

Chapter 4

Biofunctional Coatings for Dental Implants

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Abstract Success of dental implant materials depends on their integration into the adjacent soft and hard tissues where critical interactions take place at the interface between the surface of the metal and the biological components. The properties of the dental implant surface, such as surface morphology, surface energy, and chemistry affect cell responses and tissue regeneration. Therefore, modifications of the surfaces of the implant to minimize the nonspecific adsorption of proteins and to mediate bone osseointegration and tissue healing are research subjects of major interest. One promising approach consists of functionalizing dental implant materials by incorporating biological molecules with known bioactivities. Bioactive components such as extracellular matrix proteins, growth factors, and peptides have been covalently immobilized on surfaces to investigate their potential benefit in the clinical success of dental implants. The immobilization by means of primary bonds between the surface and the biomolecules can enhance stability and retention of the biomolecules on the implant and preserve biological activity compared to physically adsorbed molecules. We introduce here methodologies to covalently anchor biomolecules on the surface of dental implants. We thoroughly review the chemical strategies and biomolecules used as well as their effects on different biological responses of interest, such as osteoblasts response to improve osseointegration, antimicrobial properties, and in vivo integration. The stable immobilization of biomolecules on implants to form a bioactive surface can be an effective and novel approach to achieve implantation success in all clinical scenarios.

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4.1 Introduction

4.1.1 *Need for Improving Surface Properties of Dental Implants*

Commercially pure titanium (c.p. Ti) is the dominant material for making dental implants because it is biocompatible by combining very high corrosion resistance in contact with biological fluids and appropriate mechanical properties, namely high strength, high fracture toughness, and relatively low modulus of elasticity [1, 2]. In the last three decades, titanium dental implants have become successful for the replacement of teeth lost due to decay, trauma or disease. Generally, more than 90 % of implant success rates are achieved after 10–15 years of implantation [3, 4]. The most important factor for dental implant success is the osseointegration of the metallic device; i.e., the formation of a strong and long-lasting connection between the implant surface and the peri-implant bone that results in a stable mechanical fixation of the implant in the bone bed [5].

In spite of the high rate of dental implant success, surface modification of implants remains a very active area of research [6–8] as titanium is a bioinert material with passive interactions with the biological environment. Titanium does not trigger any specific positive reactions in the surrounding biological environment to improve the process of bone healing [9]. As a result, implants get osseointegrated following an osseoconductive process that ends up with a successful performance that entirely relies in mechanical considerations. However, the process of osseointegration is also fully dependent on the biological interactions at the metal surface. The process starts with wetting of the surface and rapid adsorption of biologically active molecules, and follows with recruitment of osseoprogenitor cells that finally orchestrate the regeneration of the tissue [10] and facilitates the reduction of the foreign body reaction [11, 12].

All of those biological interactions can hence be conditioned by the properties of the implant surface and thus, implants have room for improvement as:

- Under healthy conditions the process of bone regeneration is very slow and is far from allowing early or immediate loading of implants [13]. That has significant implications in terms of reduced patient morbidity and health care costs [14].
- It is counter indicated to place these implants in patients that present compromised clinical scenarios (in elderly, smokers, traumatic damage, systemic diseases) [15] and
- Infection of the surface of implants may result in short or long-term failure of the implants in place [6].

Surface modification of titanium dental implants in order to enhance its osseointegration, peri-implant bone regeneration, and/or antimicrobial properties includes several approaches and techniques (Fig. 4.1). Among them, the most traditional surface treatments to modify topography at the micro and nanoscale, to

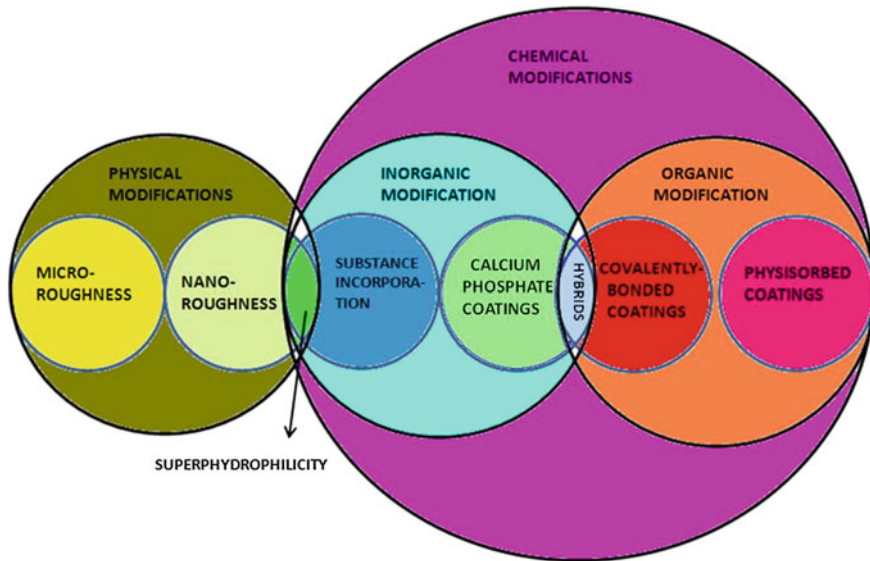


Fig. 4.1 Classification of strategies to modify or coat titanium surfaces to improve clinical performance of dental implants

increase hydrophilicity, or to obtain inorganic coatings made of calcium phosphates not only are still actively investigated but have found already room in the market. Those are briefly presented in the next section of this introduction.

4.1.2 Traditional Approaches to Modify Dental Implant Surfaces

The increase of surface micro-roughness was the first attempt to improve osseointegration with the rationale that direct contact of rough implants with newly-formed bone would result in higher micromechanical retention than a smooth or as-machined implant. As a matter of fact, this has been proved to be a successful approach, and implant surface topography is nowadays modified in commercially available products [7, 8] by chemical etching [16], by grit-blasting [17], by plasma-spraying titanium coatings [18], by electrochemical processes with different solutions [19], or by a combination of some of them [20]. Later, intensive and prolific research assessed other beneficial effects in the biological response to rough metallic surfaces, such as improved cellular attachment and osteoblast-like cell activity [21–23], selective protein adsorption, and collagen synthesis [24] as well as more intensive bone implant contact and higher mechanical retention than as-machined implants when implanted *in vivo* [12, 23, 25]. We concluded that $R_a = 4.5 \mu\text{m}$ was optimal for cell response and protein adsorption [26, 27], but

this is still a controversial subject [8] and nowadays most of dental implant systems have a microrough surface with values of $R_a = 1\text{--}5\ \mu\text{m}$.

More recently the exploration of the benefits of modifying the topography of metallic implants with nanofeatures with different size, distribution, and shape has become a hot topic of research [28, 29]. The simplest process to incorporate nanotopographical details to an implant surface is by etching titanium with a mixture of acids. Recent studies showed that surfaces with nanoscale features stimulate additional biological effects *in vitro* and *in vivo*, e.g., by producing an accelerating integration [30]. In fact, micro and nanorough surfaces have been recently used in some commercial products with good clinical results that, however, have not yet reached the clinical evidence stage [30, 31].

Increasing surface roughness influences other important physicochemical properties. For instance, it increases surface energy, which has a strong impact on the interactions of the metal with the surrounding biological system [32]. Of current clinical interest is the development of metallic surfaces that have high surface energy with superhydrophilic properties. Those surfaces are obtained by a process that delivers a metallic surface permanently free of hydrocarbons, which are hydrophobic in nature and that otherwise can be readily adsorbed on the titanium surface. The resulting superhydrophilic surfaces have been claimed to accelerate early processes of bone healing [33] as fibronectin, osteocalcin, and growth factors are preferentially adsorbed on them, thereby favoring bone growth right at the surface of the implant [34]. Other approaches to increase surface energy and hydrophilicity of titanium that produced promising *in vitro* and *in vivo* outcomes have been recently developed, such as the use of ultraviolet irradiation [35].

Another group of traditional surface treatments for dental implants has focused on coating the surface of titanium with a layer of calcium-phosphates [7, 36] as none of the previously introduced surface treatments change the intrinsic bioinert chemical characteristics of the titanium surfaces and are limited in their ability to accelerate and improve osseointegration. The deposition of bioactive calcium-phosphate minerals, such as apatite, can enhance implant performance at an early stage after implantation by an osseoinductive process of regeneration around the implant. This is because the biological nature of apatites, which represent the mineral phase in bone, have the potential to actively signal the cells that interrogate the surface after implantation.

A first generation of thick coatings applied on titanium dental implants by a plasma-sprayed or electrodeposition processes showed that response [37]. But later, it was also demonstrated that the chemical and structural heterogeneity of the layers obtained resulted in heterogeneous degradation of the layer and eventually mechanical failure and delamination [38]. As a result of the process of degradation, *in vivo* reactivity of delaminated fragments from the calcium-phosphate layer resulted in adverse tissue reactions and failure of the implants at mid- and long-term after implantation [39].

To avoid the problems of the thick calcium-phosphate layers deposited by plasma-spray at high temperatures, a series of new treatments to obtain thin layers at room temperature using biomimetically-inspired processes was published

during the 1990s [40–44]. The traditional biomimetic treatments for obtaining a bioactive layer of calcium-phosphates are widely based on etching the surface of titanium to obtain a nanoporous, highly reactive, and strongly negatively charged surface. In some cases the etched surface is thermally treated to mechanically stabilize the oxides formed [45]. Then, the treated surface is immersed at 37 °C in a solution with controlled supersaturation levels of calcium and phosphate, such as Kokubo’s Simulated Body Fluid (SBF) [46]. As a result of a cascade of ionic and electrostatic interactions between SBF and the surface, the local pH at the surface increases and thus, the relative supersaturation of the solution with respect to apatite increases. That triggers a sequential electrostatic attraction of calcium and phosphate ions to the metallic surface and precipitation of a continuous coating of carbonated apatite that is strongly bonded to the metal [47]. One important consideration is that, as described, the surfaces can be coated with apatite by immersion in SBF before implantation, but the etched surface implanted *in vivo* is also able to induce the formation of the apatite layer and thus, it is potentially bioactive [48].

As a further step, we developed a new surface treatment, *2Step*, for titanium dental implants that combines microroughness and potential bioactivity by first, grit blasting, and second, alkaline etching and thermally treating the implant surface. We demonstrated the potential bioactivity of the *2Step* surfaces [49] by growing *in vitro* HA layers in SBF and assessing that the preferential nucleation of apatite crystals took place on the bottom of the microrough features. This phenomenon accelerated the *in vitro* formation of the apatite layer in comparison to smooth surfaces with the same chemical treatment. Those implant surfaces were also tested to prove that they induce preferential differentiation of MG63 cells into the osteoblastic lineage [50]. We recently concluded that the *2Step* implants accelerated bone tissue regeneration and increased mechanical retention in mandible and maxilla of minipigs at short periods of implantation in comparison with microrough, HF-etched, and as-machined titanium implants [48]. This was mostly attributed to the ability of *2Step* implants to form *in vivo* a layer of apatitic mineral that coated the implant and could rapidly stimulate (1) bone nucleation directly on the implant surface; and (2) bone growth from the implant surface (Fig. 4.2).

4.2 Functionalization of Dental Implants with Biochemical Coatings

Currently, surface modification of dental implants using biochemical methods bring an attractive new approach to promote implant success as it aims inducing specific cell and tissue responses using critical biological components (Fig. 4.3). The regeneration of bone highly depends on the communication between cells and extracellular matrix components. Thus, the extracellular matrix proteins and its components, growth factors and bone morphogenetic proteins govern various key

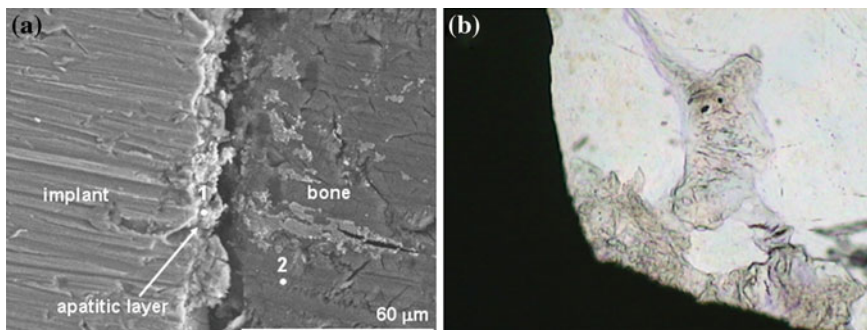


Fig. 4.2 **a** SEM picture of the interface between a 2Step implant and bone after 10 weeks of implantation. The image shows the presence of an apatitic layer on top of the 2Step cpTi implant. Locations used for microprobe chemical analysis on the apatitic layer (point 1) and bone (point 2) had Ca/P molar ratio of 1.70 and 1.66, respectively; **b** Representative histology ($\times 80$) of a 2Step dental implant after 2 weeks of implantation showing the growth of immature bone from the surface of the implant and that nucleated on top of the surface. Reproduced with permission from [48]

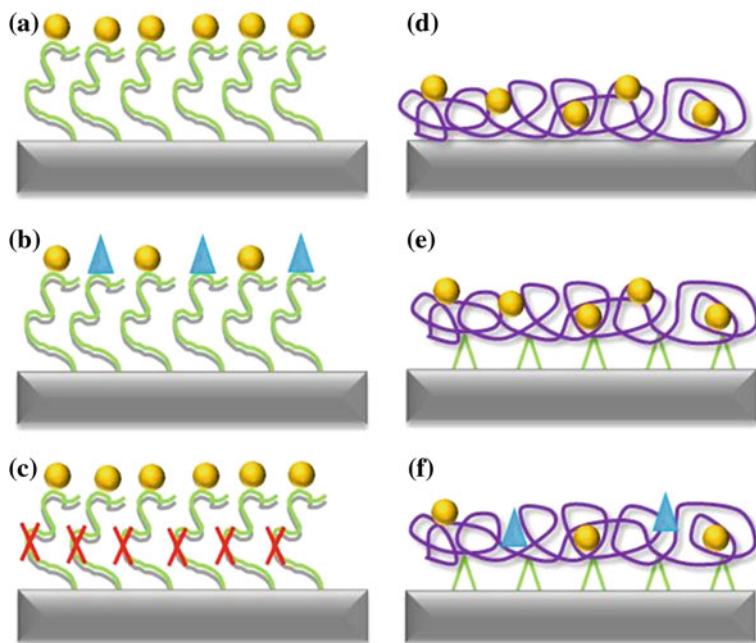


Fig. 4.3 Different approaches to coat titanium dental implants with bioactive peptides and proteins. **a** Covalently-bonded peptides with one bioactive signal (o); **b** Co-immobilization of peptides with multiple bioactivities (o, Δ); **c** Immobilization of peptides incorporating an enzyme-cleavable motif (X); **d** Physical adsorption of proteins; **e** Covalently-bonded proteins with one bioactive signal (o); **f** Covalently-bonded proteins with multiple bioactivities (o, Δ)

biological events, including cell adhesion, proliferation and differentiation. Immobilization of bioactive molecules on the surface of the implant potentially provides control over the tissue implant interface with further improvement of cell communication; tissue and bone repair.

These approaches depend on key factors for being effective on displaying biomolecules on the surface of a dental implant and retaining the molecular bioactivity. The specific chemistry used to retain the molecule on the surface and the selection and further modification of the biomolecule to also favor chemical and mechanical stability of the coating are among the most important ones. The rest of this chapter thoroughly reviews the newest strategies based on stable functional coatings made of different biomolecules of interest.

4.2.1 Methods for Immobilizing Biomolecules on Implant Surfaces

The methods for surface modification of biomaterials with biomolecules are not only an alternative to physicochemical and morphological modification, but also a supplement to improve dental implant performance. Those methods are based on current understanding of the biology and biochemistry of cellular function and differentiation, especially on lessons learned from the mechanisms of cells adherent to substrates [51] and the role of biomolecules in regulating cell differentiation and remodeling of tissues. The goal of biological surface modification is to immobilize proteins, peptides and polysaccharides on biomaterials to induce specific cell and tissue response [14] as well as minimize the unspecific adsorption of tissue fluid proteins [52]. The display of those molecules on the surface of dental implants should enhance peri-implant bone healing and/or prevent bacteria colonization of the surface [6].

To design a methodology to decorate the surface of a dental implant with biomolecules there are two main factors to be considered to improve their clinical performance: (1) the temporal stability of the bioactive molecules presented at the implant-tissue interface; and (2) the density and accessibility of the bioactive sites. Cells need to interact with the implant for a certain period of time to initiate cellular events, implying the biomolecules must be stable at the interface during that period. The concentration of the bioactive molecules must be above threshold levels to successfully induce the targeted cellular activity [53].

There are three main methods for surface immobilization of biomolecules [54] that have been extensively studied for coating surfaces for dental implant applications. The simplest one is by physical absorption of the organic molecules onto the surface, which can be achieved by immersing the substrate into a solution of the bioactive molecule. Another method consists of embedding the biomolecules into a bioresorbable material that is used for coating the implant surface. The third

method, which is the focus of this chapter relies on directly conjugate the biomolecules to the surface by covalent bonding or molecular self-assembly.

The simplicity of physical adsorption of biomolecules on the implant surface is the most attractive characteristic of this strategy. However, the physical adsorption method provides little control over both the density and the retention with time of the biomolecules on the surface. As the microenvironment around the implant changes during its time of application and the attachment of the molecules depends on secondary weak bonds—hydrogen bonds, electrostatic attraction, etc., the adsorbed molecules on the implant surface can be easily detached from the surface by desorption or displacement by other molecules. Thus, the surface can rapidly lose their bioactive properties. That makes this method far from ideal to fabricate a stable and long lasting coating. Others have proved that titanium surfaces coated with physically-adsorbed bone morphogenic protein lost 96 % of their bioactivity after the first hours in contact with biological fluids [55]. Moreover, this method can not precisely control the surface density and/or orientation of the molecules, which are vital for regulating the interactions of the implant with the biological agents—proteins, cells, tissues. The conformation of the biomolecules can also change during time and thus, lose their bioactivity [56].

Alternatively, covalently bonding biomolecules to implant surfaces provides coatings that are more stable and more resistant to disruption not only under harsh physiological conditions but also during fabrication of the coating and implantation at the time of the surgery. Overall, this can help for preserving the biological activity of the bound biomolecules [57].

The covalent bonding of the biomolecules also provides the potential to control their density and orientation, e.g., aligning and/or exposing the appropriate active sites at the interface, and thus provoking a more specific and rapid host reaction [58, 59]. The way the orientation and density of the immobilized molecules can be controlled depends on:

- the characteristics of the metallic substrate, in this case the nature of the passivating titanium oxide that naturally covers the surface and that provides plenty of hydroxyl groups under adequate fabrication conditions. Functional groups such as amino, carboxyl, and thiol can also be deposited on metallic surfaces by plasma treatment [60], by photo-initiated polymerization, by chemical etching [59], or by ion beam etching [61], which could be further used to attach biomolecules.
- the chemistry/molecule used as a linker between the surface and the biomolecule, which in some cases incorporates additional properties—hydrophobicity, electrostatic charges, etc.
- the design/modification of the biomolecule to be anchored, which in many cases includes additional chemical groups to physically separate the biomolecule from the coated surface and thus, facilitating the access of the cells to the bioactive cues.

The rest of this section presents the chemical strategies—functional groups and molecular linkers—that have been mostly used to functionalize titanium with active molecules for dental applications. The next section thoroughly reviews the biomolecules that have been investigated with strategies for designing more specific and effective bioactive coatings and, hence, the main applications of those coatings are also introduced.

4.2.2 Coupling of Biomolecules with Silane Agents

Silane coupling agents have been used to anchor peptides, enzymes and adhesive proteins on different biomaterials, such as Ti, NiTi, Ti-6Al-4V and Co-Cr-Mo [59, 62–65]. Covalent anchoring of biomolecules by silane chemistry is a simple and versatile method to modify surface properties. Silane coupling agents with different end functional groups are attached via reaction with the oxidized metal surface, which is activated to display reactive groups. The functional groups of the silane molecule at the opposite end are used to couple the bioactive molecules, either directly or via a cross-linker molecule. Generally, the process of silanization with biomolecules can be grouped into four types of directed reactions, namely thiol-, amino-, carboxyl-, and chloro- (Fig. 4.4) according to the functional end group in the silane molecule. The selection of the chemical group is based on the active residues of the biomolecules that are aimed to be used in the immobilization process. The ductile alkyl spacers on silane agents; i.e., the central part of the molecule can partially absorb the biomaterial-tissue interfacial stresses and may be also used to appropriately orientate and expose the bioactive molecules at the biomaterial interface in an arrangement to induce the desired tissue responses. Additionally, the alkyl spacer can also be used to adjust the hydrophobic properties of the final coating [66].

Silanes with different terminal functionalities deposited on model substrates such as gold can form stable self-assembled monolayers (SAMs), but the

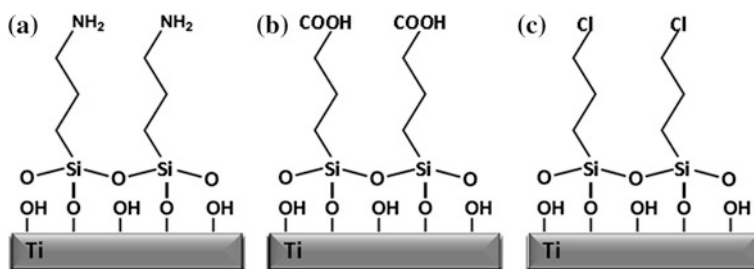


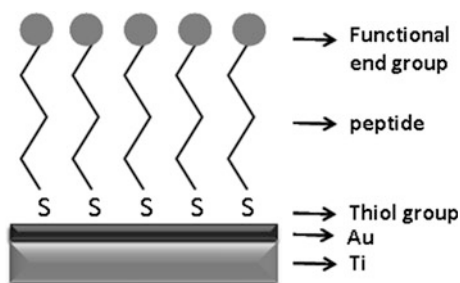
Fig. 4.4 Silane coupling agents with different functional end groups: **a** amine-, **b** carboxyl-, **c** chloro- that have been used to covalently-anchor biological molecules on Ti surfaces using appropriate chemistry and, in some cases, specific design of the biofunctional molecules

formation of SAMs on imperfect surfaces of polycrystalline metals such as dental implants, has proved to be a more difficult task. To graft silane agents to polycrystalline metal oxides the surface of the metal is first activated to form hydroxyl groups. Etching or plasma treatment of the titanium surface are often used to that purpose. Then hydrolysis of the alkoxy groups of the silane followed by condensation of the surface hydroxyls with the formed silanols at the silane molecules occur. On the one hand, in hydrous conditions, hydrolysis of the alkoxy groups occur immediately in solution, provoking condensation between silanols before contacting with the metal surface and thus, resulting in an uncontrollable and inhomogeneous polymerized silane multilayer. On the other hand, in anhydrous conditions, hydrolysis and condensation only occur when the silane molecules directly contact with the H₂O layer adsorbed on the metal oxide surface. Consequently, the silane monomers polymerize before reaching the metal oxide surface, preventing the formation of a uniform silane layer on polycrystalline metal surfaces. Nevertheless, anhydrous silanization has been found to yield better coverage and higher stability of coupled silanes compared to hydrous silanization [67].

Conventional, well established, and commercially-exploited techniques for immobilization of biomolecules on synthetic substrates use carbodiimides and glutaraldehydes as linkers to anchor terminal amino groups of biomolecules to silane agents [59, 68], or maleimides to biomolecules with cysteinethiol groups [61, 69].

The titanium surface can be also modified by surface-initiated atom-transfer radical polymerization (ATRP) of vinyl monomers [70]. Atom transfer radical polymerization (ATRP) is a recently-developed conveniently-controlled radical polymerization method [71–73]. ATRP fabricates polymers with narrowly-dispersed molecular weights because the method allows for the polymerization and block copolymerization of a wide range of functional monomers in a controlled manner. In addition, the tolerance of ATRP to polar functionality allows the direct polymerization of functional monomers without protection and deprotection procedures. Functional monomers such as poly(ethylene glycol)methacrylate, (2-dimethylamino)-ethyl methacrylate, and 2-hydroxyethyl methacrylate as well as their block copolymers can be tethered on the titanium surface to form polymer brushes by first silane coupling the surface with an agent that contains the ATRP initiator. Through the transferring of the ATRP initiator, such as chloride, functional monomers can be anchored to the surface in desired amount and orientation. The surface initiated ATRP produce a polymer-coated titanium surface with well-controlled hydrophilicity/hydrophobicity [70] that can be converted into carboxyl or amine groups. Those groups can then be further used to immobilize the target biomolecules, such as antibacterial agents or proteins of interest [74].

Fig. 4.5 Peptides with biofunctional end groups can be self-assembled on Au-coated titanium surfaces by incorporating thiol groups to covalently interact with the gold coating



4.2.3 Coupling of Biomolecules with Thiols Forming Self-Assembled Monolayers

Titanium can be coated with a thin layer of gold to further introduce alkanethiols coupling agents to the metal surface. Gold surfaces are not toxic to living cells and biocompatible with conditions used for cell culture. Covalent binding of thiols on gold surfaces is a simple and cost efficient method for surface modification [75]. Peptides with terminal cysteine groups can be covalently bonded to gold through the thiol groups of cysteine to form monolayers (Fig. 4.5).

Recently, self-assembled monolayers of alkane phosphates or phosphonates have been used on titanium or titanium alloy surfaces via reaction of phosphonic acids with their native oxides [76]. However, the harsh conditions needed, such as the use of anhydrous organic solvents and high temperatures might not be compatible with the stability of the biomolecules. The use of bisphosphonic acids with greater affinity to titanium surface to form monolayers on titanium has been also reported [77], as it requires less harsh conditions to be incorporated on the metallic surface.

4.2.4 Coupling of Biomolecules Using Tresyl Chloride Activation

Immobilizing biomolecules to various hydroxyl groups by using highly reactive sulfonyl chlorides, such as 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride) has been reported [78]. This process involves casting the tresyl chloride solution on the titanium substrate for two days followed by protein immobilization through the interaction between the amino groups of the protein and the tresyl chloride activated hydroxyl groups on the titanium surface.

4.2.5 Use of Spacer Assistant for Coupling Biomolecules

The non-treated titanium surfaces and most of the ones coated with biomolecules following the aforementioned methods lack the property of resisting unspecific protein adsorption from the biological medium. The massive uncontrolled adsorption of proteins from the medium can form an effective barrier on the metal surface that may hinder the desired interactions between the bioactive molecules and cells during the process of healing and tissue regeneration. One effective way to minimize unspecific protein adsorption onto titanium surfaces that has been now vastly investigated is the use of a poly (L-lysine)-graft-poly (ethylene glycol) (PLL-g-PEG) molecules in an assembly on the surface of the biomaterial mediated by electrostatic attractions [79–81]. Once the titanium surface is coated with PLL-g-PEG, the biomolecules can be covalently attached via vinyl sulfone-cysteine coupling reaction to the PEG side chains [82]. The result is a coating that blocks non-specific protein adsorption while promoting appropriate cell interactions.

4.2.6 Oligonucleotide Mediated Immobilization of Biomolecules

A novel and recently developed method for surface modification of titanium and titanium alloys is based on the immobilization of bioactive molecules using electrochemically fixed nucleic acids [83, 84]. In this method, the first step is the regioselectively adsorption of nucleic acid single strands on the air-formed passive layer of titanium alloys via 5'-terminally phosphorylated sites. Adsorption is followed by anodic polarization during which the single stranded nucleic acids; i.e., the anchor strands are entrapped and fixed on the titanium oxide layer by partial incorporation. The next step is the hybridization of the anchor strands with complementary strands that will be further conjugated with bioactive molecules.

This method, as a difference to all previously introduced, does not use an additional chemical agent for surface modification and thus, it is free from potential hazards that those synthetic linkers pose when applied in vivo. In addition, the release behavior of the bioactive molecules can be controlled by adjusting the hybrid stability. The successful use of this method for anchoring osteogenic growth factors on titanium and their slow release from the surfaces has been reported and positively compared to physically adsorbed biomolecules [85].

4.3 Biomolecules to Coat Titanium for Improving the Clinical Performance of Dental Implants

There is a debate on whether using short synthetic peptides or long chain extracellular matrix (ECM) proteins is the best approach for designing biomaterials that guide cell response for tissue engineering and regenerative medicine (Fig. 4.3). The ECM of bone, which is synthesized, deposited and mineralized by osteoblasts, consists of 90 % collagenous proteins (type I collagen 97 % and type V collagen 3 %) and 10 % non-collagenous proteins (osteocalcin, osteonectin, bone sialoproteins, proteoglycans, osteopontin, fibronectin, growth factors, etc.) [86]. ECM proteins mediate cell response—adhesion, proliferation and differentiation. Peptides are the functional motifs in ECM proteins which have specific bioactive functions, such as recognizing corresponding integrins on the cell membrane and thus, attach to them. Both ECM proteins [87–89] and short peptides [90–92] were proved to be effective in enhancing cell performance after being used to coat the surface of biomaterials. Both have advantages and disadvantages from both, the biological and chemical point of view.

The most advantageous property of peptides is that they are small and chemically defined [93], which implies that they can be easily synthesized, modified or reconstructed. By using techniques such as solid phase peptide synthesis (SPPS), peptides of up to 30–50 aminoacids can be routinely prepared with good yields [94]. Peptides with diverse functions, such as cell adhesion induction, enzyme-controlled, or antimicrobial properties can be precisely conjugated to biomaterial surfaces comparing with large molecules, such as proteins. Also, non-native chemistries and functional groups can also be conveniently incorporated in the peptide sequence with no much difficulty [93]. However, the short length of peptides limits their ability to selectively acquire the most desired conformation to achieve their bio-function. Also, although they might be retained in higher amounts than proteins and other larger biomolecules, the achievement of full coverage of the surface is challenging. Thus, they can be readily cleaved from the surface by proteases unless specific strategies, such as some of the ones introduced below, that aim to overcome this drawback are used to immobilize them.

Proteins have the main advantage that they carry the whole biochemical information needed for retaining the desired conformation and multiple biofunctions and not just part of it, as it is the case for short peptides. For instance, SPARC contains both hydroxyapatite bonding sequence (glutamic acid-rich sequence) and plasmin cleavable sequences; and bone sialoprotein possesses both hydroxyapatite bonding domain and cell recruitment domain. Therefore, ECM proteins have the intrinsic potential of contributing to multi-functionalization of titanium surfaces. However, most of the aforementioned advantages for the use of peptides are significant hurdles for the ease of use and appropriate performance of macromolecules attached to titanium surfaces as ECM proteins are difficult to be reconstructed, synthesized, and modified. Additionally, proteins from animal origin raise concerns about infection and immunological undesired reactions, which

requires significant attention and expenses to be highly purified [93]. The alternative recombinant synthesis is a high-investment and expensive methodology for producing these same molecules.

4.3.1 Peptides

4.3.1.1 Peptides to Enhance Cell Recruitment

RGD-Containing Peptides

Extracellular matrix governs various cellular events of cells, including cell adhesion, proliferation and differentiation [95]. A heterodimeric cell membrane receptor family known as integrins is involved in cell adhesion to extracellular matrix proteins [96] by interacting with short amino acid sequences present in molecules of the ECM. Especially, the RGD (Arginine-Glycine-Aspartate) amino acid sequence is identified as a key mediator of cell adhesion through interaction with integrins at the cell membrane [97]. RGD peptide is found in most ECM molecules including fibronectin, vitronectin, type I collagen, osteopontin and bone sialoprotein [98]. Thus, synthetic peptides that contain the RGD amino acid sequence can induce and thus, enhance cell attachment [91]. Covalently immobilized peptides with RGD sequences to implant surfaces has been recognized as a strategy for enhancing cell interaction with implants [69] and is the most used one in the category of coatings with biological molecules.

The RGD cell-adhesive sequences derived from fibronectin have been widely investigated. It has reported that the binding ability of fibronectin to cells can be due to the tetrapeptide L-arginyl-glycyl-L-aspartyl-L-serine (RGDS), a sequence which is a part of the cell adhesion domain of fibronectin [91]. Synthetic peptides containing GRGDSP (glycine-arginine-glycine-aspartate-serine-proline) can regulate cell attachment activity of the parent molecule [99] and have been used to modify the surface of titanium with enhanced cell attachment. However, these biomimetic strategies yielded only marginal enhancement in tissue healing in vivo.

Peptides with RGD motifs and sequences containing GRGDSP derived from human vitronectin are known to affect osteoblast adhesion by activating $\alpha 2\beta 1$, $\alpha 1\beta 1$, $\alpha v\beta 3$ and other integrins expressed on osteoblasts and osteoclasts [92]. Titanium alloy surfaces with immobilized RGD sequences displayed significantly increased levels of osteocalcin and pro-collagen I α 1 mRNAs, compared with the untreated Ti6Al4 V.

RGD-containing peptides derived from bone sialoprotein induced high cell adhesion strength [100]. A peptide with 15 amino acids having an RGD sequence which is unique to bone sialoprotein was linked to amino functionalized surfaces. The effects of the RGD-peptide on cell adhesion were compared with those induced by the arginine-glycine-glutamate (RGE) peptide. The cell detachment study using a radial flow apparatus showed that the RGD-grafted surface induced

significantly higher cell adhesion strength than the RGE-grafted surface. The cell contact area and focal contact patches on the periphery of the bone cells were considerably enhanced by the RGD-containing peptide surface as well.

Fibronectin type III 7th to 10th domain (FNIII7-10) attached to titanium surfaces using silanization and poly(oligo(ethylene glycol) methacrylate) polymerization was recently evaluated for in vitro osteoblastic cell differentiation and in vivo osseointegration [101]. Results demonstrated that $\alpha 5\beta 1$ -integrin-specific fibronectin fragment FNIII7-10-functionalized titanium improved implant osseointegration compared to RGD-functionalized and unmodified titanium. Moreover, bioactive peptides promoting integrin binding specificity regulated marrow-derived progenitor osteoblastic differentiation and enhanced healing response and integration.

Synergistic RGD and PHSRN Peptides

More recently, a Proline-Histidine-Serine-Arginine-Asparagine (PHSRN) sequence in the 9th type III repeating unit of fibronectin was found to have synergistic effect with RGD in improving cell adhesion. Many authors have demonstrated that multi-component peptide systems containing both RGD (the primary recognition site for $\alpha 5\beta 1$ integrin) and PHSRN (the synergistic site for $\alpha 5\beta 1$ integrins, in fibronectin 9th type III repeating unit) were more efficient in increasing cell adhesion, spreading, proliferation, and differentiation than the RGD peptide alone [102–107]. In the native conformation of fibronectin, RGD and PHSRN are spaced by approximately 40 aminoacids [103]. Therefore, the distance between PHSRN and RGD and the conformation of that part of the protein are important for the synergistic interaction of the two peptides. Ochsenhirt et al. [108] reported that the alternation of the concentration of RGD and PHSRN on the functionalized surface led to changes in the RGD-PHSRN distance and thus, influenced cell performance. They assessed that the distance which most closely mimicked the natural RGD-PHSRN distance significantly enhanced cell spreading. Vogel showed that small mechanical forces, in the range of tens of pN, can partially unfold fibronectin and change the RGD-PHSRN distance [109, 110]. A switch in integrin specificity from $\alpha 5\beta 1$ in non stretched status to $\alpha v\beta 3$ in stretched status was followed by this conformational change [109, 110]. In addition, structure, conformation, orientation and spatial distribution of RGD and PHSRN peptides are all important parameters in affecting the bioactivity of the modified surface.

Non RGD-Peptides

The triple-helical type I collagen-mimicking peptide with glycine-phenylalanine-hydroxyproline-glycine-glutamate-glycine-arginine (GFOGER) has been investigated by Reyes et al. [111]. Integrin $\alpha 2\beta 1$ can recognize the GFOGER motif in residues 502–508 of the $\alpha 1(I)$ chain of type I collagen. The adhesion of cells is

entirely dependent on the triple-helical conformation of the ligand in a similar way to what happens to native collagen. Results showed that immobilized peptides exhibited higher cell adhesion activity than physically adsorbed peptides. The GFOGER peptide promoted cell adhesion, mimicked the post-adhesion signaling characteristics of collagen surfaces that involves interaction with $\alpha_2\beta_1$ the integrins and further enhances cell differentiation.

The heparin-binding motif of human vitronectin precursor, phenylalanine-arginine-histidine-arginine-asparagine-arginine-lysine-tyrosine (FRHRNRKGY) was also investigated by being covalently immobilized on titanium surfaces [112]. Results demonstrated that FRHRNRKGY peptide preferentially promoted human osteoblast cell adhesion on the functionalized metal surfaces.

4.3.1.2 Peptides to Enhance Biomineralization

Osteoinductive surfaces that will produce osteogenesis around dental implants have been pursued to significantly improve their clinical performance. This is because it accelerates the process of osseointegration and can induce direct bonds between the surface of the implant and the newly-formed bone. The aforementioned inorganic coatings made of calcium-phosphates aim to do so by mimicking the extracellular matrix of bone in one of its components; i.e., its mineral phase. One further step to obtain biomimetic coatings incorporating calcium phosphates is the use of organic components known to have a role in ECM mineralization [113] to coat titanium surfaces and regulate nucleation and growth of the calcium-phosphates that form on the functionalized surface of the implant [65].

Benesch et al. reviewed proteins and corresponding peptides that have relevant roles in controlling biomineralization at different stages of tissue regeneration for different hard tissues in our body. Those are non-collagenous proteins that associate to collagen, which in its fibrillar assembly serves as a template for bone mineral nucleation and growth [114].

Osteopontin [115–117] and statherin [118–120] can inhibit mineral nucleation and growth by recognition of the mineral surface and adsorption on it. We have immobilized on titanium a recombinant molecule that contains the 15-aminoacid N-terminus peptide of the salivary protein statherin to take advantage of its affinity to calcium-phosphates [65]. We demonstrated that surfaces with the conjugated statherin-derived peptide were able to nucleate and control growth of calcium phosphate nanominerals and induced preferential differentiation of osteoblasts-like cells compared to non-coated surfaces and surfaces with physical adsorbed molecules.

Osteocalcin also inhibits nucleation and growth of apatite crystals in vitro, but considering its late appearance during bone formation, it might be important in bone remodeling [121, 122].

Glutamic acid rich sequences of osteonectin and, most significantly bone sialoprotein are responsible for improvement of mineralization [123, 124]. Sialoprotein is an effective apatite nucleator in vitro. The functional motifs in bone

sialoprotein are long sequences of glutamic acids –E4, E6 and E8–. Therefore, a highly negatively charged multi-Glutamic acid peptide sequence is supposed to be able to enhance surface mineralization and hence improve osteoblast differentiation.

4.3.1.3 Antibiotics and Antimicrobial Peptides

Infections are the most prevalent cause of failure for dental implants and orthopedic prosthesis. The inflammatory response to bacteria on the implant surface is called peri-implantitis, which may finally result in bone loss and implant failure. Peri-implantitis can happen immediately after oral surgery or months or years later. The literature has shown that peri-implantitis can affect up to 14 % of implants after 5 years; however, the relevant incidence may be higher due to poor clinical diagnosis and the short duration of reporting clinical studies [125]. The implant surface has a higher risk of infection comparing with natural tooth surface because it accumulates serum proteins which promote bacterial adherence and colonization faster. This is even more prone to happen on the current devices as they all incorporate microroughness surfaces that further facilitate bacteria attachment.

Existing approaches for surface modification of implants to reduce bacterial formation include the immobilization of antibiotics [126, 127]. Gentamicin have been loaded into nanotubes [128], poly (D,L-lactide) coating [129] or porous hydroxyapatite coatings [130] on titanium implants. The antibiotic-hydroxyapatite-coatings exhibited significant improvement in infection prevention [131]. Antibiotics have been normally physically adsorbed on titanium surfaces for ease of processing and prevention of degradation of the molecules that some fabrication methods would provoke; e.g., high temperatures when incorporated on plasma-sprayed hydroxyapatite coatings. The physical absorption process; however, limits the loaded amount and release characteristics of the drugs. Loading antibiotics in HA coatings, for example, led to 80–90 % of the total loaded drug being released during the first 60 min in contact with fluids [132, 133] and drugs loaded into nanotubes were fully released in 50–150 min [128]. Surfaces incorporating chlorhexidine [134], silver [135], poly lysine [136] and chitosan [137] have all been developed. Recently, Vancomycin has been successfully covalently-bonded to titanium and its antibacterial activity is retained even after incubation in PBS for at least 11 months [138–141]. Comparing with the non covalent coatings that quickly release antibiotics, covalently-bonded antibiotics remained active for notably longer periods.

Although antibiotics coated on titanium proved to be effective, their use is controversial because of their potential host cytotoxicity and bacterial resistance. For instance, Weber and Lautenbach [142] noted that 41 % of bacteria isolated postoperatively were resistant to gentamicin following the application of gentamicin-impregnated bone cement. Other investigations also showed drug resistance of bacteria isolated from orthopedic implants [143]. In addition, although

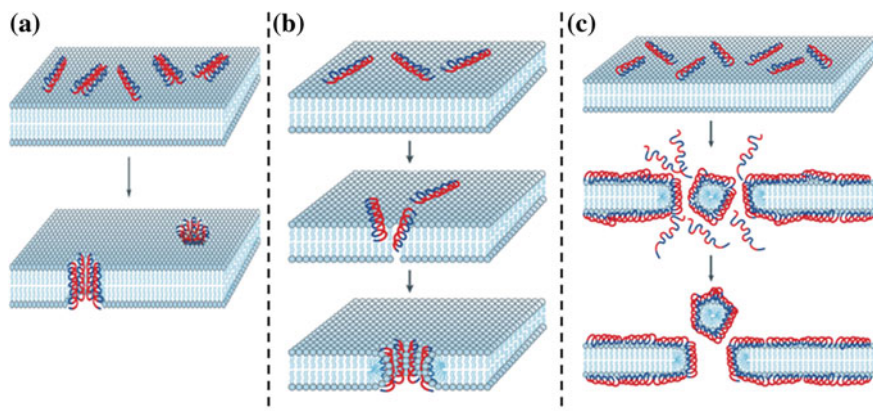


Fig. 4.6 Current models of the mechanism of antimicrobial induced-killing peptides; **a** Barrel-stave model; **b** Toroidal pore model; **c** Carpet model. Adapted with permission from [237]

antibiotics are normally thought to be biocompatible, their potential in inducing host cytotoxicity have been also widely reported [144–148].

The use of antimicrobial peptides (AMPs) have recently raised as an alternative antimicrobial approach with strong potential to improve dental implants performance when immobilized on titanium. Naturally, the bacterial flora in the oral cavity is mediated by the human innate immune system, which is rich in antimicrobial proteins and peptides [149, 150]. These AMPs can kill bacteria directly through membrane disruption, or act as immune modulators by enhancing bacteria clearance using our innate defense system [151] (Fig. 4.6). The advantages of using AMPs over antibiotics are 1) they are of human origin; hence, with potential low host cytotoxicity. This is still disputable because some reports showed that certain AMPs, such as LL-37, can freely translocate into cells and carry passenger molecules into their nuclei [152, 153]. More studies are needed to evaluate subtle toxicities of AMPs. 2) The co-evolution of AMPs with bacteria suggested low bacterial resistance. In addition, the immunomodulatory properties of AMPs would not be affected by antimicrobial resistance because of the irrelevance of direct bacteria killing. 3) The exceptionally broad activity of AMPs indicates that a single peptide can have activity against gram-negative and gram positive bacteria, fungi, and even viruses and parasites [151]. However, the disadvantage of AMPs involves the potential liability to proteases, which indicates the possibility of being proteolytically degraded by enzymes secreted by the microbial flora.

Over 45 AMPs with different antimicrobial mechanisms have already been identified from the human immune system, ranging from small cationic peptides to enzymes and large agglutinating proteins [149]. They may act as metal ion chelators, protease inhibitors, or promoters of enhanced bacterial agglutination [149].

A few AMPs are in current clinical use, such as polymyxin B, which is in clinical use for ophthalmic infections. But there are few reports regarding the application of AMPs on titanium surfaces to prevent peri-implant infection.

Kazemzadeh-Narbat et al. [154] used physically-adsorbed AMPs on micro-porous calcium-phosphate coated titanium surfaces and proved that had efficient antimicrobial activity and acceptable biocompatibility. However, again, physical adsorption of AMPs resulted in a rapid burst out of the agent from the surface, which quickly lost its antimicrobial ability. In addition, non-covalent coatings may develop a concentration gradient from the surface that would lead to the development of drug resistance as bacteria get the possibility of gradually respond to the antimicrobial challenge [155, 156]. Recently, the same research group built up a covalently anchored antimicrobial coating on titanium surfaces based on hydrophilic polymer brushes conjugated with the AMPs [157]. The hydrophilic polymer brushes were tethered on titanium using ATRP. The surfaces were maleimide functionalized, and cysteine modified AMPs were finally conjugated to the coatings. These tethered AMPs demonstrated excellent in vitro and in vivo antimicrobial activity with no toxicity to osteoblasts. We have also immobilized AMPs derived from the parotid secretory protein using silane chemistry. Our results proved the sustained antimicrobial activity of the AMPs, resistance to form bacteria biofilm, and appropriate cytocompatibility [158].

It is worth noting that AMPs may suffer conformational changes after being tethered on the surface. It has been previously suggested that soluble AMPs change their conformation when interacted with bacterial membrane and their subsequent incorporation is one mechanism for their antimicrobial activities [159, 160]. Gao et al. [157] confirmed this hypothesis by demonstrating the alteration of CD spectra of soluble AMPs after interacting with a modal bacterial lipid membrane. Most interestingly the unlikely soluble AMPs, such as polymer brush conjugated AMPs changed conformation to a substantially less degree. The spatial confinements of AMPs after being tethered could hinder their complete penetration into the bacterial membrane. Therefore, the induced disturbance to the bacteria membrane may trigger other cell death mechanisms that finally confer their antimicrobial effect.

4.3.1.4 Enzyme-Cleavable Peptides

The importance of proteolytic susceptibility of peptides relies on their ability of building up a degradable system conducted by enzyme secreted by cells. The degradation of coatings can trigger controlled release of important motifs that would add another level of sophistication to the coatings on dental implants. For example, many implant related infections occur not only as a consequence of the initial exposure during the surgery but also after a long time of implantation, from months to years, as bacteria enter into the body through the lungs or wounds and find the surface of the implant an ideal location to be colonized. A coating with controlled and sustained release of antimicrobial agents is an obvious improved system to work towards the long-term success of dental implants.

Biodegradable polymers, such as poly(lactic-*co*-glycolic acid) (PLGA), have been widely utilized as degradable carriers in drug delivery and the application of

antibiotics loaded on PLGA for periodontitis treatment have been also investigated [161–163]. PLGA degradation is mediated by hydrolysis and thus, with no specific control. On the contrary, given that most of enzymes are cell secreted, the release of antimicrobials mediated by enzyme-cleavable peptides is therefore “cell mediated”, in a process that simulates the natural degradation of the ECM.

Two main categories of proteolyzable peptides are matrix metalloproteases (MMP)-cleavable and plasmin-cleavable peptides [93]. MMP family are produced by plenty of cells such as activated inflammatory cells (neutrophils and macrophages), epithelial cells and fibroblasts. Besides, osteoclasts can secrete MMPs to degrade collagen and other components of the ECM of soft tissues, which is the main problem related to periodontitis. Therefore, with the continuous secretion of MMPs in the peri-implant environment by inflammatory cells in early implantation stages and osteoclasts in late bone formation stages, MMP-cleavable peptides are suitable candidates for antimicrobial agent controlled-release systems. Plasmin acts during wound healing to degrade provisional fibrin matrix generated during clot formation. It is secreted by blood endothelial cells and platelets. The wound healing process following implantation may activate plasmin in the peri-implant environment and thus, cleave suitable plasmin-cleavable peptides.

The most widely used MMP-cleavable peptides are GPQG↓IAGQ and GPQG↓IWGQ. GPQG↓IAGQ is the MMP substrate site found within the alpha chain of type I collagen, and GPQG↓IWGQ incorporates an amino acid substitution (A/W) to enhance enzymatic activity [164]. Other important peptides include VPMS↓MR and its modified VPMS↓MRG and VPMS↓MRGG sequences [93]. Patterson and Hubbell [165] used a combinatorial method of oriented peptide libraries to test degradation rate of 17 MMP sensitive aminoacid sequences. They used GPQG↓IAGQ (GPQG↓IWGQ) as reference peptides, and they assessed different degradation times for all tested MMP sensitive sequences ranging from less than 2 days to more than 10 days when incubated with MMPs. When incubated with cells, EGTKKGHK was degraded after 26 days, which constituted the fastest time for degradation, whereas GPQG↓IAGQ took the longest time to be fully degraded. Therefore, proper selection of the MMP-cleavable peptide can be done according to the degradation time needed to optimize the antimicrobial delivery process. It is worth noting that 3D hydrogels incorporating the MMP-cleavable peptides with the fastest degradation times increased cell spreading and proliferation.

The most widely used plasmin-cleavable peptide is HPVE↓LLAR. This peptide is also sensitive to a number of MMPs (MMP-2, MMP-7, MMP-9 and MMP-13) [93]. Many of the plasmin-cleavable peptides are derived from secreted protein acidic rich in cysteine (SPARC). SPARC, also referred to as BM-40 or osteonectin, is a ECM protein that has been discovered to bind hydroxyapatite to type I collagen in bone. As a large protein with 4 domains, SPARC contains both hydroxyapatite bonding sequence (glutamic acid-rich sequence) and plasmin-cleavable sequences which makes this protein a target for further research. Patterson [166] also tested the degradation time of a total of 12 peptides derived from SPARC and demonstrated their different degradation rates when submitted to

plasmin-rich media. Again, this shows the potential for time control of antimicrobial agent release. However, peptide sensitivity to proteases is not the only factor that may determine release rates of the molecules of interest. For instance, the concentration of protease on the surface and the number of cleavable sites tethered on the implant surface should also be considered [167].

MMP- and plasmin-cleavable systems have been already tested in several hydrogel models. Aulisa et al. [168] developed a self-assembly method for peptides that were organized with triblock ABA structure in which the central B domain contained alternating hydrophilic and hydrophobic amino acids. On the one hand, the hydrophobic amino acids can drive peptides to be packed together to form “hydrophobic sandwich” structures. On the other hand, the hydrophilic amino acids are the functional motifs, such as MMP-cleavable sites. Proteolytic degradation was confirmed by mass spectrometry and transmission electron microscopy [169]. Galler et al. [170] tested self-assembled peptide-amphiphile nanofibers as scaffolds for pulpal stem cells. These peptide amphiphiles were designed with 4 functional groups: an alkyl tail, an enzyme-cleavable site, a glutamic acid for calcium binding, and a RGD group for cell recruitment. Other researches used polyethylene glycol diacrylate (PEGDA) monomer with MMP sensitive crosslinkers to form a degradable PEGDA hydrogel network [167]. Those experiments, either through self assembly [171] or UV crosslinking, were aimed to be applied as scaffolds for tissue engineering and regenerative medicine. The covalent bonding of MMP-cleavable peptides on solid substrates has been just rarely investigated. Tokatlian et al. reported on the immobilization of GPQG↓IAGQ coated nanoparticles on tissue culture plastic surfaces using biotin and streptavidin as cross-linking system [172].

4.3.1.5 Multifunctional Coatings with a Combination of Peptides

As previously discussed, surface modification of titanium surface to improve osseointegration of dental implants by improving cell recruitment and differentiation, biomineral formation, or antimicrobial activity has been widely investigated. However, the fabrication of advanced multifunctional coatings that bear both bone regenerative and antimicrobial signals is an ambitious and desired goal that has been seldom pursued. It is a challenging task, though, as it requires original designs for the bioactive molecules as well as the methodological steps to obtain a robust and active coating.

The competition between bacterial adhesion and tissue integration was described by Gristina [173] as the “race for the surface”. The general concept is that if the bacteria first colonize the surface of the implant to form a biofilm they win the race as bacteria in biofilms are difficult to eradicate and become resistant to antimicrobial agents. Thus, cells from the tissue in regeneration can not displace bacteria from the surface, which leads to decreased tissue integration, occurrence of infection, and failed osseointegration. Therefore, the simultaneous prevention of bacteria adhesion and promotion of cell recruitment and differentiation on the

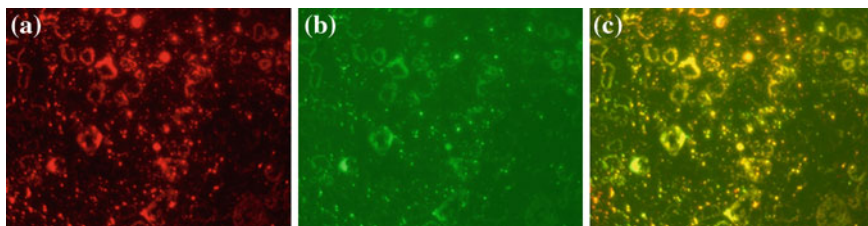


Fig. 4.7 Dual-functional coatings on Ti by co-immobilization of PHSRN- (*red/dark* tag-labeled in **a**) and RGD-containing (*green/light* tag-labeled in **b**) oligopeptides. The overlapping of the two fluorescent signals at the surface of the dual-functional coating gave a *yellow/brighter* figure shown in **(c)**

implant surface can be regarded as crucial in facilitating cells to win the race and improve clinical performance of dental implants [174].

A post-implantation period of 6 h has been identified as the “decisive period” when an implant is particularly susceptible to bacteria colonization [175]. To properly balance osseointegration and antimicrobial activity on the surface of the implant, prevention of bacterial adhesion and stimulation of cellular response should be accomplished in early stages after implantation. A barrier of hydrophilic polymers, either anionic (dextran [176] or hyaluronic acid [137, 177]) or highly flexible (polyethylene glycol (PEG) [178, 179]) have been used on Ti surfaces to control bacterial adhesion. They notably hinder protein adsorption as well as bacterial and cell adhesion due to their non-fouling properties that are acquired by either electrostatic repulsion or formation of a hydration shell. Those polymers are susceptible to further functionalization with bioactive molecules to stimulate osseointegration. By further incorporating cell integrin specific receptors, such as RGD or bone morphogenetic proteins, which are not recognizable by main bacteria in implant infection [180], simultaneous increased of osteoblastic functions and decreased bacteria adhesion were observed [181, 182].

Multifunctional coatings with a mixture of peptides with other combinations of bioactivities have been also recently studied. For instance, we fabricated silanized titanium surfaces with combination in parallel of RGD and PHSRN peptides that demonstrated the targeted synergistic effect of the two peptides on osteoblast adhesion [183] (Fig. 4.7).

4.3.2 ECM Proteins

4.3.2.1 Collagen

Collagen is the major component of connective tissues and accounts for approximately 30 % of all proteins in the human body. Collagen is often found in every major tissue that requires strength and flexibility, such as tendons, skin, bones, and

fascia. Collagen plays a critical role in the evolution of large complex organisms where it provides an insoluble scaffold for shape and form, for the attachment of macromolecules, glycoproteins, hydrated polymers and inorganic ions, as well as cell attachment. Collagen type I and its active peptides play an important role in osteoblast response [96]. Collagen can accelerate cell adhesion [184] and promote osteoblast proliferation and differentiation [185].

Collagen and collagen fibrils have been covalently bonded to the surface of titanium implants by silane chemistry. Collagen type I immobilized to titanium surfaces using acrylic acid grafting enhanced early osseointegration in *in vivo* studies [186]. A significant increase rate of bone growth and bone-to-implant contact in rabbit femur on collagen modified titanium implants was assessed in comparison to non-coated surfaces. That was even though a lower proliferation rate of SaOS-2 and a non-significant difference in alkaline phosphatase production was previously detected for the collagen-coated titanium surfaces.

The effect of collagen-modified surfaces on osseointegration of trabecular bone has also been studied [187]. Results indicated that a significant increase in bone-to-implant contact and enhanced bone in-growth can be achieved with collagen-coated implants when compared to control titanium surfaces. Since bone formation around implants is highly dependent on the recruitment of undifferentiated mesenchymal cells with osteogenic differentiation capability, it was suggested that the collagen layer on implants provided biological advantages over the non-coated surfaces to adhere and proliferate mesenchymal cells.

The covalent immobilization of type I collagen on implant surfaces can increase their enzymatic and mechanical stability [188]. It has been recently reported that the stability of the collagen on the substrate plays an important role on cellular response [189]. Fibrillar type I collagen was covalently attached via activated metal surfaces and aspartic and glutamic acid residues in type I collagen that were activated using an EDC/NHS cross-linking system. An increase in cell adhesion was found on collagen modified surfaces in comparison to non-coated oxidized surfaces. Osteoblast-like cells proliferation was also significantly enhanced on collagen modified titanium and cobalt alloys. However, a significantly increased cell proliferation on collagen-coated commercially pure titanium was not found compared with non-coated surfaces of the same metal. In this case, it was suggested that type I collagen is an attractive material for orthopedic and dental implant coatings due to its osteoconductivity and its potential to serve as a biocompatible carrier for bioactive molecules.

4.3.2.2 Non-Collagenous ECM Proteins

Fibronectin and Laminin

Fibronectin is a high molecular weight (400 kDa) glycoprotein having two homologous subunits which are held by two disulfide bonds near the carboxyl termini. Fibronectin is expressed during early stages of bone development to

promote bone mineralization [190, 191]. Endo et al. [62] reported on covalent-immobilization of human plasma fibronectin on NiTi substrates using silane chemistry and its effect on cell response. The results showed a significant increase on fibroblast spreading on the coated surfaces. We recently also assessed that fibronectin-coated titanium surfaces significantly influenced adhesion and differentiation of SaOS-2 cells both at the gene expression and protein production levels [44, 192].

Laminin-5, a component of basement membranes, plays a crucial role in the assembly and maintenance of hemidesmosomes which connect the gingival epithelium to dental implants [193]. Laminin has been used to promote the formation of a biological seal around the transmucosal portion of dental implants. The laminin-5-derived peptide coating strongly favored the formation of hemidesmosomes [194, 195]. Lange et al. [196] immobilized laminin and human epidermal growth factor (EGF) to promote adhesion of epithelial cells. Laminin and EGF showed inhibition on adsorption of salivary proteins and bacteria while enhanced epithelial adhesion.

Growth Factors

While RGD-peptides have been the molecules most largely investigated to mediate adhesion of cells to substrates, immobilized growth factors have been the selected ones to study modulation of subsequent cell functions, such as proliferation, differentiation, and activity on biomaterial surfaces [197]. However, compared to investigations with peptides, only a few reports have focused on chemical immobilization of growth factors on implant surfaces. Most of the published work is on physical adsorption and/or local administration of these proteins both in *in vitro* and *in vivo* experiments.

Platelet-derived growth factor and insulin-like growth factor have been applied to the insertion site of titanium dental implants and implanted in dogs with a notable increase in bone regeneration [198]. EGF was immobilized on polystyrene plates and induced phosphorylation of the RGF receptor [199]. Immobilized EGF was as effective as a soluble growth factor in stimulating DNA synthesis in hepatocytes, too [200].

Bone morphogenetic proteins (BMP) belong to the TGF- β superfamily. They were originally identified as active components in bone extracts capable of inducing bone formation at ectopic sites. Three members of the family, namely BMP-2, BMP-4 and BMP-7 are expressed in bone. BMPs are able to regulate Runx2 activity through several ways, for example, through Protein Kinase D [201] or SMAD pathway [202], to regulate osteoblast differentiation.

BMP-2 has been known to play an important role in bone-healing processes and to enhance therapeutic efficacy. BMP-2 is one of the most effective osseoinductive factors as demonstrated in many works in the literature [203]. It has been successfully applied in repairing segmental defects and alveolar bone defects in conjunction with implants [204, 205] and Seol et al. [206] used a synthetic binding

motif to BMP-2 and showed that surfaces modified with BMP-2 enhanced cell attachment and significantly increased bone growth when implanted in canine mandibles in vivo compared to untreated titanium surfaces.

BMP-4 can induce osteoblastic responses in non-osteoblastic cells [207]. BMP-4 was immobilized on NH₂-rich metal surfaces. Significant amounts of BMP-4 were retained on the functionalized surfaces, which resulted in higher osteoblastic activity when compared with surfaces with no biomolecules or with lower density of initial NH₂ groups [60]. Some researchers have suggested that BMPs are better to regulate bone growth around dental implants than the widely used RGD peptide because RGDs are cell unspecific [14, 208] whereas BMPs are not. However, as a locally distributed growth factor, the long term effects of BMPs to systemic organs need to be further investigated [209].

ECM Glycosaminoglycans

Proteoglycans and their glycosaminoglycans interact with ECM molecules like collagen and bone cells like osteoblasts and osteoclasts. These molecules further mediate the attachment of cytokines and growth factors to the ECM or the cell surface [210]. However, the interactions of proteoglycans and glycosaminoglycans with cytokines and growth factors are complex in nature. A glycosaminoglycan and chondroitin sulphate enhanced bone remodeling at peri-implant interfaces when co-adsorbed with collagen [211].

Hyaluronan, also called hyaluronic acid, is a linear polysaccharide with bio-activity during cell proliferation, repair and regeneration [212]. Even though hyaluronan-functionalized titanium surfaces inhibited osteoblast adhesion, the interaction of hyaluronan with its receptors on cell surface is modulated by interactions with cytokines transiently expressed on wound healing [213]. Hyaluronan covalently coupled to titanium surfaces induced a significant improvement in bone-to-implant contact and bone ingrowth after 4 weeks of implantation in vivo studies [214]. Those improvements were more notable on trabecular bone than on cortical bone.

Biom mineralization-Related Proteins with Potential Application as Coatings for Dental Implants

Glycosylated proteins with RGD motifs. Investigations on ECM proteins with bone/teeth specific functions led to the discovery of the SIBLINGS family (Small Integrin-Binding LIgand N-linked Glycoprotein), which included Bone sialoprotein (BSP), dentin matrix protein 1 (DMP1), dentin sialophosphoprotein (DSPP), enamel (ENAM), matrix extracellular phosphoglycoprotein (MEPE), and osteopontin (OPN) [215]. All proteins of the SIBLINGS family contain an RGD sequence which is able to bind to integrin receptors on the cell membrane, as described in a previous section of this chapter. The family is also characterized by

extensive post translational modifications including N- and O-linked oligosaccharides, many of them rich in sialic acid [216]. Two members of the SIBLINGS family that have specific roles in bone regeneration are OPN and BSP.

OPN is expressed in osteoblasts just prior to mineralization. It has high affinity for calcium and it can mediate cell matrix interactions as it contains the RGD motif. OPN plays a notable role on binding osteoclasts to hydroxyapatite, thus causing bone resorption. An osteopontin knock-out mouse model showed significance resistance to bone resorption [217]. Other reports indicated that OPN is required for bone resorption as it increases angiogenesis, vascularization, and accumulation of osteoclasts [218].

BSP is a highly phosphorylated protein which is rich in polyglutamic regions. Thus, BSP is an effective apatite nucleator in vitro [219, 220]. BSP has been shown to be linear with minimal secondary structure [221, 222], but it contains several spatially segmented motifs that can bind collagen [223, 224], matrix metalloproteinases [225], hydroxyapatite [226] and cell membrane integrins [227, 228]. The spatiotemporal expression of BSP at sites of de novo bone indicates its effect in the onset of mineralization [229]. Studies showed that interference of BSP and osteoblast binding by addition of anti-BSP antibody led to compromised osteoblast differentiation and reduced mineralization [230]. In contrast, increased BSP expression improved osteoblast differentiation as well as mineralized nodule formation [231].

gamma-carboxylated (GLA) proteins. Vitamin K dependent γ -carboxylase can add carboxyl groups to glutamic acids and thus form GLA proteins. Two acidic carboxyl groups in GLA contribute to calcium binding activity. Two major members in this family are Matrix Gla protein (MGP) and osteocalcin (OCN). OCN is specifically secreted by osteoblasts and osteoclasts in bone while MGP is also highly expressed in cartilage and arteries. MGP is a powerful mineralization inhibitor. MGP deficient mice showed severe calcification of arteries and cartilage [232]. Osteocalcin also inhibits nucleation and growth of apatite crystals in vitro, but considering its late appearance during bone formation, it might be important in bone remodeling [121].

Alkaline phosphatase (ALP) has a prominent role in bone formation with multiple functions, including local regulation of phosphate supersaturation [233, 234]. ALP has been already covalently-anchored to an oxide layer of titanium using silane chemistry without losing its enzymatic activity [59].

Osteonectin was one of the first ECM proteins postulated to have bone specific functions [235]. The name “osteonectin” means bone connector because this protein has a strong affinity for both collagen and inorganic calcium-phosphate minerals. It is theorized to be a positive regulator of bone formation by improving mineral nucleation. Experiments in mice showed that mutation of the osteonectin gene caused osteopenia—resulting from low bone turnover—with defects in both osteoblast and osteoclast activity [236]. A glutamate-rich region in osteonectin has been suggested to be the site for hydroxyapatite bonding [123].

Elastin-like recombinamers with bone-mineralizing peptide sequences. Recombinant polymers with bio-mimetic activities can be covalently coated on Ti

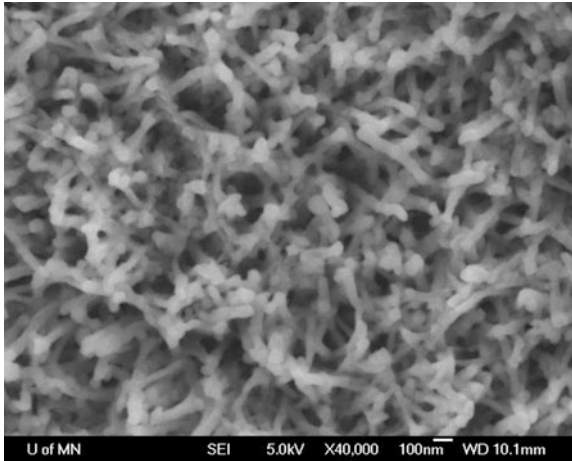


Fig. 4.8 SEM image of a Ti surface etched, coated with a recombinant carrying a peptide sequence derived from statherin, and mineralization during 7 days through an enzymatically-controlled biomimetic process. The image shows a coarse mineralized nanorod-like texture that suggested that calcium phosphate minerals formed specifically around the surface of functionalized Ti preserving the original nanorough texture

surfaces. In our lab, we have covalently-functionalized nano-rough Ti surface with a genetically synthesized elastin-like recombinamers carrying a peptide sequence derived from statherin (HSS), a multifunctional salivary protein. We further mineralized the HSS coatings using a enzymatically-controlled biomimetic mineralization process, in which the obtained homogeneous layer of mineral preserved the nano-rough topography of the metallic substrate (Fig. 4.8) [65]. This mineral layer was composed of amorphous calcium phosphate nanoparticles of 10–40 nm in diameter. Cell culture studies demonstrated that the cellular adhesion was improved on the HSS-coated Ti after 7 days of biomineralization. Furthermore, cell differentiation to osteoblasts (expression of ALP and OCN) was stimulated during 21 days of culture. The biomimetic mineralized Ti surfaces that were covalently-functionalized with statherin derived recombinant biopolymers possessed compositional and topographical features mimicking the natural bone, and are of potential interest for implant applications.

4.4 Outlook

Even though titanium has been now used for several decades to produce dental implants there is still significant research dedicated to improve its interactions with cells and tissues. Among the different strategies reviewed here, we believe that those focusing on obtaining multifunctional coatings are the ones with the highest

potential to significantly impact clinical performance of dental implants in the near future. In that respect, the use of biomolecules to be covalently anchored to the metal surface is a very attractive strategy as it provides with the desired versatility and extraordinary specificity. However, notable achievements are still to be accomplished before these coatings will be available in the dental office. For instance, the most effective chemical strategies for the immobilization of the molecules to increase implant shelf life and avoid rapid degradation of the coating after implantation as well as the methods of sterilization to be applied need to be further studied. In years to come it is expected that researchers of different disciplines—engineering, chemistry, biology, dentistry—will continue developing together new dental implants to expand their application and to improve their clinical outcome both at short and long term after implantation.

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