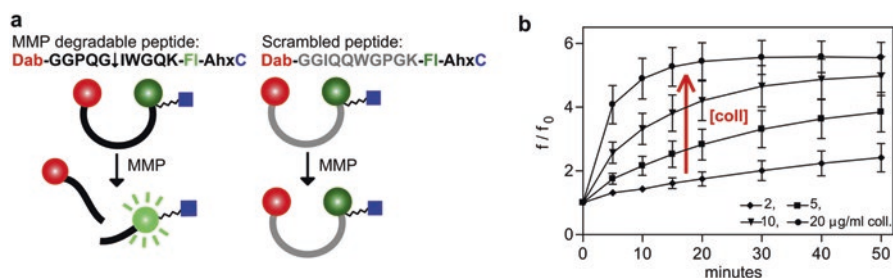


Fig. 4 Design and characterization of quenched fluorescent peptides as enzyme substrates. (a) Sequence and design of cleavable peptide and non-degradable control. (b) Degradation kinetics tracked by monitoring fluorescence over time. (Reprinted from *Biomaterials*, 34, Leight JL et al., Direct measurement of matrix metalloproteinase activity in 3D cellular microenvironments using a fluorogenic peptide substrate, 7344–52, ©2013 with permission from Elsevier [51])

Peptide Functionalization

In addition to synthesis of the desired peptide, it must be able to be chemically bound to or mixed in with the biomaterial while maintaining its biological activity. This can also influence the design of the peptide. For covalent binding of the peptide, an appropriate reactive group or additional amino acids must be added to allow the reaction to occur. One example of this is the use of a peptide substrate, which can be covalently cross-linked through the action of an enzyme. One such substrate is NQEQVSP(L), which is derived from α_2 -plasmin inhibitor and is a substrate for the transglutaminase enzyme factor XIIIa. This peptide allows covalent cross-linking with some natural biomaterials, such as fibrin [52, 53], or with synthetic biomaterials that have a complementary substrate (FKGG) recognized by the enzyme [54]. For covalently bound peptides that act as a ligand, spacer amino acids may need to be added within the peptide to prevent steric hindrance interfering with receptor recognition. Cyclization of the peptide to constrain its presentation may also help to improve ligand–receptor interactions, as has been demonstrated with the RGD peptide [55]. For peptides that are active in soluble form, an MMP substrate can be added in the peptide sequence between the covalent attachment point and the peptide of interest to allow enzymatic cleavage and release of the peptide. For example, this has been done by adding the MMP substrate sequence PVGLIG to the N-terminus of osteogenic growth peptide (OGP) and then using carbodiimide chemistry to link the peptide to a partially oxidized form of alginate. Release of the peptide from the alginate matrix through the action of MMP-2 was confirmed [56].

In the PEG hydrogels prepared by a Michael-type addition reaction described above, a covalent bond was formed between the polymer and the peptides through the functionalization of the PEG with vinyl sulfone groups and the incorporation of cysteine residues, which have a reactive thiol group, in the peptides. This allowed both the functionalization of the PEG with the cell adhesive RGD peptide and the cross-linking of the PEG into an enzymatically degradable network using the substrate peptides [22, 23, 38, 39]. Similar conjugation reactions can also be performed between maleimide groups and thiol groups [57]. This strategy has been used to functionalize surfaces with peptides, for example, with layer-by-layer films of peptide-grafted poly(allylamine hydrochloride) (PAH) [58] and maleimide self-assembling monolayers on quartz substrates [59].

Depending on the reactive groups present on the material, peptides can also be covalently attached via an amide bond using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) as coupling reagents to materials such as maleic anhydride-modified poly(lactic acid) (PLA) [60] or the carboxyl groups of partially oxidized alginate [56]. Typically, this approach relies on the reaction of an amine group coming from the N-terminus or a lysine side chain of the peptide with a carboxyl group on the material. Further, copper-catalyzed azide alkyne cycloaddition reactions can be used with azide-functionalized peptides and, for example, propargyl functionalized L-phenylalanine-based poly(ester urea)s [61]. Alternatively, the reactive groups can be switched between peptide and

polymer, as in the case of azidopropyl hyperbranched poly(arylene oxindole) functionalization with *5-Hexynoic*-RGDS [62].

For noncovalent binding of peptides, the peptides may be allowed to adsorb to the material or be mixed in during processing, so that they are entrapped in the matrix. Alternatively, the peptide can be modified with an affinity domain that promotes noncovalent interactions with the material. For example, the addition of a highly negatively charged E7 domain, consisting of seven glutamic acid residues in a row, enables peptides to bind to calcium-based materials [63–66] or to pre-mineralized materials, such as PLGA/collagen/gelatin nanofibers [49]. For an inorganic bovine bone graft material, coupling of a BMP-2-derived peptide to E7 led to increased loading and greater retention, even after 8 weeks of implantation in vivo [63]. Alternatively, the hydroxyapatite binding domain of statherin (N15 domain) was used to bind either PGRGDS or PDGEA (a cell adhesive peptide derived from type I collagen) to hydroxyapatite surfaces. The peptide-coated materials bound MC3T3-E1 osteoblasts via the $\alpha_v\beta_3$ integrin (for PGRGDS) and $\alpha_2\beta_1$ integrin (for PDGEA) [67].

Other linkers, such as polydopamine, can be used to mediate peptide attachment to surfaces [68–72]. In one approach, the surface can be coated with polydopamine, and then the peptide can be bound to the polydopamine coating [68]. Polydopamine coatings can be formed by dip-coating of a material in an aqueous solution of dopamine, which self-polymerizes, and they allow for further functionalization via secondary reactions [73]. This methodology is inspired by the adhesive proteins in mussels, which contain 3,4-dihydroxy-L-phenylalanine and lysine amino acids, and the covalent and noncovalent interactions that can occur with catechol compounds, and it is particularly interesting because it is compatible with a number of different inorganic and organic materials, such as metals, oxides, polymers, and ceramics [73]. Alternatively, peptides can be functionalized with a short polydopamine tag that can then interact with surfaces via the catechol groups (Fig. 5) [70]. This approach has been used to functionalize otherwise inert titanium (Ti) surfaces with RGDS and OGP, which enhanced the attachment, proliferation, and osteogenic differentiation of bone marrow-derived mesenchymal stem cells (BM-MSCs) in vitro as well as improved osteogenesis and mechanical stability of peptide-coated screws implanted in the femoral condyles of New Zealand White rabbits in vivo [70].

Osteoinductive Peptides

Peptide-functionalized materials provide an interesting approach to stimulate bone regeneration. In the fields of orthopedics and dentistry, peptides are relevant in two major ways. The first is to provide surface functionalization of the frequently Ti-based implants that are used as permanent implants. The use and characterization of Ti implants [74] as well as their combination with biopolymers [75] have been previously reviewed. The second is to use peptides in tissue engineering approaches. Typically, tissue engineering aims to combine cells, biomaterials, and biological

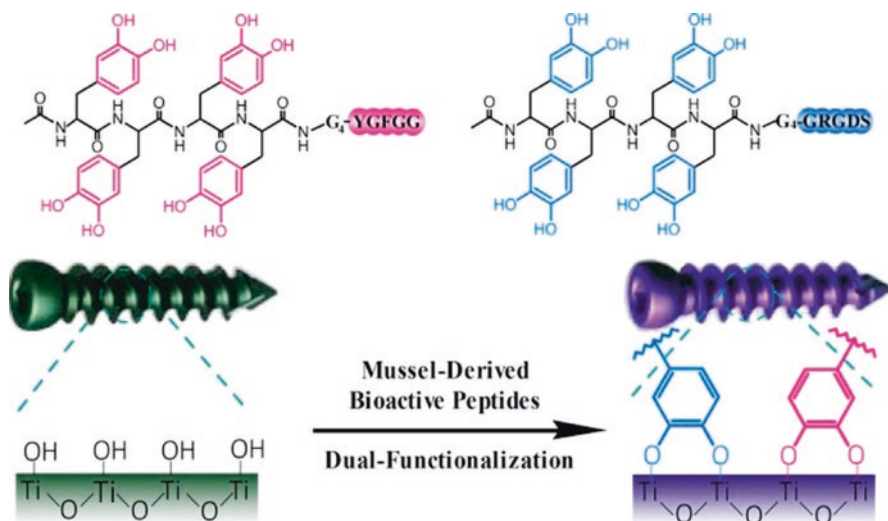


Fig. 5 Cell adhesive $(\text{DOPA})_4\text{-G}_4\text{-GRGDS}$ and osteoinductive $(\text{DOPA})_4\text{-G}_4\text{-YGFGG}$ peptides that mimic mussel-derived proteins with catechol groups to enable coordination between the catechol groups and titanium oxide for surface functionalization of Ti cortical bone screws. (Reprinted with permission from [70]. ©2016 American Chemical Society)

factors to create living implants that can repair or regenerate tissue function [76], and peptides are one means of providing these biological signals. In this case, tissue engineering scaffolds of a variety of materials have been modified and examined for their effects both *in vitro* and *in vivo*.

This section focuses on the application of peptides with osteoinductive capabilities, that is, peptides that can promote the osteogenic differentiation of progenitor cells *in vitro* and can lead to *de novo* bone formation *in vivo*. While the cell adhesive and enzymatically degradable peptides discussed above as well as other peptides, such as pro-angiogenic peptides, can support tissue remodeling and repair more generally and thus are interesting to consider as part of treatments to regenerate bone, as considered in recent reviews [77, 78], in this section, peptides that influence bone progenitor cells are specifically considered. These peptides are derived from a number of sources including identification of active domains in BMP-2, BMP-7, and other osteogenic proteins. A number of osteogenic peptides, their sequences and variants, and their effects are briefly summarized in Table 5.

OGP Peptides

Osteogenic growth peptide (OGP; ALKRQGRTLYGFGG) is a 14 amino acid peptide derived from the C-terminus of histone H4. It is found naturally in soluble form in serum and has been shown to influence osteoblast proliferation and differentiation [79]. OGP increased alkaline phosphatase (ALP) activity of MC3T3-E1 pre-

Table 5 Osteoinductive/osteogenic peptides and their effects

Peptide	Sequence	Effects	Reference
OGP	ALKRQGRTLYGFGG YGFGG (shorter variant)	Osteogenic differentiation in vitro; bone formation in vivo	[70, 79, 80]
BMP-2 derived	NSVNSKIPKACCVPTELSAI	Ectopic bone formation in vivo	[81]
	KIPKASSVPTELSAISTLYL	Osteogenic differentiation in vitro; ectopic bone formation in vivo	[82]
	DWIVA	Osteogenic differentiation in vitro; increased bone formation in vivo	[83]
BMP-7 derived	TVPKPSSAPTQLNAISTLYF	Osteogenic differentiation in vitro	[84]
	GQGFSSYPYKAVFSTQ (BFP-1)	Osteogenic differentiation in vitro	[85]
	ETLDGQSINPKLAGL (BFP-3)	Osteogenic differentiation in vitro	[86]
BMP-4 derived	RKKNPNCRRH	Osteogenic differentiation in vitro (ERK1/2 activation); bone formation in vivo	[87]
BMP-9 derived	KVGKACCVPTKLSPISVLYK	Osteogenic differentiation in vitro; ectopic bone formation in vivo	[88, 89]
PTH ₁₋₃₄	SVSEIQLMHNLGKHLNS- MERVEWLRKKLQDVHNF	Orthotopic bone formation in vivo	[90]
Others	GTPGPQGIAGQRGVV (P-15)	Proliferation in vitro; bone formation in vivo	[91]
	GLRSKSKKFRFPDIQY- PDATDEDITSHM (CBM)	Mineralization in vitro and in vivo	[92]
	PFSSTKT (BMHP1)	Osteogenic differentiation in vitro	[59]

osteoblasts in vitro and improved bone remodeling in vivo in a rat mandibular condyle model when delivered systemically [79]. In addition, systemic administration of OGP has been shown to improve the structural and mechanical properties of the fracture callus in a rat model [93].

OGP has also been used with several biomaterials, both as a 2D surface modification and for incorporation in a 3D scaffold [94, 95]. When OGP and its shorter variant, OGP(10–14) (YGFGG) [80], were tethered to 2D surfaces via click chemistry between an azide-functionalized peptide and an alkyne-terminated self-assembled monolayer (SAM), they led to increases in MC3T3-E1 attachment and proliferation in vitro [96]. A similar approach has been used with azide-functionalized OGP and poly(ester urea) scaffolds via propargyl groups on the surface of the scaffolds. This functionalization increased the differentiation of human mesenchymal stem cells (hMSCs), as demonstrated by increases in ALP activity, calcium deposition, and expression of osteogenic genes [61]. Carbodiimide chemistry has been utilized to link OGP(10–14) to maleic anhydride-modified PLA scaffolds, improving the proliferation, differentiation, and mineralization of neonatal rat calvarial osteoblasts [60]. Moreover, OGP has been used to functionalize the surface of Ti implants through a polydomamine linker. In combination with the cell adhesive peptide RGD, the OGP-functionalized surfaces promoted

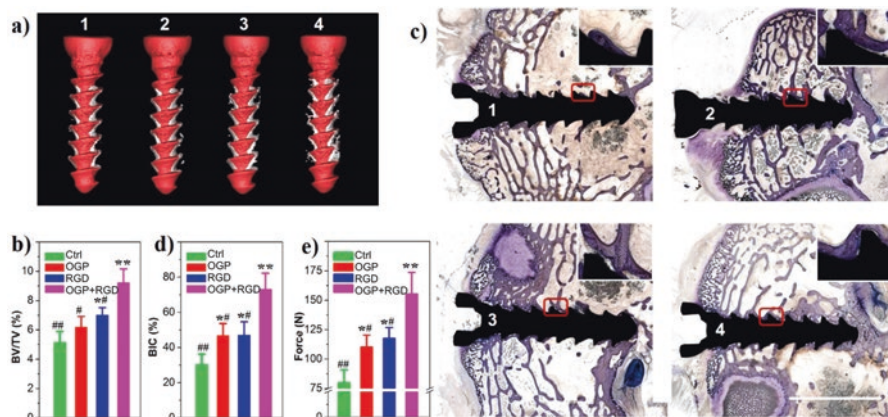


Fig. 6 Effect of OGP surface treatment of Ti bone screws on osteogenesis and mechanical stability in a rabbit femoral condyle. (a) Reconstructed microCT images. (b) Quantitation of bone volume (BV)/total volume (TV) from microCT images. (c) Representative histological images stained with toluidine blue with 1 = untreated control, 2 = OGP, 3 = RGD, and 4 = OGP/RGD (3,1). (d) Quantification of bone-implant contact (BIC) from histological images. (e) Biomechanical pull-out testing. (Reprinted with permission from [70]. ©2016 American Chemical Society)

cell attachment and mineralization *in vitro*, and functionalized Ti screws led to increased osseointegration, as shown by increased bone volume, bone-implant contact, and pull-out force (Fig. 6) [70].

Additionally, hydrogels have been formed using OGP(10–14) tethered to alginate matrices by a protease-sensitive linker. Exposure to enzymatic treatment led to the release of the peptide and osteogenic differentiation of treated hMSCs. The MSC-laden, OGP-functionalized alginate hydrogels also resulted in ectopic bone formation *in vivo* [56]. Moreover, functionalization with OGP increased MC3T3-E1 proliferation and alkaline phosphatase activity for cells cultured on hydrogels formed from the self-assembling peptide RADA16 [97].

Peptides Derived from BMP-2

As BMP-2 is one of the most potent osteoinductive growth factors [98], peptides derived from BMP-2 have also been studied for their ability to stimulate osteogenic differentiation *in vitro* and bone formation *in vivo*. One of the first peptides derived from BMP-2 was a 20-amino-acid-long sequence (NSVNSK-IPKACCVPTLSAI), which led to ectopic bone formation in the calf muscle of rats when linked to an alginate hydrogel [81]. A peptide containing the motif DWIVA has also been demonstrated to promote proliferation and osteogenic differentiation of MC3T3-E1 cells *in vitro* and to increase bone growth when conjugated to the surface of Ti implants used in mandibular bone defects in beagle dogs [83].

The peptide KIPKASSVPTELSAISTLYL, derived from residues 73–92 of the knuckle epitope of BMP-2, has promoted ALP activity and osteocalcin gene expression by C3H10T1/2 cells in culture and, when combined with an alginate hydrogel, has led to ectopic bone formation in rat muscle tissue [82, 99] and accelerated bone formation in a rat tibial defect [100]. Entrapping the peptide in chitosan microspheres that were then embedded in nano-hydroxyapatite/collagen/poly(lactic acid) (PLA) scaffolds provided controlled release of the peptide, which still retained its activity [101]. Further, grafting the peptide to self-assembled monolayers in combination with the cell adhesive RGD peptide led to the upregulation of bone sialoprotein expression as well as promotion of mineralization, even in the absence of other osteogenic supplements [102]. As has been shown with OGP, polydopamine has also been used to link the BMP-2-derived peptide to PLGA scaffolds [69, 71]. For example, the modified scaffolds promoted osteogenic differentiation of human adipose-derived stem cells (hADSCs) in culture and resulted in bone formation in calvarial defects in vivo [69].

The BMP-2-derived KIPKASSVPTELSAISTLYL has also been incorporated into surface coatings using electrostatic interactions. One way this has been achieved is by forming layer-by-layer films of PAH and poly(sodium 4-styrenesulfonate), with the peptide grafted on the PAH. The films were coated on electrospun membranes of nano-hydroxyapatite and PLGA, and they promoted alkaline phosphatase activity by MSCs in vitro and bone formation in a rat calvarial defect in vivo [58]. A second method to immobilize the peptide is through the E7 calcium binding domain described earlier. E7-modified BMP-2-derived peptides have been bound to anorganic bovine bone [63], α -tricalcium phosphate (α -TCP) scaffolds [64], and even nanofibrous membranes of PLGA/collagen/gelatin that were pre-mineralized [49]. Nanofibrous aerogels of electrospun PLGA/collagen/gelatin and Sr-Cu-doped bioactive glass fibers were also functionalized with E7-BMP-2 peptides. The addition of the peptide increased bone formation in vivo in rat calvarial bone defects (Fig. 7) [50].

Peptides Derived from BMP-7

Bone forming peptide-1 (BFP-1; GQGFSYPYKAVFSTQ) is derived from residues 100–114 of BMP-7. Treatment with soluble BFP-1 led to increased ALP activity and calcium deposition by bone marrow stromal cells, and further these BFP-1-treated cells increased bone formation when injected subcutaneously in mice [85]. Likewise, hMSCs also showed increased ALP activity, calcium deposition, and osteogenic gene expression when cultured on nanofibrous scaffolds consisting of polycaprolactone (PCL) functionalized with the peptide via a polydopamine linker. This enhancement in osteogenic differentiation was also seen when the cells were cultured in medium lacking other osteoinductive factors [68]. Further, human-induced pluripotent stem cells (hiPSCs) also increased ALP activity, calcium deposition, and osteogenic gene expression when cultured on 2D surfaces that had been functionalized with BFP-1 and carboxymethyl chitosan [72]. A second osteoinductive peptide,

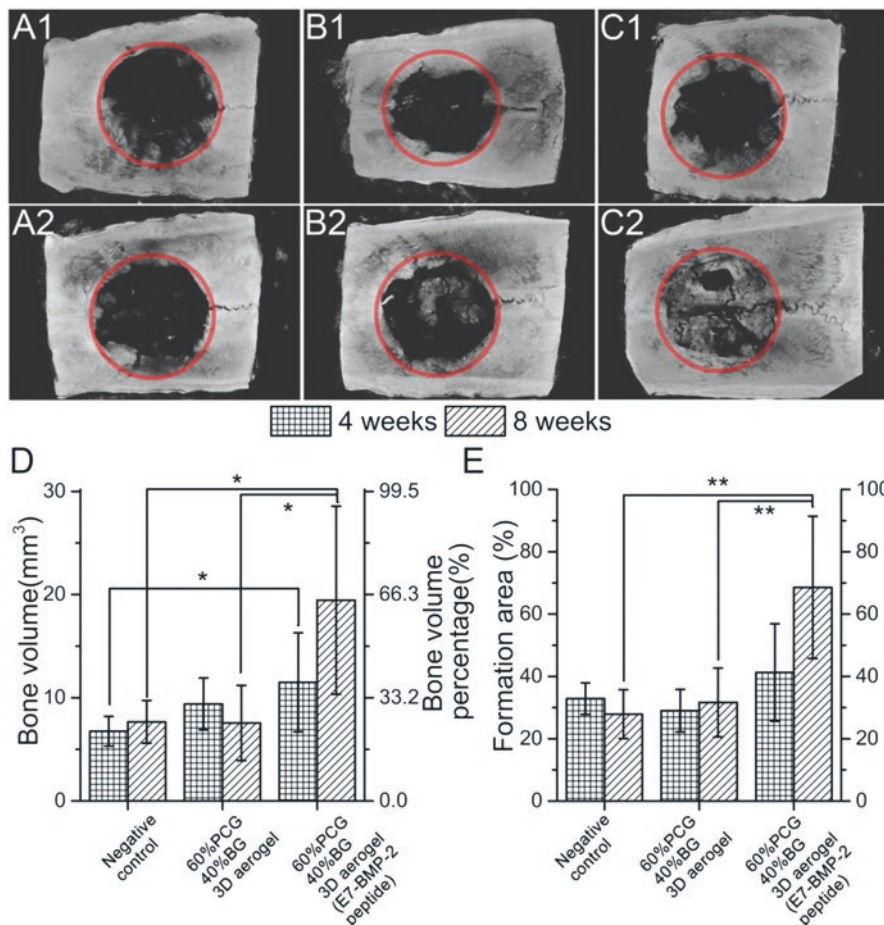


Fig. 7 Calvarial bone regeneration induced by E7-BMP-2 peptide loaded aerogels. Radiographs of (A) untreated controls, (B) aerogel only control, (C) E7-BMP-2 peptide loaded aerogel group. Quantification of (D) regenerated bone volume and (E) bone formation area. (Reprinted with permission from [50]. ©2018 John Wiley and Sons)

BFP-3, was also determined from BMP-7 residues 250–265 (ETLDGQSI-NPKLAGL). It increased in vitro osteogenic differentiation of bone marrow stromal cells by increasing ERK1/2 and Smad1/5/8 phosphorylation [86].

Peptides Derived from BMP-4 and BMP-9

Osteoinductive peptides have also been identified within BMP-9, including from the knuckle epitope, residues 68–87 (KVGKACCVPTKLSPIVLYK) [103]. In vitro, this peptide-induced gene expression of Runx2, osterix, type I collagen, and osteo-

calcin by MC3T3-E1 pre-osteoblast cells, and it activated the Smad pathway [89]. The phosphorylation of Smad1/5/8 was further enhanced when this peptide was presented in combination with a fibronectin-derived peptide containing both PHSRN and RGD ligands and functionalized on films of PCL [104]. When combined with a chitosan-based delivery system, the peptide induced ectopic bone formation in the quadriceps of mice. Interestingly, a collagen-based delivery system abrogated this response [88].

A heparin binding domain from residues 15–24 of BMP-4, RKKNPNCRRH, has been shown to have osteogenic activity. Administered in soluble form, the peptide promoted the osteogenic differentiation of hMSCs, as shown by increased matrix mineralization as well as upregulation of the osteogenic genes, ALP, osteopontin, and osteonectin. Phosphorylation of Smad 1/5/8 and MAPK was demonstrated by Western blotting and confirmed the activation of the ERK1/2 pathway. Further, when combined with an alginate hydrogel matrix, the peptide was able to stimulate *in vivo* bone formation in a rabbit calvarial defect model [87].

Peptides Derived from Parathyroid Hormone

A peptide derived from parathyroid hormone residues 1–34 (PTH_{1–34}; SVSEIQL-MHNLGKHLNSMERVEWLRKKLQDVHNF) has been used clinically as treatment for osteoporosis [105]. For stimulating bone regeneration, it has been covalently bound to an RGD-functionalized PEG hydrogel and been shown to lead to increases in *in vivo* bone formation, at levels similar to treatment with autologous bone, when implanted into alveolar bone defects surrounding standard Ti implants in a dog model [90]. Similarly, PTH_{1–34} was covalently bound to a fibrin matrix via an enzymatically degradable peptide linker, and this led to increased bone formation in defects of the femur and humerus of sheep [106]. The soluble form of PTH_{1–34} was also administered via injection to determine dosing amount and frequency to improve bone healing in a mouse femoral allograft model [107]. Moreover, it was shown to protect against radiotherapy-induced trabecular bone loss in a rat model [108].

Other Peptides

A number of other peptides have shown osteoinductive activity. Bone marrow homing peptide 1 (BMHP1; PFSSTKT), which was discovered from screening a phage display library, led to osteogenic differentiation of MSCs cultured on quartz substrates functionalized with the peptide [59]. The peptide P-15 derived from residues 766–780 of the type I collagen α chain (GTPGPQGIAGQRG-VV) accelerated bone formation in a rabbit calvarial defect model when it was coated on deproteinized bovine bone [91]. A collagen-binding motif (CBM) from osteopontin (GLRSKSKKFRRPDIQYPDATDEDITSHM) in combination with a type I collagen

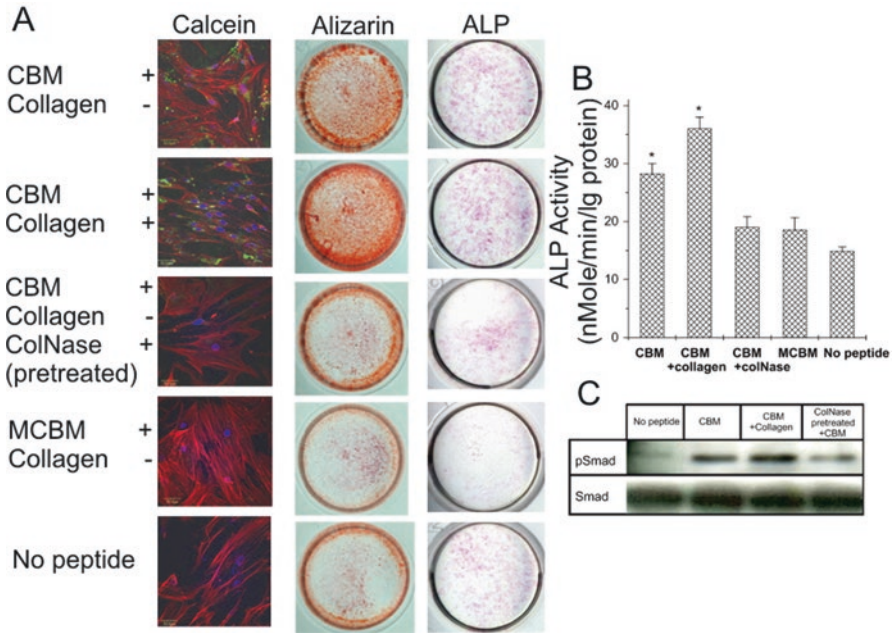


Fig. 8 Effect of CBM on bone marrow stromal cells. (A) Micrographs of calcein staining (left panel), Alizarin Red S staining (middle panel), and ALP expression (right panel). (B) Quantification of ALP activity. (C) Western blot analysis for phosphorylated-Smad expression. (Reprinted from *Biomaterials*, 28, Lee J-Y et al., Assembly of collagen-binding peptide with collagen as a bioactive scaffold for osteogenesis in vitro and in vivo, 4257–67, ©2007 with permission from Elsevier [92])

matrix increased mineralization by cultured bone marrow stromal cells, as shown by calcein, Alizarin Red S, and ALP staining, as well as phosphorylation of Smad1/5/8 (Fig. 8). The peptide functionalized matrices also led to increased bone formation in rabbit calvarial defects [92].

Use of Osteoinductive Peptides Clinically

The majority of peptides used to stimulate bone healing and regeneration have only been evaluated in vitro or in preclinical animal studies [109, 110]. Of the osteoinductive peptides discussed in this chapter, two have seen more extensive clinical use, PTH₁₋₃₄ and P-15. In addition, chrysalin, also known as thrombin peptide 508 (TP508), has been evaluated in Phase I/II clinical trials to treat distal radial fractures, showing reduced time to healing [111]. PTH₁₋₃₄ has been approved for the prevention and treatment of osteoporosis clinically [105]. In a clinical study for the treatment of distal radial fractures, PTH₁₋₃₄ shortened the time to healing with the lower of two doses tested [112] and appeared to improve early callus formation [113]. The use of

PTH₁₋₃₄ in bone healing has also been documented in several case reports, including for sternal nonunion [114] and hip fracture [115], among others [110]. P-15 was combined with anorganic bone mineral and a hydrogel carrier material and tested in a clinical study for anterior cervical discectomy and fusion in comparison with autograft. The radiographic fusion rates, clinical and patient-reported outcomes, and safety profile of the P-15 formulation were all similar to autograft bone [116]. In a first example of its use for orthopedic applications, again combined with anorganic bone mineral, P-15 has been tested in a pilot clinical trial to treat patients with malunion or delayed union fractures, leading to full consolidation in 90% of the cases [117]. In the oral cavity, P-15 in combination with anorganic bone mineral has been shown to lead to improved defect fill results when compared to anorganic bone mineral alone in a multicenter trial with 33 patients [118].

Anti-biofilm Peptides

Biofilms are formed by the colonization of bacteria on surfaces and represent a leading cause of chronic and implant-associated infections clinically [119, 120]. They are characterized by the formation of aggregates of bacteria that encapsulate themselves in a dense extracellular matrix composed of polysaccharides, extracellular DNA, proteins, and lipids [121]. As a result, bacteria in biofilms are 10- to 1000-fold more resistant to antibiotics than planktonic (motile) bacteria [122, 123]. Of relevance to biomaterials, biofilms can form on the surface of implanted medical devices, such as catheters, valves, stents, and orthopedic prostheses, and even of contact lenses [124]. In dentistry, the plaque that develops on the surface of teeth is also a biofilm [125]. Peptide-based treatments for preventing or eliminating biofilms are becoming interesting due to the development of antimicrobial peptides, which demonstrate broad-spectrum activity against planktonic Gram-positive and Gram-negative bacteria. A subset of these antimicrobial peptides have also demonstrated activity against bacteria in biofilms, both on their own and in combination with other antibiotics [126–132], and a number of examples are summarized in Table 6.

Antimicrobial peptides, a subset host defense peptides, are naturally produced by organisms including animals, fungi, plants, and bacteria, resulting in the identification of more than 2000 such peptides [152]. Antimicrobial peptides typically act rapidly and have a broad-spectrum antimicrobial activity against planktonic cells, while host defense peptides also include those peptides that act as innate immune modulators through anti-infective, anti-inflammatory, wound healing, and/or anti-biofilm activities [153, 154]. Thus, these natural peptides are interesting to explore as potential therapeutics against biofilms. Alternatively, peptide screening methods can be used to identify peptides with antibacterial activity [155]. As the literature on antimicrobial peptides is vast, this chapter will focus on a few key examples of antimicrobial peptides that are naturally derived or identified from peptide screening studies and have shown anti-biofilm activity. Additional emphasis will be placed on

Table 6 Anti-biofilm antimicrobial peptides and their in vitro and in vivo effects

Peptide sequence	Bacteria	Effect	Reference
3002 ILV/RWRIRW-NH ₂	MRSA	Mouse cutaneous abscess model in vivo	[133]
AS10 KLKIAQKIKNFFQKLVLP	<i>C. albicans</i> <i>P. aeruginosa</i> <i>E. coli</i>	Inhibition in vitro	[134]
Battacin 4-Methylhexanoyl-D-Dab-Dab-Dab-L-D-F-Dab-Dab-L-NH ₂	<i>P. aeruginosa</i> <i>P. syringae</i> <i>pv. actinidiae</i> <i>S. aureus</i>	Inhibition/eradication in vitro	[135]
BMAP-27-melitlin KFKKLFKCLSPVIGAVLKVLT	<i>S. aureus</i> <i>P. aeruginosa</i>	Inhibition/eradication in vitro	[136]
BMAP-28 GGLRSLGRKILRAWKKYGPPIIPIRI-NH ₂	<i>S. aureus</i> <i>E. faecalis</i>	Rat ureteral stent infection model in vivo	[137]
CAMA KWKLFKKIGIKFLOSAKKF-NH ₂	MRSA	Inhibition in vitro	[138]
Cyclic lipopeptide 3 12-Aminododecanoic-Dap-D-L-L-D-I-N-D-K	<i>S. aureus</i> <i>P. aeruginosa</i>	Inhibition/eradication in vitro	[139]
DJK-5 vqwrtrvir (D-amino acids) DJK-6 vqwrtrvir (D-amino acids)	<i>P. aeruginosa</i> <i>E. coli</i> <i>A. baumannii</i> <i>K. pneumoniae</i> <i>S. enterica</i>	Inhibition/eradication in vitro <i>C. elegans</i> nematodes in vivo <i>G. mellonella</i> larvae in vivo Mouse cutaneous abscess model in vivo	[140, 141]
D-RR4 wlrrikawlrrika-NH ₂ (D-amino acids)	<i>P. aeruginosa</i> <i>A. baumannii</i>	<i>C. elegans</i> model in vivo	[142]
hep20 ICIFCCGCHRSCHGMCKCT	<i>S. epidermis</i>	Inhibition in vitro	[143]

(continued)

Table 6 (continued)

Peptide sequence	Bacteria	Effect	Reference
IDR-1018 VRLIVAVRIWRR-NH ₂	<i>P. aeruginosa</i> <i>E. coli</i> <i>A. baumannii</i> <i>K. pneumoniae</i> <i>S. enterica</i> MRSA	Inhibition/eradication in vitro	[133, 144]
KT2 NGVQPKYKWWKWWKWW-NH ₂	<i>E. coli</i>	Inhibition/eradication in vitro	[145]
LL-37 LLGDFRKSKEKIGKEFKRIVQRIKDFLRNLRVPTES	<i>P. aeruginosa</i>	Inhibition in vitro	[146]
P10 LAREYKKIVEKLRWLRQVLRTLR	MDR <i>S. aureus</i>	Inhibition/eradication in vitro	[147]
RT2 NGVQPKYRWWRRWW-NH ₂	<i>E. coli</i>	Inhibition/eradication in vitro	[145]
SAAP-148 LKRWWKRVFKLLKRYWRQLKQPVR	<i>A. baumannii</i> MRSA	Mouse skin wound model in vivo	[148]
Tachyplesin III KWCFRCYRGICYRKCR-NH ₂	<i>P. aeruginosa</i>	Inhibition in vitro and rat ureteral stent infection model in vivo	[149]
UP-5 RBRBR (B = biphenylalanine)	MRSA	Inhibition in vitro	[150]
WRL3 WLRAFRRRLVRRARGLRR-NH ₂	MRSA	Mouse infected burn wound in vivo	[151]

peptides that have shown anti-biofilm activity *in vivo* in animal models or that have been used to modify biomaterial surfaces. Anti-biofilm activity is typically demonstrated *in vitro* by the ability to kill multiple species of bacteria that can be present in biofilms, including *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species [131]. *In vivo* activity has primarily been tested in mouse skin wound or cutaneous abscess models as well as rat ureteral catheterization models. In addition to killing planktonic bacteria that can form or detach from biofilms as well as killing embedded bacteria, antimicrobial peptides can also act to interfere with bacterial adhesion and gene expression as well as influence the host response to the biofilm [127]. These latter activities can occur at concentrations much lower than the minimum inhibitory concentration (MIC), which is usually used to characterize the effects of antimicrobial agents on planktonic bacteria [127] and provides a measurement of anti-biofilm activity.

LL-37, P10, AS10, and hep20 Peptides (Naturally Derived)

Of the many antimicrobial peptides, cathelicidin LL-37 (LLGDFFRKSKEKIG-KEFKRIVQRIKDFLRNLPRTES) was one of the first human-derived peptides to demonstrate anti-biofilm activity. It can be found at mucosal surfaces and in the granules of phagocytes, and its concentration at sites of chronic inflammation is higher than baseline levels in most bodily fluids [146]. It is able to prevent biofilm formation *in vitro* at concentrations well below its MIC, and it can also reduce existing *P. aeruginosa* biofilms [146]. Shorter peptides derived from LL-37, such as P10 (LAREYKKIVEKLKRWLRQVLRRL-R), have also shown antimicrobial activity. P10 was more effective at killing the methicillin-resistant *S. aureus* (MRSA) strain LUH14616 than LL-37, and it could eradicate MRSA strains LUH14616 and LUH15051 from thermally wounded human skin equivalents *in vitro* without showing toxicity towards the fibroblasts and keratinocytes within the tissue models [147].

Likewise, the mouse cathelicidin-related antimicrobial peptide (CRAMP) has been shown to inhibit fungal biofilm formation [156]. Shorter peptides based on CRAMP, such as AS10 (KLKKIAQKIKNFFQKLVP), were also able to inhibit *Candida albicans* biofilm growth on Ti disks as well as formation of biofilms from other bacteria [134]. The study also tested the effect of AS10 on human osteoblasts, mesenchymal stromal cells, and endothelial cells *in vitro* and demonstrated no negative effects on cell viability. Further, AS10 did not interfere with calcium deposition by the osteoblasts or mesenchymal stromal cells or with tube formation by the endothelial cells, which suggests that AS10 could be used as an anti-biofilm coating on implants without affecting cells in the surrounding tissues [134].

A second human-derived antimicrobial peptide is hepcidin 20 (hep20; ICIFCC-GCCHRSHCGMCCKT), a 20-amino-acid peptide that is found in the liver. The related hepcidin 25 (hep25) is involved in the regulation of plasma iron levels by binding ferroportin on macrophages, enterocytes, and hepatocytes [157]. Both hep25

and hep20 have demonstrated antimicrobial activity against several bacterial strains [158] with hep20 also showing antifungal activity [159]. Hep20 inhibited biofilm formation of polysaccharide intercellular adhesion (PIA)-positive and PIA-negative clinical isolates of *Staphylococcus epidermis*, most likely by inhibiting the accumulation of extracellular matrix within the biofilm [143].

IDR-1018 and 3002 Peptides (from Peptide Library Screenings)

Innate defense regulator peptide 1018 (IDR-1018) and 3002 are two examples of peptides that have been optimized by peptide library screening methods. Starting from the bovine bacterenecin derivative Bac2a (RLARIVVIRVAR-NH₂), a library of over 100 peptides, each 12 amino acids long, was generated by performing point mutations, scrambling, and deletion of amino acids [160]. The immunomodulatory potential of the peptides from the library was evaluated by measuring the ex vivo induction of monocyte chemotactic protein 1 (MCP-1) and MCP-3 by human peripheral blood mononuclear cells, and IDR-1018 (VRLI-VAVRIWRR-NH₂) resulted in a >50-fold increase compared to Bac2a [160]. IDR-1018 also exhibited broad-spectrum anti-biofilm activity towards *P. aeruginosa*, *E. coli*, *A. baumannii*, *K. pneumoniae*, MRSA, *S. typhimurium*, and *B. cenocepacia* when tested with sub-MIC concentrations [161]. It could also inhibit or eliminate biofilms, as demonstrated by growing *P. aeruginosa* biofilms in continuous-culture flow cells in a minimal medium. The medium flowing through the cells was supplemented with IDR-1018 either during or after biofilm establishment, and in both cases, the treatment led to thinner biofilms that lacked the structural features of mature biofilms or eliminated them entirely (Fig. 9) [161].

The sequence specificity of IDR-1018 was further explored by generating a second peptide library that contained 96 peptides with single amino acid substitutions [133]. This library was SPOT-synthesized on cellulose arrays, and the ability to prevent MRSA biofilms was evaluated. The resulting data were fed into a quantitative structure–activity relationship (QSAR) model, which predicted new peptides with anti-biofilm activity from an in silico library of 100,000 peptide sequences. The identified peptide 3002 (ILVRWIRWRIQW-NH₂) was more effective than IDR-1018 in inhibiting biofilm growth and in eradicating pre-formed biofilms. Further, peptide 3002 was able to reduce abscess size in a mouse cutaneous model of high-density bacterial infection, an MRSA chronic infection model [133].

DJK-5, DJK-6, and D-RR4 Peptides (D-Enantiomeric Peptides)

While peptides represent a potentially powerful therapeutic option, they have some drawbacks for use in vivo including degradation by proteases and inactivation in bodily fluids. The incorporation of non-natural amino acids into synthetic peptides

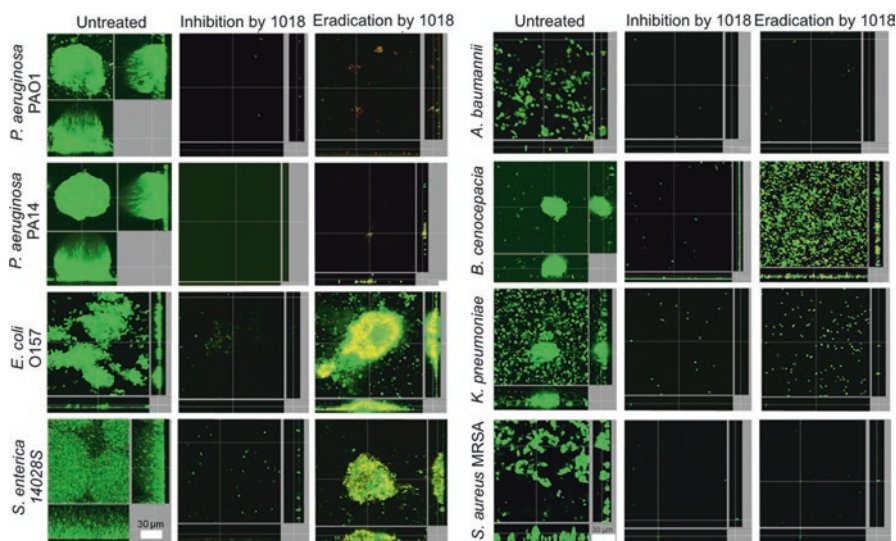


Fig. 9 Inhibition and eradication/reduction of biofilms of different bacterial strains by sub-inhibitory concentrations of peptide IDR-1018, as demonstrated in a flow cell assay. The scale bars are 30 μm . (Reprinted under the terms of the Creative Commons Attribution License from de la Fuente-Núñez et al. ©2014 [161])

can address some of these limitations by providing more stable variants, which may lead to increased activity [140–142, 162]. The synthetic antimicrobial peptides DJK-5, DJK-6, and D-RR4 have been developed including the D-enantiomeric form of amino acids and have shown strong anti-biofilm activity both in vitro and in vivo [140–142]. DJK-5 (vqwrairvrvir; D-amino acids) and DJK-6 (vqwrirvw-vir; D-amino acids) were identified from screening a library of 12-amino-acid-long peptides with the following design constraints: D-enantiomeric forms of only 9 amino acids (A, F, I, K, L, Q, R, V, W), 4 charged residues, 7–8 hydrophobic residues, and 0–1 Q residues. DJK-5 and DJK-6 were identified as being able to inhibit biofilm formation by several common bacterial strains, at concentrations below their MIC, and to eradicate established biofilms [140]. Further, DJK-5 was shown to decrease abscess size and reduce bacterial burden in a mouse cutaneous abscess model induced by *P. aeruginosa* [141].

The peptide D-RR4 (wlrrikawlrrika-NH₂; D-amino acids) was developed to be resistant to proteolytic digestion, as confirmed by stability in the presence of both bacterial (proteinase K) and mammalian (trypsin) proteases, and it remained active in the presence of physiologic concentrations of salt, serum proteins, and acidic pH [142]. It also showed reduced toxicity towards macrophages and keratinocytes when compared to the L-enantiomeric form of the peptide. The in vivo antibacterial activity of D-RR4 was demonstrated by increased survival and decreased bacterial burden of *Caenorhabditis elegans* worms infected with *P. aeruginosa* or *A. baumannii*, after treatment with the peptide [142].

BMAP28, Tachyplesin III, WRL3, and DRGN-1 Peptides (Evaluation In Vivo)

In vitro activity to inhibit or kill relevant bacterial strains as well as to inhibit or eradicate biofilms helps to demonstrate the potential of anti-biofilm peptides. However, ultimately, they must be effective in vivo, and thus the development of relevant animal models is important. As mentioned previously, the survival of *C. elegans* worms and the reduction of cutaneous abscesses in mice are two examples of such in vivo models. However, they lack some of the complex features of implant-associated biofilm formation and chronic infection. A more challenging model is an infected mouse burn wound model. In this case, a region of skin is first scalded and then infected with MRSA. In addition to the size of the wound and the bacterial burden, the effects on host response [interleukin (IL)-6, IL-10, tumor necrosis factor (TNF)- α , and MCP-1 production] as well as angiogenesis can be evaluated. Using this model, the antimicrobial effects of an engineered amphiphathic peptide, WRL3 (WLRAFRRLVRRRLARGLRR-NH₂), was demonstrated (Fig. 10). This confirmed its potential as an anti-biofilm agent as also shown by antimicrobial activity against MRSA and inhibition of biofilm activity in vitro [151]. Likewise, DRGN-1 (PSKKTTPVKPKKVA) enhanced healing in a mouse skin wound model that was infected with a biofilm containing both *P. aeruginosa* and *S. aureus* [163].

As catheters frequently develop biofilms, in vivo models have also been developed to study the effects of antimicrobial peptides on ureteral stent infection. These rat models have involved the implantation of ureteral stents either subcutaneously or in the bladder. The subcutaneous model was used to assess the effectiveness of stents coated with Tachyplesin III (KWCFRVCYRGICYRKCR-NH₂), which is derived from horseshoe crabs and exhibits antimicrobial activity against *P. aeruginosa*. After implantation of the Tachyplesin III-coated stent, *P. aeruginosa* was injected onto the implant surface. The Tachyplesin-III-treated group showed a reduction in bacterial count compared to uncoated stents, and this effect was further enhanced by co-treatment with intraperitoneal injection of piperacillin-tazobactam (TZP) [149]. On the other hand, the bladder implantation model was used with BMAP-28 (GGLRSLGR KILRAWKKYGPIIVPIIRI-NH₂), a cathelicidin peptide. After implantation of the BMAP-28-coated stent, *S. aureus* or *E. faecalis* was inoculated into the bladder. The BMAP-28 coating reduced the bacterial counts for both strains compared to uncoated controls, and this level was further reduced by coadministration of vancomycin intraperitoneally [137].

Immobilization of Antimicrobial Peptides

A final consideration in the development of anti-biofilm peptides is their delivery mechanism. As biofilms tend to form on the surface of implanted devices, peptide immobilization techniques become relevant [164]. Antimicrobial peptides can simply be adsorbed on the surface of materials by soaking them in solutions of the peptides

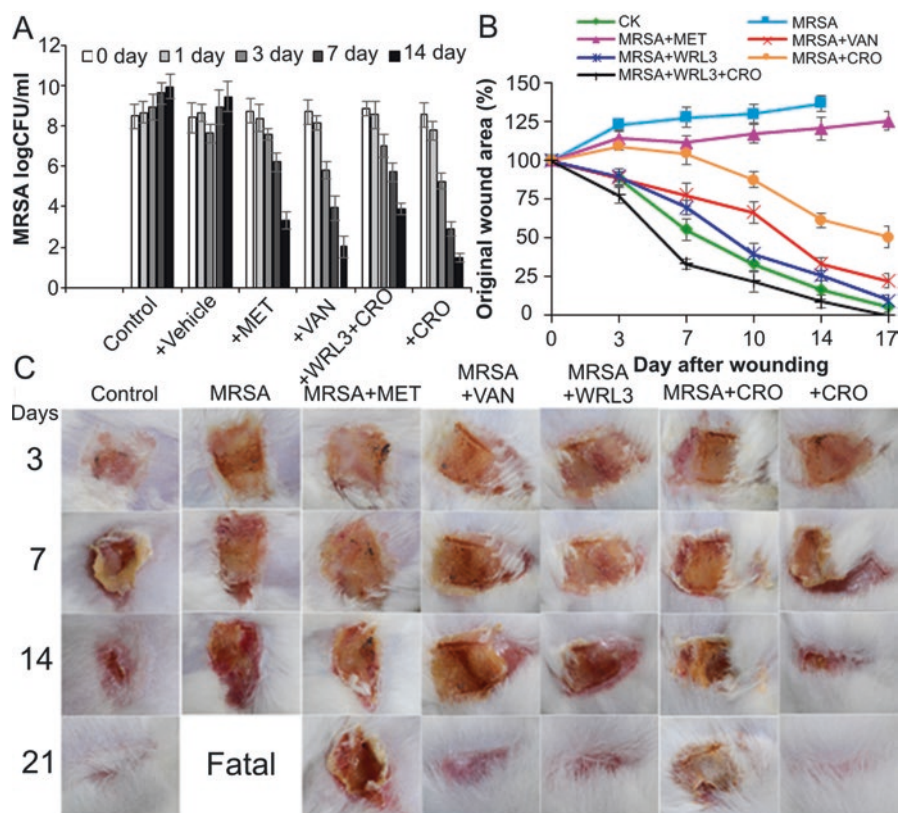


Fig. 10 In vivo antimicrobial activity of WRL3 as demonstrated in a mouse infected burn wound model. (A) Viable bacterial counts in colony-forming units (CFU). (B) Wound area measurements. (C) Images of wounded regions. (Reprinted with permission from [151]. ©2016 American Chemical Society)

[137, 149, 165]. Layer-by-layer films provide a next step in complexity of preparation and controlled release of the peptide. These films are formed from alternating deposition of cationic and anionic molecules (polymers or peptides) [166, 167]. For example, the thickness and stability of the coating, amount of peptide loaded, and the release rates of the antimicrobial peptide ponericin G1 (GWKDWAKKAGGWLKKKGPGMAKAALKAAMQ-NH₂) were affected by the choice of polyanion used, and the film composition also influenced resistance to attachment of *S. aureus* [167].

Polymer brushes provide a means for covalent conjugation of antimicrobial peptides. Ti and quartz surfaces have been functionalized by surface-initiated polymerization of *N,N*-dimethylacrylamide and aminopropyl methacrylamide hydrochloride, resulting in a primary amine functionality. These amine groups were modified to maleimide groups, which then allowed the reaction with cysteine-containing peptides [168, 169]. The presentation of antimicrobial peptide

Tet-20 (KRWIRVRVIRKC) inhibited *P. aeruginosa* and *S. aureus* growth in vitro and *S. aureus* adherence in vivo on subcutaneous implants [169]. Similarly, an allyl glycidyl ether polymer brush with PEG-maleimide spacer was used to bind cysteine-containing peptides to polydimethylsiloxane (PDMS) surfaces [170].

As reduction in biofilm formation and improved osseointegration are important considerations for Ti orthopedic and dental implants, loading of antimicrobial peptides in porous calcium phosphate (CaP) coatings on Ti surfaces has been explored. Coatings containing the peptide Tet213 (KRWWKWRRC) demonstrated antimicrobial activity against *S. aureus* and *P. aeruginosa* bacterial strains while remaining cytocompatible towards MG-63 osteoblast-like cells [171]. A similar coating loaded with HHC36 (KRW-WKWRRC) did not interfere with bone growth around press-fit grafts in a rabbit femoral defect model [172]. A more complex coating combining the CaP with vertically oriented TiO₂ nanotubes and a phospholipid film were also able to provide sustained release and antimicrobial activity of HHC36 [173].

Use of Anti-biofilm and Antimicrobial Peptides Clinically

Similar to the osteoinductive peptides, anti-biofilm peptides as well as antimicrobial peptides more generally have not yet resulted in many products for clinical use [174, 175]. Of the peptides discussed herein, LL-37 has been tested in Phase I/II clinical trials to treat hard-to-heal venous leg ulcers. In this case, the LL-37 improved the healing rate constants [176], but the application was a topical treatment for wound healing and not treatment of biofilms. Polymyxin B and Polymyxin E (colistin) have been used clinically to treat drug-resistant infections resulting from Gram-negative bacteria [177–179]. However, the majority of antimicrobial peptides tested clinically have been used topically in clinical trials to treat infected diabetic foot ulcers, catheter infections, and skin and fungal infections [175] and not for treatment and/or prevention of biofilms. While several anti-biofilm peptides show promise, clinical translation of antimicrobial peptides has met a number of regulatory hurdles [174].

Conclusion/Summary and Future Perspectives

This chapter started out by introducing basic concepts in peptide design, from learning from nature and deriving peptides from active domains within proteins to utilizing peptide library screening methods to identify peptides with a desired response. Standard methods for peptide synthesis, most commonly solid-phase peptide synthesis, and various techniques for functionalizing biomaterials with peptides were summarized. These chemical methods for peptide synthesis bring about additional opportunities in peptide design, as non-natural amino acids and fluorescent tags, among other molecules, can be incorporated during the synthesis. This allows for the

development of peptides that may be more stable or have higher activity than their naturally derived counterparts, and the possibility of adding dyes further provides simplified methods for detecting the peptides. These so-called peptidomimetics have started to be explored for their antimicrobial activities [126, 132, 180], and it would be interesting to explore further osteoinductive peptidomimetics. Covalent and non-covalent binding of peptides to biomaterials also provides a means to localize the peptide and in some cases provide controlled release. It would also be interesting to explore osteoinductive and antimicrobial peptides in the context of other controlled release systems. For example, research is rapidly advancing in the development of stimuli-responsive systems that release their cargo in response to changes in temperature, pH, light, etc., and conjugation to polymers or encapsulation in micro-/nano-particles also provide a means for extended delivery of compounds [181].

Finally, this chapter focused on the development and application of two classes of peptides, those with osteoinductive and anti-biofilm activity. The osteoinductive peptides have been derived for a large part from the active domains of the different BMPs. Their activities have been demonstrated *in vitro* by stimulating osteogenic differentiation of different types of progenitor cells and *in vivo* by promoting bone growth, ectopically or orthotopically. These peptides show promise to support osseointegration when functionalized on the surface of Ti or other inert implant materials. Further work to combine these peptides with newly developed biomaterial scaffolds [182, 183] would help to advance the field of bone tissue engineering. The anti-biofilm peptides presented have been developed from naturally occurring antimicrobial peptides as well as from screening of peptide libraries. Candidate peptides have shown activity against several strains of bacteria that are involved in biofilm formation, and they have also been shown to reduce bacterial burden or wound size in *in vivo* models. Development of more complex animal models that mimic the clinical conditions of biofilm infection would help to further identify and characterize peptides with anti-biofilm activity. Last but not least, the combination of osteoinductive and anti-biofilm peptides would allow for the development of materials that stimulate bone formation while simultaneously protecting against chronic infection. Anti-biofilm peptides have clearly been intended for orthopedic [184] and dental applications, as they have been studied while functionalized on Ti surfaces. Full-length BMPs have been combined with antibacterial agents, such as vancomycin and silver (Ag⁺) [185], so the combination of osteoinductive and anti-biofilm peptides, functionalized on the surface of permanent implants or implemented in tissue engineering scaffolds, is a logical next step.

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