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Review

A review of chemical surface modification of bioceramics: Effects on protein adsorption and cellular response

Wing-Hin Lee[a,b], Ching-Yee Loo[a,b], Ramin Rohanizadeh[a,*]

[a] Advanced Drug Delivery Group, Faculty of Pharmacy, University of Sydney, NSW 2006, Australia
[b] Woolcook Institute of Medical Research, NSW 2037, Australia

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ABSTRACT

Calcium phosphates (CaPs) are ideal biomaterials for bone repair because of the similarities between their chemical structure and the mineral phase of hard biological tissues (e.g., bones and teeth). Since CaP bone grafts exhibit superior biocompatibility and strong osseointegration properties, they have been widely investigated for use as an in situ carrier for delivery of anti-resorptive and osteogenic drugs. The surface properties of CaP govern the affinity and the binding mechanisms between biological macromolecules (e.g., proteins) and the CaP surface, which indirectly determines the interactions between bone cells and implanted CaP biomaterials. These surface properties ultimately play a pivotal role in determining the success of CaP as bone implants and/or drug carriers. This review provides an in-depth discussion of the current methodologies used to regulate the surface chemistry of CaP and their subsequent effects in regards to protein adsorption and delivery, as well as cell/materials interactions.

Contents

1. Introduction

Bone autografting and allografting are commonly used approaches for replacing damaged or diseased bone [1,2]. However, several limitations have been identified and associated with these approaches. In particular, the availability of healthy bone from patients is often limited, and autograft procedures result in additional surgical trauma at the donor site. In addition, the risk of host reaction due to genetic differences and disease transmission from a donor cadaver are potential problems associated with allograft procedures [3,4]. Synthetic bone substitute materials have, therefore, been developed to overcome the significant problems associated with natural graft materials (autografts and allografts).

Among bone substitute materials, calcium phosphate (CaP)-based materials are one of the most suitable for bone-filling, tissue

* Corresponding author at: Faculty of Pharmacy (A15), University of Sydney, Sydney, NSW 2006, Australia. Tel.: +61 2 9351 2355.
E-mail address: ramin.rohanizadeh@sydney.edu.au (R. Rohanizadeh).

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engineering, and drug delivery devices [5]. CaPs are very biocompatible, bioactive, easy to prepare, and inexpensive [6]. In addition, the degradation products of CaP are Ca^{2+} and PO_4^{3-}, which are the major constituents of bone and enamel in the human body [7,8]. Once placed in the body, a CaP implant undergoes a dissolution/reprecipitation process, leading to the formation of bone-like apatite crystals on the surface of implants, which contribute to better osseointegration of CaP implants [9–11]. Depending on the CaP phase, in general, CaP has low solubility under physiological conditions (pH 7.4) but undergoes marginally higher dissolutions in acidic conditions (pH 6.5). This dissolution property makes CaP a suitable drug carrier (e.g., antibiotics) for bone applications, such as surrounding an injured or diseased bone, which is generally associated with a sub-neutral pH [12,13]. Several criteria are taken into consideration during the fabrication or selection of suitable carrier: (i) specific targeting to a selected site in the body, (ii) sustained release of the drug while avoiding burst release, (iii) minimizing pre-mature degradation or clearance of the drug, and (iv) retaining the drug bioactivity.

Today, the scope of the term "drug" in the field of bone tissue engineering has broadened from just antibiotics to include biological macromolecules such as growth factors, proteins, enzymes, and genes. Specifically, delivery of growth factors and drugs, such as bone morphogenetic protein (BMP) [14] and bisphosphonate (BP) [15], respectively, to regenerate bone in defective areas is widely studied. Because of the high affinity for BMP to the CaP surface, CaP has been used as a carrier for BMP, providing slow release of BMP into surrounding bone [16,17]. A list describing the application of CaPs in drug delivery is provided in Table 1. Significant efforts have been devoted to developing CaPs with great ability for delivery of drugs to promote osteogenic activity.

1.1. Different phases of CaP

Depending on temperature and pH during synthesis, several different phases of CaP with varying crystallinity and Ca/P ratios may be synthesized. This includes monocalcium phosphate, dicalcium phosphate, amorphous calcium phosphate, and hydroxyapatite (HA) (Table 2). The various bioactivities and degradation behaviors of these CaPs are listed in Table 2.

1.2. Factors affecting protein adsorption and cell adhesion to CaP

The complex interactions between proteins and the CaP surface are primarily governed by physicochemical properties of the CaP surface (topography, roughness, particle size, porosity, pore size, and charge), functional groups, and the protein conformation. In general, the smaller the CaP particle sizes, the higher the total surface area; and, thus, the greater the number of available binding sites for protein adhesion [36]. Numerous studies have reported that CaP with a higher surface area could be fabricated by modulating the surface roughness, porosity, and pore size [37,38]. Pore size and pore distribution have a significant impact on the total surface area of CaP and, subsequently, their protein adsorption capacities [39]. Meanwhile, the effect of pore size on protein adsorption is size dependent. For example, if the pore size is larger than a protein molecule, the protein can enter into the pores, thereby increasing protein adsorption. Surface roughness and topography influence protein adsorption by affecting the spatial distribution of adsorption sites [40]. Independent of the total surface area, increasing surface roughness on the nanometer scale improves protein adsorption [41]. This increase is attributed to a change in the geometrical arrangement of protein molecules on the surface of materials, and it has been shown that the adsorption is less correlated to the roughness for globular proteins such as bovine serum albumin (BSA) [41]. Protein adsorption is less influenced by roughness on the micrometer scale as the surface topography may appear smooth to proteins. Furthermore, CaPs with lower crystallinity have higher solubility, which in turn causes an inevitable increase in ionic strength in the surrounding medium. Proteins or drugs in solutions with high ionic strength undergo structural conformational changes, exposing more polar ionized charges on the surface of the protein, which promotes a higher adsorption rate onto the material surfaces [42]. Proteins are known to favorably adsorb onto a hydrophobic surface than a neutrally charged hydrophilic surface. Proteins tend to adsorb to a hydrophobic surface by unfolding their hydrophobic core over the surface of the materials [43].

The influence of CaP surface charge and charge density on protein adsorption has been studied in-depth, as many believe that the electrostatic attraction between CaP and proteins is the primary force that drives protein adsorption [44–46]. The charged CaP sites (Ca^{2+} and PO_4^{3-}) can bond with any charged functional groups on protein molecules, such as amino, carboxyl, carbonyl, and aromatic groups [39]. It should also be noted that each protein possesses its own isoelectric point (p*I*) that is determined by the degree of de-protonation and protonation of the carboxyl (COO^-) and amide (NH_3^+) groups, respectively, at various pH values [47]. Bovine serum albumin (BSA), whose p*I* is less than 7 (p*I* = 4.5), is often used as a model acidic protein, while a basic protein is represented by lysozyme with a p*I* above 7 (p*I* = 11.1) [48,49]. Manipulating the surface charge of CaP by selectively immobilizing the surface with biomolecules, such as amino acids, carboxylic acid, or bisphosphonates (BPs) is intended to provide stronger electrostatic interactions between proteins and CaP (Table 3) [49–51].

Surface energy and surface wettability are other factors affecting protein adsorption. The interactions between proteins and the surface of biomaterials are mainly through energetic interactions, such as van der Waals, electrostatic, hydrogen, and hydrophobic bonding. Change in surface energy is, therefore, believed to directly modulate the availability of free charged groups on the surface of biomaterials. An increase in the polar component and a decrease in the dispersive component of a surface will improve surface wettability, which in turn, makes the surface more favorable for protein adsorption [38]. Notably, surface wettability is a direct function of both surface chemical composition (polar and dispersive components) and surface nanoscale topography; therefore, changes in topography also affect surface wettability.

The number and conformation of protein molecules adsorbed onto the CaP surface have been shown to influence cell adhesion. Proteins are rapidly adsorbed onto CaP biomaterials when in contact with blood or physiological body fluids after implantation. These proteins might then serve as ligands (cell adhesion proteins) to bind to cell membrane protein receptors (integrins) leading to cell/materials adhesion. Osteoblasts preferentially bind to a specific amino acid sequence arginine-glycine-aspartic acid (Arg-Gly-Asp; RGD), a region that is widely present on cell adhesion proteins. These cells adhesion proteins (i.e. fibronectin, vitronectin) [52] have a total negative charge at physiological conditions (pH 7.4). When they are adsorbed onto the CaP surface with a positive site (Ca-rich site), the positively charged groups of the protein surface are exposed, which, in turn, promotes cell adhesion on the materials and thereby accelerates tissue regeneration. Cell adhesion is also influenced by properties of CaP such as crystallinity, surface charge, and CaP phases (Table 4). In the following section of this review, we will focus in detail on the effects of surface modification on protein adsorption and cell adhesion.

2. Surface modification

In cases where electrostatic interactions between biomaterials and proteins are the predominant factors, the immobilization

Table 1
Delivery of drugs by calcium phosphates (CaP).

Drug	Drug application	Calcium phosphate	Incorporation methods	Release profile	Effect on microbial/cellular	References
Gentamicin	Antibiotic	Mixture of DCPA and (TTCP).	Encapsulated by PLGA and mix with calcium phosphate.	16–17% of release after 96 days.	Negative effect on fibroblast cell viability.	[18]
		Mixture of β-TCP and MCPM.	Adsorption from liquid.	Between 50 and 100%, depending on poly(acrylic acid) contents.	–	[19]
		Mixture of β-TCP and MCPM.	Adsorption from liquid.	Almost 100% in 80 h.	–	[20]
		Mixture of β-TCP, DCPD and TTCP.	Mix with calcium phosphates or poly-lactic acid.	Average release was 23 μg/day for 2 months.	High concentration of drug was accumulated in rabbit's bone marrow.	[21]
		Mixture of calcium phosphate and alginate	Added during the preparation.	80–90% in within 4 to 6 days.	Reduced the expression of cyclooxygenase-2. Promoted cell viability.	[22]
Vancomycin	Antibiotic	Mixture of TTCP and DCPA.	Added during the preparation.	70% in 7 days.	No significant effect against *Staphylococcus aureus*.	[23]
		Mixture of DCPA, HA and α-TCP (Biopex).	Impregnated into the Biopex.	Release rate from Biopex is slower than PMMA.	The antimicrobial activity is maintained.	[24]
Kanamycin	Antibiotic	Mixture of α-TCP, DCP, TTCP and HA.	Added during the preparation.	Drug delivery profile depended on preparation methods.	The elution was above the minimum inhibitory concentration (MIC).	[25]
Ciprofloxacin	Antibiotic	Equimolar of β-TCP + MCPM	Added during the preparation.	Depended on the porosity of cement system.	–	[26]
Recombinant transforming growth factor beta-1 (rhTGF-β1)	Treatment of bone defects	Mixture of α-TCP, DCPA, calcium carbonate and HA	Mixing during the preparation		Improved 50% of bone volume compared to control.	[27]
Recombinant bone morphogenetic protein-2 (rhBMP-2)	Treatment of bone defetcs with impaired healing	Calcium phosphate cement (CPC) and gelatin	BMP loaded into gelatin microspheres and mix with CPC	Formulation with gelatin had higher BMP release	With the presence of gelatin, higher rate of bone healing in osteoporotic patients could be achieved	[28]
	Treatment of bone defects	Mixture of α-TCP, DCPA, calcium carbonate and HA	Administrated onto the CaP implants material		Bone formation was only observed on CaP loaded rhBMP-2	[29]
Recombinant human basic fibroblast growth factor (rhbFGF)	Treatment of bone defects	TCP	Mix with TCP		Inhibited new bone formation	[30]

Abbreviations: Dicalcium phosphate anhydrous (DCPA); Tetracalcium phosphate (TTCP); β-Tricalcium phosphate (β-TCP); Monocalcium phosphate monohydrate (MCPM); Dicalcium phosphate dihydrate (DCPD); Hydroxyapatite (HA); α-Tricalcium phosphate (α-TCP); Dicalcium phosphate (DCP).

Table 2
Properties of different phases of CaP [8,31–35].

Phases of CaP	Chemical formula	Ca/P ratio	Solubility equilibrium (g/L)[a]
Monocalcium phosphate monohydrate (MCPM)	$Ca(H_2PO_4)_2{\cdot}H_2O$	0.5	~18
Dicalcium phosphate dihydrate (DCPD)	$CaHPO_4{\cdot}2H_2O$	1.0	~0.088
Dicalcium phosphate anhydrous (DCPA)	$CaHPO_4$	1.0	~0.048
Octacalcium phosphate (OCP)	$Ca_8(HPO_4)_2(PO_4)_4{\cdot}5H_2O$	1.33	~0.0081
α-Tricalcium phosphate (α-TCP)	$\alpha\text{-}Ca_3(PO_4)_2$	1.5	~0.0025
β-Tricalcium phosphate (β-TCP)	$\beta\text{-}Ca_3(PO_4)_2$	1.5	~0.0005
Amorphous calcium phosphate (ACP)	$Ca_XH_Y(PO_4)_Z{\cdot}nH_2O$, $n = 3–4.5$	1.2–2.2	[b]
Calcium-deficient hydroxyapatite (CDHA)	$Ca_{10-X}(HPO_4)_X(PO_4)_{6-X}(OH)_{2-X}$ $(0 < X < 1)$	1.5–1.67	~0.0094
Hydroxyapatite (HA)	$Ca_{10}(PO_4)_6(OH)_2$	1.67	~0.0003

[a] Measured at 25 °C in water.
[b] Solubility depends on the stoichiometry.

Table 3
Effect of CaP properties on protein adsorption.

Properties	Influence on protein adsorption	References
Surface roughness	Depending on protein structure, in general, protein adsorption increases with increasing surface roughness on the nanometer scale. This is beyond the accompanying increase in surface area.	[41]
Microporosity and particles size	Higher microporosity and smaller particles size induce better protein adsorption due to the higher surface area. Therefore more binding sites are available for protein adsorption.	[37]
Surface charge and ionic strength microenvironment	The distribution of charge on the surface of CaP provides strong electrostatic interaction between materials surface and protein. Optimum ionic strength could promote protein adsorption through regulation of protein surface charge and its structural stability.	[48–50]
Crystallinity and types of CaP	Composition and crystallinity of CaP greatly influence its solubility that could further change the ion concentration and local pH, affecting protein adsorption.	[36]

Table 4
Effect of CaP properties on cell adhesion.

Properties	Effect on cell adhesion	References
Surface roughness	Cell adhesion is higher on rougher surface. However, effects of roughness at nano- or micron-scale are not conclusive due to the conflicting data.	[53]
Crystallinity and type of CaP	Crystallinity and type of CaP govern solubility and thereby ionic strength, affecting cell adhesion.	[54,55]
Protein and peptide	The presence of protein or peptide could be an intermediate linker to cell adhesion proteins such as fibronectin or vibronetin.	[6,56]
Surface charge	Cells generally have slight negative charges. By introducing net positive charges on CaP surface, the interactions between cells and materials could be stronger. In addition, charged surfaces could also bind to cells adhesive proteins. Surface charge of biomaterials imparted by different functional groups results in variable cells response.	[39,57]

of charged biomolecules onto biomaterials is beneficial because, through this, proteins may be oriented in a manner that is optimal for binding to their respective charged sites. With this technique, a wide range of drugs or proteins could be loaded onto the surface of biomaterials and could minimize some drug delivery-associated drawbacks, such as premature degradation and rapid systemic clearance. In general, surface modification may be classified into three groups: (i) physical, (ii) chemical, and (iii) biological [58].

2.1. Physical immobilization

Direct immobilization or physical immobilization is the simplest way to impregnate target protein onto the CaP surface in which proteins are adsorbed via weak non-covalent interactions, such as electrostatic, van der Waals, hydrogen bonding, and/or hydrophobic interactions. As previously mentioned, electrostatic interactions are attributed to the attractive or repulsive forces between charged sites of both proteins and CaP, which is determined by the p*I* of proteins and the charged state of the adsorbent (CaP). Therefore, the regulation of environmental conditions (i.e. pH, ionic strength, protein concentration) [50] could be highly effective to modulate a materials' capacity for protein adsorption. The advantages of physical immobilization methods include the structural preservation of protein molecules, and in theory, these methods could be applied to most types of native surfaces. Due to the non-specific nature of this method, the binding affinities of proteins are often relatively low. The blockage of binding sites and non-specific interaction of proteins with their surroundings adversely lead to either low adsorption or loss of protein activity. It has also been shown that desorption of proteins into surrounding medium occurs easily as this is a weak interaction between the protein and the CaP surface. It is often possible to observe heterogeneous, multilayered adsorbed proteins onto the surface of CaPs in which the outer layers have weaker bonding and are easily diffused into the medium [48]. This binding stability is further exacerbated with variations in pH and ionic strength in the microenvironment. Therefore, reproducibility and reliability of results is relatively low (Fig. 1).

2.2. Chemical immobilization

Chemical modification is based on the concept of altering the surface energy, charge, and composition of a material. This may be achieved with methods, such as covalent immobilization of organic functional groups, ion-bombardment, sol–gel, and treatment with acid/alkaline. Through covalent immobilization, proteins are strongly bound onto the surface of CaP with linkers, such as silanes, mercaptans, and catechol [58–60]. The functional groups of proteins present in side chains, such as of amines, carboxylates, thiols, or hydroxyls, attach to the linkers on the surface of materials [61]. The toxicity of these linkers (coupling agents) varies based on their molecular structures, dictating their potential biomedical applications. For example, using a silane coupling agent, aminopropyl-triethoxysilane (APS), collagen fibers were immobilized on the surface of tantalum [62]. The prepared materials showed excellent biocompatibility both in vitro and in vivo, and the linker system did not affect cell proliferation. Chemical immobilization is not selective as more than one copy of functional groups is present on proteins. Moreover, in chemical immobilization, strong chemical bonding between the protein functional groups and the surface of the material limits the release of the attached protein.

2.3. Bioaffinity immobilization

Bioaffinity immobilization of a biomolecule (enzyme/protein) occurs when a biomolecule is immobilized via bioaffinity interactions with other biomolecules. In general, this type of immobilization is not widely desired as it could result in enzyme/protein inactivation due to the blockage of their active sites [63]. However, in some scenarios, bioaffinity immobilization of biomolecules on the surface of bioceramics could be beneficial as the interactions between these biomolecules can improve the overall biological activity of the bioceramic. For example, it was shown that HA precoated with RGD peptide then over-coated with serum increased mesenchymal stem cell (MSC) attachment and spreading on HA, compared to either coating alone [64]. Interestingly, the added stimulatory effect of RGD and serum on MSCs was only observed when HA was coated with low concentrations of RGD. The authors suggested that at high densities, RGD changed the conformation

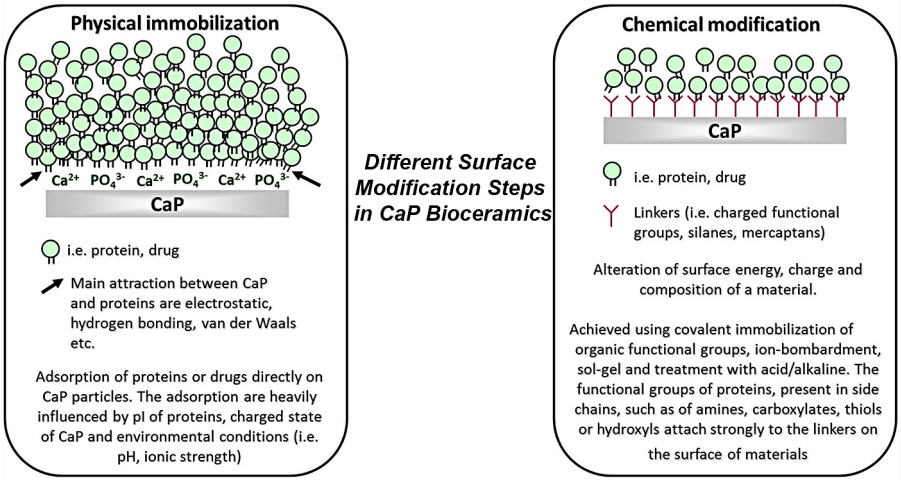

Fig. 1. Types of surface modification processes commonly used for modifying CaP particles to achieve optimal protein or drug adsorption and cells–CaP interactions.

of absorbed osteogenic proteins (e.g., fibronectin) such that the proteins did not engage or fully activate cell-surface receptors.

2.4. Surface functionalization with bisphosphonates (BPs) and phosphate-based molecules

BPs are widely used as anti-resorptive agents for bone disease as they have high binding affinity toward bone and could act as physiological regulators of calcium metabolism [14,65,66]. The binding affinities of six clinically used BPs to HA are ranked in increasing order as follows: clodronate < etidronate < risedronate ∼ ibandronate < alendronate < zoledronate. This variation in their binding affinities to HA is a phenomenon best explained by the differences in the BP side chain, interfacial tension, and HA surface charge [67]. These differences have an apparent effect on the potency and pharmacokinetics of respective BPs in clinical settings [67].

BPs could be applied as protein linkers either through: (i) direct immobilization of BP onto the CaP surface followed by protein adsorption [68,69], or (ii) conjugation of BP with the protein followed by immobilization onto the CaP surface [51,70,71]. In a previous study, HA was functionalized with alendronate via adsorption and subsequently characterized for its affinity toward myoglobin, a neutral protein at pH 7.4 [69]. Alendronate-functionalized HA effectively hindered the adsorption of myoglobin while maintaining the coordination state of the heme moiety in myoglobin. Schuessele and co-workers compared the protein adsorption behavior and biological activity of an HA surface modified using two different approaches, including aminobisphosphonates pamidronate/alendronate immobilization or amino-silanization [68]. With regard to the amount of adsorbed proteins (lysozyme or BMP-2), both modification procedures led to similar results. However, the bisphosphonate-based surface modification resulted in higher lysozyme enzymatic activity and osteoblastic differentiation compared to the silanized HA [68].

HA modified with pyrophosphoric acid showed tremendous affinity toward both acidic (e.g., BSA) and basic proteins (e.g., lysozyme) even though the modified HA particles were negatively charged [72,73]. This enhancement of BSA adsorption suggested that the interaction was due to hydrogen bonding between oxygen and OH^- groups of pyrophosphoric acid and BSA, respectively. Meanwhile, stronger electrostatic affinities have been expected to occur between lysozyme and modified HA due to the differences in the net charge on both surfaces, leading to the higher adsorption rate of lysozyme when compared to BSA [72]. Simultaneously, pyrophosphoric acid-anchored HA particles were shown to exhibit a branched structure, which indirectly increased their surface binding sites available to proteins. As observed, proteins with a smaller molecular weight and size, such as lysozyme and myoglobin (Mw ∼ 20 kDa), were entrapped within the pyrophosphoric acid branches while these branches effectively blocked larger proteins (i.e. BSA) [72]. The same group further clarified the interactions between 'free' pyrophosphate ions and various model proteins on a material's adsorption behavior. The presence of pyrophosphate ions, irrespective of whether additional sequences were added either before, after, or simultaneously with proteins, essentially delayed the adsorption of acidic proteins (i.e. BSA) onto HA by rigorously competing for C-sites (Ca-rich sites) [72]. Since the affinity of pyrophosphate ions toward HA is much higher than that of BSA, pyrophosphate ions preferentially adsorb onto the HA surface while simultaneously the BSA molecules desorb from HA [72]. For basic proteins such as lysozyme, the compression of the electric double layer around the protein molecules by pyrophosphate ions resulted in reduction of the lateral electrostatic repulsions between lysozyme molecules and the HA surface, which in turn enhanced the adsorption of closely-packed monolayer lysozyme molecules [73].

Phosphate-based organic modifiers demonstrated enhanced effects on protein adsorption onto CaPs. Oleyl phosphate-grafted HA regulated the amount and type of proteins that could be adsorbed onto HA. As the amount of grafted oleyl phosphate increased, the surface of modified HA became more hydrophobic and carried a greater net negative charge. However, the increase in electrostatic repulsive forces between negatively charged BSA and oleyl phosphate-grafted HA was compensated by the significantly higher increase of hydrophobic interactions between them, which, thus, unexpectedly promoted BSA adsorption. As speculated, for proteins with opposite charges, the dual beneficial factors (stronger hydrophobic and electrostatic interactions) contributed to even greater protein binding to HA [74,75]. In addition, the correlation between the oriented immobilization of proteins and the preservation of their bioactivity was recently reported [61].

2.5. Surface functionalization by amino acids and carboxylic-based molecules

Amino acids are important biological compounds of amphoteric nature, characterized by the presence of NH_3^+ and COO^- functional groups along with side chains specific to each amino acid. Based on these functional and side chain groups, each amino acid possesses a

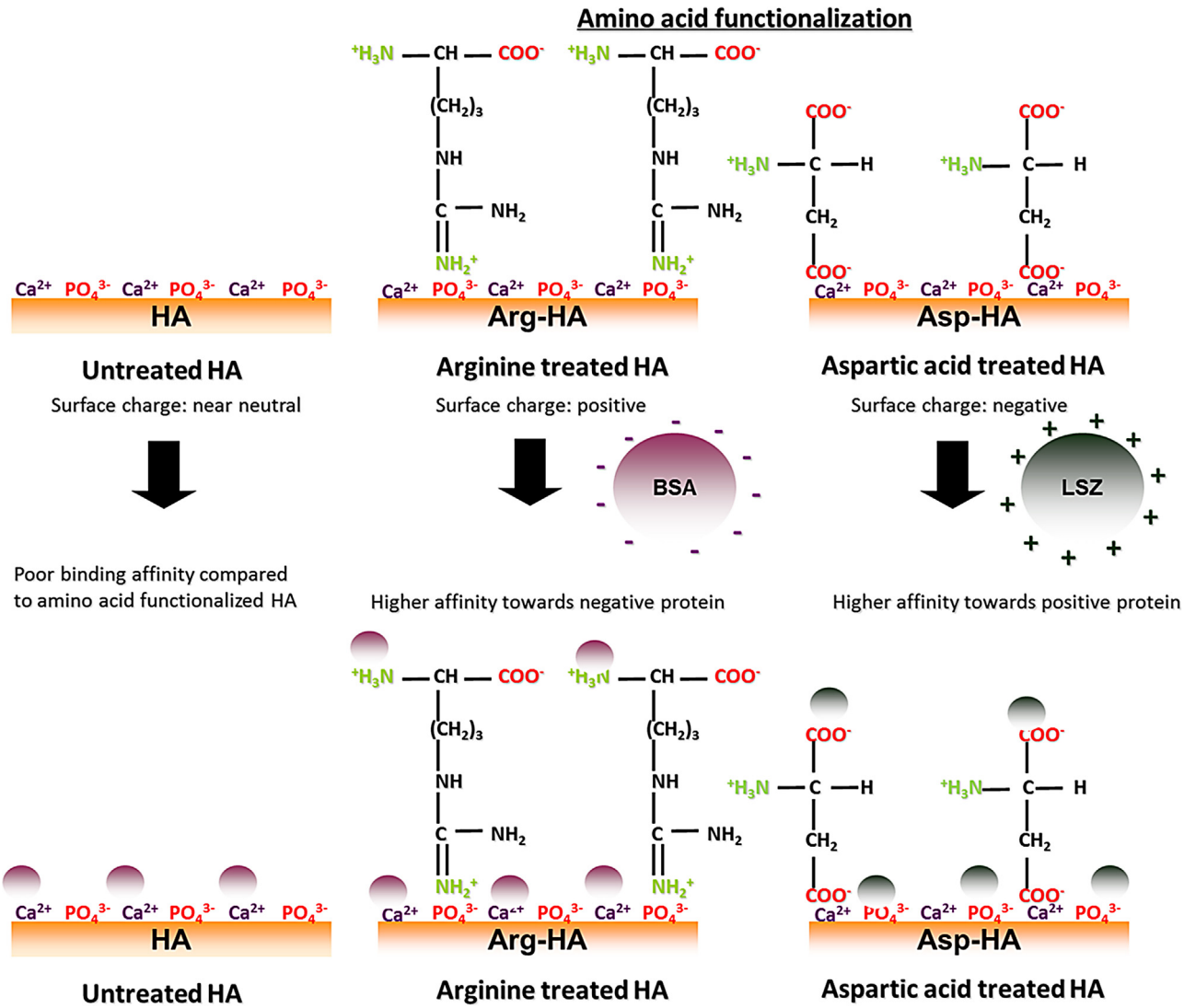

Fig. 2. The effects of functionalizing HA with amino acids possessing different charges at physiological conditions. Since Arg and Asp are positively and negatively charged, respectively, treatment of HA with these amino acids alters the particles' surface charges. Arg-treated HA has higher affinity toward BSA due to the presence of additional NH_2^+ groups contributed by Arg. Meanwhile, Asp-treated HA demonstrated higher affinity toward lysozyme.

different p*I* and, hence, variable net surface charge. The immobilization of acidic or basic amino acids onto HA during HA precipitation can be modulated by adjusting the pH of medium to favor the electrostatic attraction between the amino acid and the HA surface. It was shown that the presence of charged amino acids on HA surface increases or decreases the uptake/adsorption of polar proteins based upon electrostatic interactions between charged surfaces [48,49,76]. Uddin and co-workers demonstrated that the affinity of cytochrome C was increased when HA was functionalized with aspartic acid (Asp). They also confirmed the role of COO^- in promoting cytochrome C adsorption by blocking its functional groups with excess NH_3^+, which resulted in the reduction of protein adsorption [76].

HA was also functionalized with amino acids with three different surface polarities: neutral (serine; Ser, asparagine; Asn); positive (arginine; Arg), and negative (aspartic acid; Asp) [48]. The immobilization of an acidic amino acid (Asp) onto the HA surface enhanced the negative charges of the surface (Fig. 2), which was consistent with the findings in another study by Jack and co-workers whereby amino acid addition on carbonated apatite significantly varied the zeta potential [47]. These changes in the net surface charge of HA upon immobilization of amino acids reflected the complex interactions between the Ca^{2+} and PO_4^{3-} sites of the HA surface with the COO^- and NH_3^+ termini of amino acids. Palazzo and co-workers suggested that when HA was precipitated in the presence of Asp, Asp bound to the Ca^{2+} ions in the precipitating solution through its COO^- groups, thereby decreasing the number of free Ca^{2+} in the solution, which increased the PO_4^{3-}, NH_3^+, and unbound COO^- groups on the Asp-HA surface [77]. The exact opposite trend was observed for positively charged amino acids such as Arg [48]. Interestingly, HA particles treated with amino acids via a simple physical precipitation process demonstrated selective uptake of proteins depending on the surface charge of the prepared materials. Based on the experimental results, high binding affinity of lysozyme toward amino acid-treated HA carrying a negative charge was observed, while BSA preferentially adsorbed onto positively charged amino acid-treated HA particles [48].

Treating HA with amino acids possessing longer side chains also indirectly influenced both lysozyme and BSA adsorption onto HA [49]. Due to the steric hindrance of 'long and bulky' side chains, the arrangement of HA crystals was disrupted, thus, lowering the crystallinity of amino acid functionalized-HA. Furthermore, manipulation of external factors, such as pH and ionic strength, during protein adsorption can significantly improve its binding affinity.

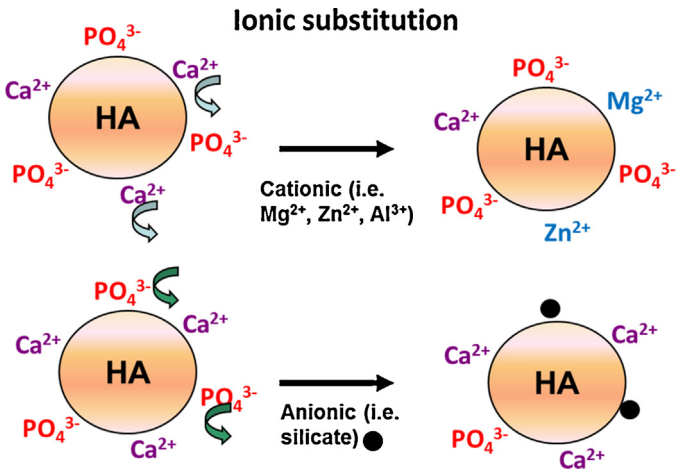

Fig. 3. A schematic representation of ion substitution (cations or anions) in CaP.

For instance, slightly acidic medium conditions (pH 6.4) resulted in increased adsorption of lysozyme glycine functionalized-HA [49].

Positively charged HA nanocrystals were synthesized in the presence of β-alanine (β-Ala). It was reported that by increasing the β-Ala-to-Ca^{2+} ratio during HA preparation, the resulting rod-like HA particles showed an increase in length with no appreciable changes in width. As expected, the affinity of β-Ala-HA toward BSA was dependent on the amount of β-Ala used, in which the highest BSA adsorption occurred for the HA particles synthesized with a β-Ala-to-Ca^{2+} ratio between 0.4 and 1.0 [78].

By immobilizing a compound consisting of one or more COO^{-} groups onto the HA surface, it was expected that the uptake of a basic protein would be significantly enhanced [50,79]. Ishihara and co-workers showed that cytochrome C adsorption onto HA functionalized with mercaptosuccinic acid (Mer) increased as a function of Mer concentration, while demonstrating suppressed binding toward BSA. The Mer-treated HA particles also had a higher protein desorption rate due to their low crystallinity and, thereby, a high dissolution rate [79]. In a recent study, HA treatment using citric acid (CA) also resulted in a similar outcome whereby the affinity of lysozyme toward CA-functionalized HA increased by ~2 fold compared to the untreated HA [50].

2.6. Ion substitution

HA is susceptible to ionic substitution (Fig. 3). Many studies have reported the substitution of Ca^{2+} with other cations, such as monovalent (Ag^{+}), divalent (Mg^{2+}, Zn^{2+}, Cu^{2+}, Sr^{2+}, Ca^{2+}, Pb^{2+}), and trivalent (Al^{3+}, Fe^{3+}) [80–85] ions. The metal ion-doped HAs stimulated osteoblast proliferation and differentiation and, thereby, bone formation. In addition, HA doped with antibacterial ions, such as Ag^{+}, Zn^{2+}, and Cu^{2+}, displayed excellent bactericidal effects [84]. Substitution of PO_4^{3-} in the HA structure with anionic compounds has also been reported [86–89]. Zn^{2+} is a trace element essential for bone growth stimulation in vivo. Studies have shown that other trace elements such as Mg^{2+} and Sr^{2+} also behaved in a manner similar to Zn^{2+} to promote bone growth. The incorporation of Zn^{2+} into the HA lattice structure has also been shown to affect protein adsorption [37,80,83,90,91]. Independent studies by Webster's, Ergun's, and Fujii's groups showed that BSA binding affinities decreased with increasing Zn content in HA [37,90,91]. However, Dasgupta and co-workers showed that Zn-doped HA exhibited preferential uptake of BSA compared to Mg-doped HA, and undoped HA [80]. Since the ionic radii of both Zn^{2+} and Mg^{2+} are significantly smaller than Ca^{2+}, their substitution into the HA lattice results in crystal lattice distortion; as evident from the reduction of the *a*-axis and the increase in the *c*-axis of doped HA. Consequently, the interplanar distance between Ca and Ca in the HA structure was increased, which suggested a reduction in lateral electrostatic repulsion and allowed stronger electrostatic interactions between C-sites of HA and larger molecules like BSA [80,92].

In a study by Fujii and co-workers, two basic proteins, BSA and β_2-microglobulin (β_2-MG), demonstrated different adsorption profiles to Zn-doped HA [37], indicating that surface charge, surface area, and particle crystallinity are not the only factors that control protein adsorption onto ion-doped HA. In addition, despite the higher specific surface area of doped HA particles, the amount of BSA adsorbed onto Zn-doped HA decreased, ruling out the effect of surface availability for binding. Meanwhile, Hayakawa and co-workers also observed that the affinity of β_2-MG toward Zn-doped HA increased with higher Zn content at physiological conditions (pH 7.4), while BSA adsorption remained constant under the same conditions [83]. The electrostatic interactions did not seem to favor the binding between BSA molecules and Zn-doped HA. Two possible reasons were suggested: (i) although BSA and β_2-MG molecules are substantially larger than Zn-doped HA, the mesopores of the particles could still accommodate the penetration of β_2-MG, and the postulated enhanced adsorption effect due to the mesopores is, therefore, negligible in the case of BSA [37]; (ii) the Zn-for-Ca substitution yielded more active sites for β_2-MG binding possibly through the formation of electrical double layers around the HA particles [83].

HA doped with trivalent metal ions, such as aluminum (Al^{3+}), ferric (Fe^{3+}), and lanthanum (La^{3+}), also offered an alternative route for regulating protein adsorption by affecting the mean particle length and hydrophilicity of HA particles [93]. Among these trivalent ions, Fe-doped HA resulted in 2.7 fold higher BSA adsorption compared to undoped HA. This was because of the elongation of mean particle length of Fe-doped HA and the surface hydroxyl ions, such as $Fe(OH)^{2+}$ or $Fe(OH)_2^{+}$, inducing hydrogen bonding between BSA and Fe-doped HA particles [93].

Kandori's group has published several studies on the adsorption of acidic and basic proteins on strontium (Sr)-doped HA [81,82,94–96]. The incorporation of Sr^{2+} into the HA lattice is advantageous to promote protein adsorption as extremely high affinity of Sr-doped HA toward BSA was reported [82,96]. This is attributed to the larger size fraction of Sr-doped HA particles than the undoped HA, as well as the conformation of exposed crystal planes on the particles' surface [82,96,97]. For instance, according to Kawasaki and co-workers, the positively charged C-sites present in the *bc* or *ac* planes, while the P-sites (PO_4-rich site) formed by PO_4^{3-}, were clustered at the *ab* crystal planes [97]. Sr^{2+} influenced the crystal growth along the *ac* planes, which inadvertently resulted in larger crystal fractions at the C-sites [82,97]. Interestingly, myoglobin, a neutral protein at pH 6, adsorbed onto Sr-doped HA at a faster rate compared to charged protein molecules such as BSA and lysozyme. Presumably, the interactions between myoglobin and HA relied on van der Waals force without involvement of electrostatic interactions [81]. In the case of lysozyme, their findings demonstrated that the amino groups of lysozyme molecules were anchored on the negatively charged site of Sr-doped HA formed by six oxygen atoms of PO_4^{3-} on the *ab* crystal planes [94].

Silicon (Si) is the most abundant trace element found in the human body, and it plays a crucial role in promoting calcification during early bone formation. It was previously reported that Si-for-PO_4 substitution caused significant modifications in the surface topography of HA (wettability, porosity, roughness, surface charge), such that these changes resulted in variable interactions with proteins [86,98–100]. To provide further understanding on how proteins behave in textured, Si-doped HA surfaces, a combination of three simulation approaches (molecular dynamics, steered molecular dynamics, and density functional

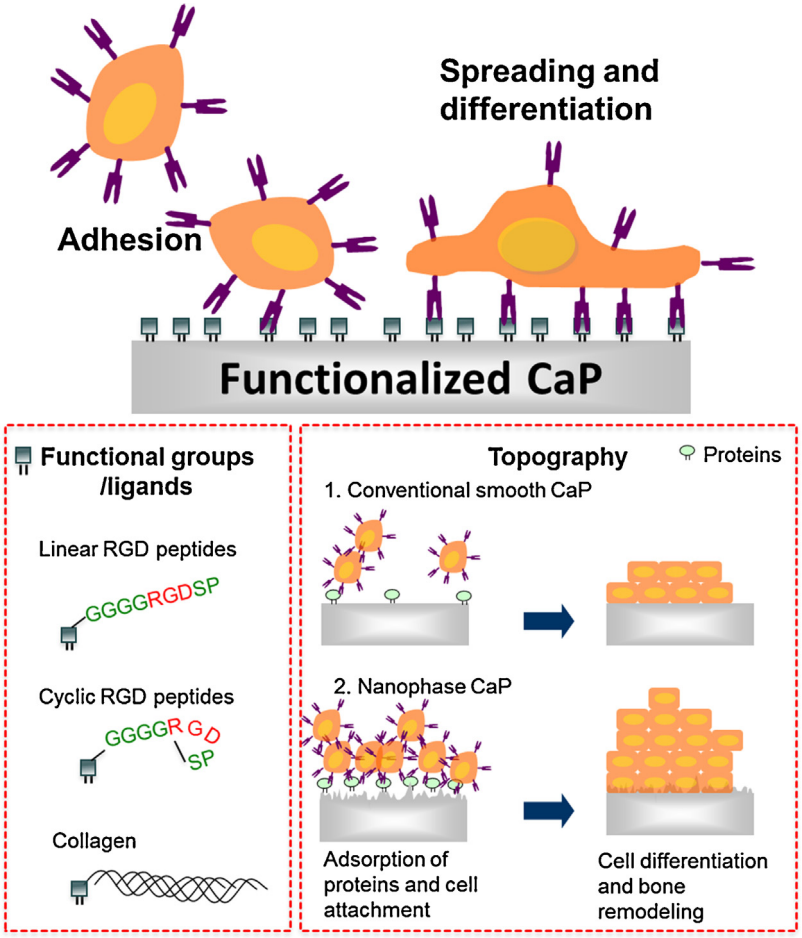

Fig. 4. Surface modification of CaP to improve cell attachment, spreading behavior, and differentiation.

theories) were used in conjunction with leucine-rich amelogenin protein as the model protein [86]. The authors found that the excess amount of incorporated Si ion on the HA surface contributed to a shielding effect composed of charge repulsion and steric hindrance. The pool of Si ions on HA surfaces increased the surface negative charge, which then drew neighboring H^+-based groups toward the surface while simultaneously repelling COO^- groups. In addition, 'bulky' Si ions provided a steric effect and prevented tight coupling between Ca^{2+} and COO^- groups [86]. Si-doped HA containing 0.8 wt% Si showed preferential adsorption of serum and fibronectin compared to undoped HA [98,100]. Recently, a microporous Si-doped HA that mimicked the interconnected struts and microporosity of bone were fabricated to establish a relationship between protein adsorption and topography of HA struts [99]. The microporosity of Si-doped HA did not abolish the enrichment of the protein layer adsorbed onto the Si-doped HA but, rather, appeared to accelerate this action. Similarly, both fibronectin and vitronectin appeared to selectively adsorb onto Si-doped HA compared to undoped HA [99].

Substitution of ions for Ca or PO_4 alters the Ca/P ratio of bioceramic implants, changing the solubility rate and thereby the concentration of extracellular Ca^{2+} around the implants in vivo [101]. It has been shown that the extracellular Ca ions modulate the behavior of bone cells and differentiation of MSCs to osteoblasts [101,102]. Thus, the difference between the Ca/P ratio of a bioceramic and that of bone will make the bioceramic a less "bone-like" material, which affects bone cell proliferation and differentiation.

2.7. Other surface functionalization methods to modulate protein adsorption to CaP

Recently, altering the surface charge of HA to accelerate bone growth has been a topic of interest in biomaterials research. Tarafder and co-workers used an electrical polarization technique to study BSA adsorption onto and its release from biphasic CaP [103]. BSA adsorption was the highest on the P-poled surface (positive charges) and the lowest on the N-poled surface (negative charges). A low-energy nitrogen ion implantation method was also employed to provide selective modification and enhance osseointegration, cell adhesion, and proliferation [104,105]. Ion-implanted HA showed negligible changes in crystallite size and lattice parameters but revealed a significant decrease in resistivity, surface roughness, and wettability. More importantly, modified HA using this approach demonstrated higher bioactivity and protein adsorption in vitro [106].

Zurlinden and co-workers reported that model proteins ubiquitin and recombinant human (rh) BMP-2 could be effectively non-covalently and covalently immobilized on APTES-silanized HA surfaces [107]. While only a limited amount of protein was adsorbed to non-functionalized HA, the APTES-silanized HA surface supported up to 9.7 mg/g protein adsorption. In desorption experiments, it was shown that proteins attached via a covalent linking method and had a higher half-life compared to the passive adsorption method. Schickle and co-workers tailored composites of tricalcium phosphate (TCP) and bioactive glass with high loading efficiency for rhBMP-2. The immobilization of rhBMP-2 was

achieved via two different strategies: (i) covalent linking by treating the surface with chrome sulfuric acid/APTES carbonyldiimidazole; and (ii) non-covalent linking by treating the surface with chrome sulfuric acid and APTES [108]. At least a 2-fold higher adsorption rate of rhBMP-2 was observed on the surface treated with carbonyldiimidazole compared to the non-covalently linked surface [108].

3. Effects of CaP surface modification on cellular response

Today, several mediators or active compounds that possess the ability to promote cell proliferation, adhesion, and differentiation have been identified [109]. Proteins containing an RGD sequence and amino acids are the early agents that were applied to induce a desired cellular response [110] (Fig. 4). In comparison with proteins, peptides are easier to use since they are inexpensive, smaller, easily synthesized, resistant to degradation, and contain specific amino acids responsible for particular cell activities [110]. Okamoto and co-workers demonstrated that an RGD sequence regulated the specific adhesion behavior of osteoblasts on different materials. Based on their findings, the tight adhesion of cells onto HA was inhibited in the presence of RGD peptides, while this was not observed for titanium surfaces, thereby indicating that cell adhesion to HA was tightly regulated by RGD domains, but not for titanium [111].

Considerable efforts have been given to synthesize and/or immobilize recombinant peptides onto CaP and to evaluate their stability and release [54,64,111–116]. One of the strategies used to functionalize the surface of HA with these synthetic peptides was ionic immobilization, in which the linkage closely mimics the natural mechanism of protein attachment found in bone sialoprotein, osteonectin, and statherin [117–119]. These peptides typically contain a sequence of negatively charged amino acids, which act to bind to the positive regions of bone mineral [115]. Synthetic peptides with a polyglutamate sequence, E7 or E_7PRGDT, were used to functionalize the HA surface. It was assumed that E_7PRGDT was bound to HA via the polyglutamic acid sequence while exposing the RGD domains of the outer layers [112]. An early report by Fujisawa and co-workers hinted that HA immobilized with E_7 peptide showed marked improvement in MC3T3-E1 osteoblastic cell adhesion to the peptide-treated HA [112]. Concurrently, this peptide also favored the attachment and differentiation of murine KUSA/A1 osteoblasts and subsequently improved HA osseointegration [120]. Sawyer and co-workers evaluated the MSC adhesion on RGD immobilized-HA with and without E_7 conjugation [115]. They found: (i) E_7RGD adsorption was significantly higher and more tightly bound to HA after implantation in vivo than compared to non-E_7 conjugated RGD; (ii) E_7 conjunction with RGD did not interfere with the bioactivity of RGD; (iii) MSC adhesion occurred through RGD domains, thus, demonstrating that E_7 not only improved the RGD binding to HA, but it also did not disrupt the ability of the RGD sequence for cell recognition, and (iv) the presence of serum coupled with a low concentration of E_7RGD increased cell binding and spreading behavior compared to E_7RGD or serum alone, thus, indicating that the ionic linkage provided more available sites for potential adsorption of non-specific serum proteins [115]. However, high concentrations of E_7RGD with serum coating (100 μg/mL) hindered spreading of cells. It was suggested that, using a higher peptide concentration could alter the cell behavior by changing the conformation of the serum. The same group further investigated the reason behind the diminishing effect of high peptide/serum concentration on cell spreading and adhesion behavior [64]. For this, MSCs were used as a model to evaluate cell adhesion to RGD-functionalized HA coated with serum [64]. Similar to their previous findings, MSC attachment and spreading was stimulated in the RGD and serum coating groups; but only at low concentrations of RGD. MSC attachment was completely inhibited at high concentrations of RGD, thus, again suggesting that the RGD density could be detrimental to the osseointegration of HA-based materials [64]. In another study to evaluate the effects of collagen mimetic peptides on osteoblastic differentiation of MSCs, the surface of HA was coated with DGEA, P15, and GFOGER. Osteoblast-specific markers, such as osteocalcin and alkaline phosphatase, were upregulated in MSCs adhering to the DGEA and P15-coated HA in the presence or absence of differentiation-inducing media [121]. Bone formation on HA implants was also increased around implants coated with these collagen mimetic peptides. The authors concluded that the improved osseointegration of HA coated with collagen mimetic peptides was likely due to enhanced osteoblastic differentiation of MSCs rather than increased adhesion of MSCs on the surface of the implants.

In recent years, immobilization of peptides to HA using covalent linking has been developed. Durrieu and co-workers grafted RGD peptides containing different conformations (linear GRGDSPC and cyclo-DfKRG) onto HA by attaching a hetero-cross linker to the HA surface [56]. To achieve this, a three step reaction was used: (i) silanization with APTES, (ii) cross-linking with N-succinimidyl-3-maleimido- propionate, and (iii) peptide immobilization via thiol bonds [56]. Adhesion of human bone marrow stromal cells (HBMSCs) on HA was significantly higher for the RGD-functionalized HA when compared to the untreated HA. Cyclo-DfKRG favored cell adhesion during short incubation times and promoted cell differentiation simultaneously, while linear GRGDSPC only showed higher cell adhesion at 24 h. The observed differences could be due to the expression of different integrin subunits by the cells and the accessibility of these peptides by integrin receptors [56]. In another study, immobilization of RGD peptide to HA nanoparticles via covalent linking was performed by Balasundram and co-workers in an attempt to promote cell adhesion using nanometer particulates with decreased crystallinity [54]. Peptide immobilization was accomplished via a silanization technique involving *N*-succinimidyl-3-maleimido propionate conjugation with the peptide to the HA nanoparticles. The adhesion of CRL-11372 osteoblasts was, as expected, better for HA nanoparticles functionalized with the RGD peptide compared to non-functionalized HA nanoparticles. Comparatively, HA nanoparticles functionalized with RGD promoted the highest cell adhesion while, surprisingly, conventional (micron-sized aggregates) HA functionalized with RGD was on par with non-functionalized amorphous nanoparticulate CaP. This could be attributed to the greater total surface area available with binding sites for nanoparticulates compared to the micron-sized HA agglomerates [54].

CaP cements functionalized with different biofunctional agents [RGD, fibronectin, fibronectin-like engineered polymer protein (FEPP), extracellular matrix Geltrex, and human platelet concentrate] were synthesized, and the behavior of human umbilical cord mesenchymal stem cell (hUCMSCs) was studied on the prepared materials. Cell adhesion and proliferation on the functionalized-HA were relatively better than on the unmodified HA. In addition, cell activities such as alkaline phosphatase, collagen I, osteocalcin, runt-related transcription factor 2 (Runx2) expressions, and bone mineralization increased with the presence of these functional groups on the CaP cement [122].

Collagen is one of the main proteins in extracellular matrix of bone, skin, and tendons, which is widely used as a natural biomolecule for various biomedical applications, particularly in bone tissue engineering as it contains RGD sequences that interact with the $\alpha_v\beta_3$ integrin receptors of cell membranes [123,124]. Hong and co-workers immobilized collagen to CaP using two different immobilization methods and investigated the physicochemical properties of the materials and osteoblast response

[123]. In the first method, collagen was incorporated directly into CaP during the precipitation process (collagen-incorporated CaP films). Meanwhile, the second method relied on the physical adsorption of collagen onto pre-formed CaP films (pre-adsorbed collagen-CaP films). The following trend was observed for the adhesion and differentiation of MG63 cells: pre-adsorbed collagen-CaP < collagen-incorporated CaP films < CaP. The authors also found that the presence of collagen regulated the expression of phenotypic genes, such as osteopontin, alkaline phosphatase, and transforming growth factor-β (TGF-β) irrespective of the immobilization method. Recently, the coupling of HA with a collagen mimetic peptide (DGEA) modified with an E_7 peptide showed enhanced performance in terms of MSC proliferation and differentiation as well as implant osseointegration [125]. Consistent with other findings, cell adhesion was highly promoted for HA functionalized with collagen-E_7 peptide but cell spreading behavior was impeded at high peptide density [125]. In general, osteoblastic differentiation of MSCs on HA exhibited a decreasing trend as follows: E_7-DGEA > collagen > DGEA > HA alone [125].

It is well known that cell adhesion is regulated by transmembrane proteins that consist of an α subunit and β subunit, whereas the substrate specificity is regulated by the combination of subunits. For instance, $\alpha_5\beta_1$ binds to fibronectin, while $\alpha_v\beta_3$ has broader protein binding specificities including vitronectin. Kilpadi and co-workers aimed to establish the mechanisms and integrin species involved in the adhesion of primary human MSCs (hMSCs) and osteosarcoma-derived cells (Saos-2) to HA [126,127]. Interestingly, Saos-2 used both α_5- and α_v-containing integrins for adhesion, but primary cells only used the α_v-containing integrin, despite the presence of α_5-containing integrins. This is further exhibited in the adhesion behavior of both hMSCs and Saos-2 to HA coated with serum, fibronectin, or vitronectin. Primary cells (hMSCs) were found to adhere equally well on fibronectin- and vitronectin-coated HA but had the highest adhesion on serum-coated HA. The adhered cells spread better with serum compared to fibronectin or vitronectin alone. On the other hand, Saos-2 demonstrated equal adhesion to all materials but failed to spread on serum-coated HA [126,127].

Lode and co-workers functionalized CaP bone cements (biocement D, BioD) with vascular endothelial growth factor (VEGF) to promote angiogenesis [128]. This cement has high binding affinity to VEGF but caused a reduction in the biological activity of VEGF once released [128]. Further modification of BioD with collagen or with a combination of collagen, O-phospho-L-serine, and sodium citrate did little to resolve the high burst release of VEGF, however the biological activity of released VEGF on endothelial cells was maintained or, in some cases, was even significantly higher than 'free' VEGF (medium supplemented with VEGF and not released from material) [128]. Further modification of the BioD/collagen composite with heparin substantially decreased the toxic effects of an initial burst release of VEGF and simultaneously promoted the adhesion of endothelial cells on the cement [129]. The enhanced cell adhesion was believed to be due to the differences in surface morphology between heparin-modified BioD/collagen composites and BioD/collagen alone (without heparin), whereby the former demonstrated a finer microstructure with smaller HA particles and higher surface area [129].

4. Conclusions

Surface functionalization is by far one of the most effective tools to improve material properties for specific applications in medicine, biotechnology, optical, and environmental-based technologies. It is undeniable that modifying the chemistry and the functionality of a biomaterials' surface can regulate protein adsorption and, thereby, cell responses to implanted materials in the human body. However, several issues still remain unresolved regarding the use of biomaterials with chemically or physically modified surfaces. One of the more immediate concerns is the limited knowledge and understanding of the effects of modified surfaces on human health. For example, there is a significant lack of proper long-term assessments evaluating cells exposed to degradation by-products from modified surfaces, such as linkers and chemical modifiers. In addition, the degradation of ion-substituted CaP could lead to excess local concentration of these ions in surrounding bone. In regards to the preparation of functionalized surfaces with tailored activity, we should also consider the parameters that could be manipulated to optimize the functionalization efficiency while simultaneously maintaining the activity of immobilized molecules. In addition, another challenge that remains to be addressed concerns the tailoring of selectivity and specificity of functionalized surfaces. Currently, most approaches use non-specific binding to cell-adhesive molecules. While surface functionalization with cell-adhesive proteins has been proven beneficial to increase cell response, saturation of these proteins on a material surface may indeed render the desired response due to their non-specific nature.

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