Chapter

Perspective Chapter: Hydroxyapatite - Surface Functionalization to Prevent Bacterial Colonization

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Abstract

Microbial colonization is one of the main causes of implant loosening and rejection. Pathogenic contamination and the subsequent biofilm formation reduce the implant's chance of survival and can be life-threatening to a patient. Among the many strategies employed to reduce the infection probability of bioceramics, surface functionalization plays a key role. This chapter is dedicated to describing the different strategies available to prevent bacterial colonization and the proliferation of hydroxyapatite-coated implants. Moreover, the factors intervening in the bacteria-implant interaction will be described, detailing the mechanisms involved during the contact, adhesion, and proliferation of bacteria. Finally, the characterization methods will be discussed, emphasizing the bioactivity and antibacterial assays.

Keywords: antibacterial, hydroxyapatite, functionalization, bioactivity, implants

1. Introduction

Bioceramics, particularly hydroxyapatite (HA), are used massively to produce ceramic biomaterials and coatings for metallic implants. Implant infection is a serious medical complication and socioeconomic concern.

The economic burden can be quantified by the increased time in the hospital, rehospitalizations, additional surgeries, and the total cost of outpatient care. There are also intangible costs associated with implant infections, such as physical limitation, mental trauma, and reduction in quality of life for the patient.

An infected medical device can be difficult to treat with only antibiotic therapies. When these therapies are ineffective, then required the use of surgical procedures, such as debridement or implant replacement. Once the infection has spread to the bone, known as osteomyelitis, it may lead to limb amputation threatening the patient's life.

The first part of this chapter describes the mechanisms of interaction between the bacteria and the surface of hydroxyapatite. The second part is a review of the

different strategies to develop an antibacterial functionalization. The last part describes the main methods of studying bioactivity and antibacterial properties.

2. Bacteria-hydroxyapatite interaction mechanism

Infection of HA implants is a complex problem in clinical medicine. The large number of pathogens that infect medical devices, their resistance to antibiotics, and the strategies that microorganisms used to resist treatments are some of the reasons.

The infection probability is dependent on several aspects, such as the environmental conditions in which the attachment occurs, the type of microorganism, the properties of the substrate, and host characteristics. Furthermore, preexisting conditions, such as diabetes, obesity, and the use of immunosuppressant drugs, can increase the probability of an infection event.

There is limited research about the effect of material composition on the probability of infection. Hailer et al. [1] studied hip implant infection, and they detected no significant difference in infection events comparing hydroxyapatite-coated with micro-rough titanium implants.

The data available show that factors, such as the local immunological environment, the type of surgical intervention, the healing time, the fluid in contact with the implant and the microorganism contained, are more important to determine the infection probability than the chemical composition of the implant [2].

2.1 Implant infection classification

Infections of medical devices can be classified by the timeline of the infection event as early postoperative, late chronic, and hematogenous infection [3]. Alternative classifications are related to the microorganisms detected (bacterial, fungal, and polymicrobial) or by the substrate infected.

Early postoperative infections, also known as surgical site infection (SSI), are associated with postoperative wound infection and nosocomial infections. The symptoms appear during the first three months after surgery because of contamination during implantation or hospitalization before the wound is closed. The most common organism isolated from early infection is *Staphylococcus aureus* [4], but depending on the type of implant and the surrounding tissues, other organisms can also be found, such as *Klebsiella* spp., *Pseudomonas* spp., and *Escherichia coli* [5].

Late chronic infections are delayed postoperative infections and the symptoms normally emerge after the third month and up to two years after the surgery. These infections normally develop after months of apparent implant stability and their treatment includes surgical intervention and implant exchange [6].

Hematogenous infection generally occurs after a symptom-free period and is caused by bacteria originating from a secondary infection that spread through the bloodstream infecting other tissues [7]. This type of infection is a threat to the patient's life years after surgery.

2.2 Bacteria's life cycle

Suitable environmental conditions are necessary for bacterial attachment to the implant surface. During unfavorable or stressful conditions, the bacteria can enter an intermediate or "starvation survival" which allows them to survive long periods of

nutrient deprivation [8]. During this stage, the bacteria morphology changes dramatically adapting to a spore-like shape, known as ultramicrobacteria [9], reducing significatively the size and the metabolic activity until the conditions allow for active growth [10].

The bacteria life cycle on a substrate is a process genetically regulated that occurs in four main stages: attachment, growth, proliferation, and dissemination [9, 11]. Starting within the first few seconds after the implant insertion, the bacteria's reversible attachment to the substrate is mediated by Van der Waals forces and determined by the surface charge, the degree of hydration, the topography, and the surface's roughness [12, 13].

An inactive bacterium, also denominated swimmer or planktonic cell, can interact with a surface using a flagellum as a mechanoreceptor. Once a suitable surface is detected, a gene expression allows the bacteria to change phenotypically attaching irreversibly to the substrate [9, 14].

Immediately after the irreversible adhesion, the bacteria begin to grow and proliferate, creating microcolonies of one or several species embedded in an extracellular polymeric matrix or slime [11]. This polymeric slime is composed of exopolysaccharides, proteins, lipids, and extracellular DNA [13, 14]. The biofilm has matrix-enclosed bacterial colonies adherent to each other and surfaces. Biofilms contain open channels that facilitate nutrient and water diffusion from the bulk phase to bacteria in the biofilm [15].

Biofilm generation is a survival strategy that protects bacteria from changes in environmental conditions or antimicrobial agents. Biofilms can protect bacteria by different mechanisms. First, by acting as a barrier that can dilute reactive species before they can reach the bacterial wall. Second, by creating a stationary phase to reduce the effectiveness of antibiotics. Third, by increasing the survival of bacterial subpopulations with antibiotic-resistant phenotypes.

During the initial formation stages, the biofilm is still unstable and susceptible to elimination, but once maturation is achieved it gains an increased thickness (up to 50 μm), a mushroom or column-like morphology, and higher resistance to antibiotics [16]. The dissemination is the culmination of the "bacterial life cycle" by biofilm dissolution and detachment of free-living bacterial cells, which will spread to other locations [9, 16]. **Figure 1** presents a schematic representation of the implant infection timeline.

2.3 Common microorganisms infecting implants

Implants infections are mainly caused by *Staphylococcus* bacteria, the coagulase-positive *S. aureus,* and the coagulase-negative *Staphylococcus epidermidis* [13, 17].

S. aureus can release enzymatic virulence factors, such as exfoliative toxins and nucleases, to avoid the immune response and toxins like hemolysins and leucocidins to destroy host cells [18]. Indeed, considerable concern has recently been raised because of the resistance to antibiotics of methicillin-resistant *Staphylococcus aureus* (MRSA) as the cause of the most important incidents of infectious diseases [19].

In healthy people, *S. epidermidis* is a symbiotic microorganism that inhibits the colonization of more virulent bacteria in the skin and mucous membranes. *S. epidermidis* adheres exceptionally well to indwelling catheters and is founded in early postoperative infections [20].

Other bacterial strains found frequently in early postoperative implant infections are aerobic gram-negative bacilli, such as *Pseudomonas aeruginosa* and *Escherichia coli*. These microorganisms are especially threatening due to their high virulence and resistance to antimicrobial agents, mainly because they can produce a mature biofilm in 5–7 days [21]. *P. aeruginosa*, an opportunistic pathogen, found in soil or water is one of the major causes of nosocomial infections [22]. The pathogenesis of *P. aeruginosa*

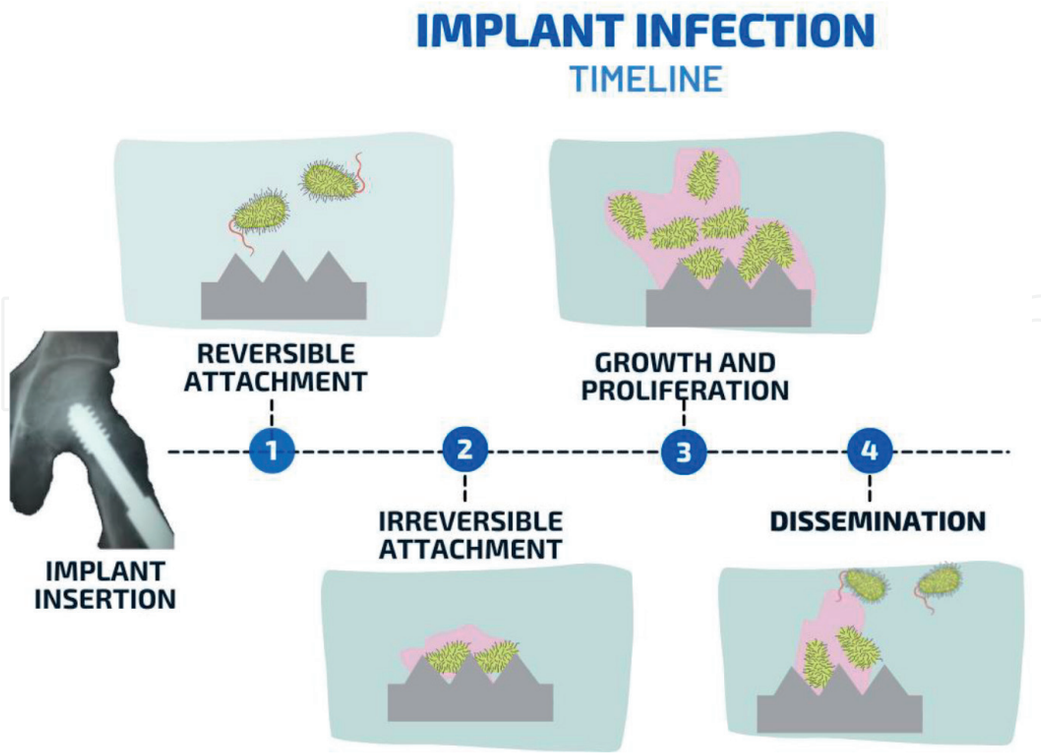

Figure 1.
Implant infection timeline. The Figure created by author using parts of figures from Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

is related to their high adherence to mucus and injured-epithelial cells [23]. *E. coli* is a symbiotic organism found in the gut flora of healthy people and can cause more than 80% of urinary tract infections (UTI). *E. coli* is the second highest cause of gram-negative orthopedic implant infection [22].

Other microorganisms found infecting orthopedic devices are *Streptococcus* and *Enterobacteriaceae* strains [7, 24]. If the implant is in direct contact with mucus or sores on the skin *Streptococcus pyogenes*, *Streptococcus agalactiae,* and *Streptococcus pneumoniae* can also be present [18, 25].

Polymicrobial infections occur generally in the early stages after surgery but can be discovered months afterward in immunocompromised patients. When two or more microbes colonize the same substrate, they interact releasing small molecules that change the host's environment. These biomolecules can increase the proliferation of the microbes improving their resistance to antimicrobial drugs resulting in a challenge for treatment [26, 27].

Fungal infections of implants are rare events, but their treatment involves more medical problems and aggressive surgical treatments than normal bacterial infections. Most fungal infections are caused by candida species, such as *Candida albicans* and *Candida parapsilosis* [28, 29].

3. Mechanisms against bacterial infections

Meticulous aseptic methods during surgery and prophylactic actions, such as antibiotic treatment, are not enough to prevent an implant infection. Several strategies can be used to improve the resistance to an infection event. These strategies can

be broadly differentiated by the production of an implant with intrinsic antibacterial properties and by the functionalization of the surface.

3.1 Intrinsic antibacterial properties of HA

Intrinsic antibacterial properties are dependent on the chemical composition, structure, and morphology of the material. The obtention of HA with intrinsic antibacterial properties can be achieved by multi-cationic/anionic substitution, also known as ion exchange [30].

Substitutions of calcium ions, phosphate, and hydroxyl groups in the HA lattice are possible because the HA-crystal can incorporate several elements with different atomic radii and charges [31]. Cations, such as copper, iron, magnesium, manganese, potassium, strontium, and zinc, among others, can substitute some calcium cations. Similarly, anions, such as chlorine, fluorine, and carbonate groups, can substitute hydroxyl and phosphate lattice positions.

The exchange of atoms opens a wide range of possibilities for customizing the properties of HA. The atomic substitution alters the solubility, reactivity, and biological properties of HA. Also, the amount of substituting atoms modify the percentage of the amorphous/crystalline phase ratio affecting the dissolution rate and the duration of the antibacterial properties. A co-doping strategy has been used to stabilize the crystalline structure after the introduction of atoms with a very big difference in radii and charge [32–35]. The objective is to improve bioactivity while maintaining good antibacterial properties.

There is a wide range of transition metals exhibiting antimicrobial activity being eligible for the cationic substitution of HA. Some examples are silver [33, 36–39], zinc [40–42], copper [34, 43, 44], and gallium [45–47]. The antibacterial characteristic of transition metals is produced by their oxidated forms [48]. The mechanism proposed is based on the inhibition of enzymes and cytoplasmatic proteins by the reaction with electron donors, creating M-thiolate bonds [49]. The inactivation of cytoplasmatic proteins can produce the disturbance of membrane potential, increasing permeability and the leaking of cellular contents [50].

Metallic ions can induce oxidative stress by increasing the production of reactive oxygen species (ROS) via the Fenton and Haber–Weiss reaction. These reactive oxygen species can react with DNA molecules and proteins, triggering condensation and denaturalization reactions, reducing the replication capacity [51]. **Figure 2** illustrates the main mechanisms of ROS toxicity.

The synthesis of multi-substituted HA can be achieved by different methods, such as sol-gel synthesis [35], co-precipitation [37, 41, 52, 53], hydrothermal [54, 55], and ball-milling [56, 57], among others [39, 42, 58–61].

The main advantage of the use of atomic substitution to increase the antibacterial resistance of implants fabricated with hydroxyapatite is the straightforward adaptation of the production facilities to prepare substituted HA. The atomic substitution can be achieved either during the synthesis of the HA or during the posttreatment of pure HA.

The challenge in the use of intrinsic antibacterial properties is related to the precise control of composition and percentage of the crystalline/amorphous phases. These variables simultaneously regulate the solubility and the release control of the antibacterial compound.

It is necessary to select the best variable combination to ensure long-term antibacterial properties and optimal concentration to avoid toxicity problems. The

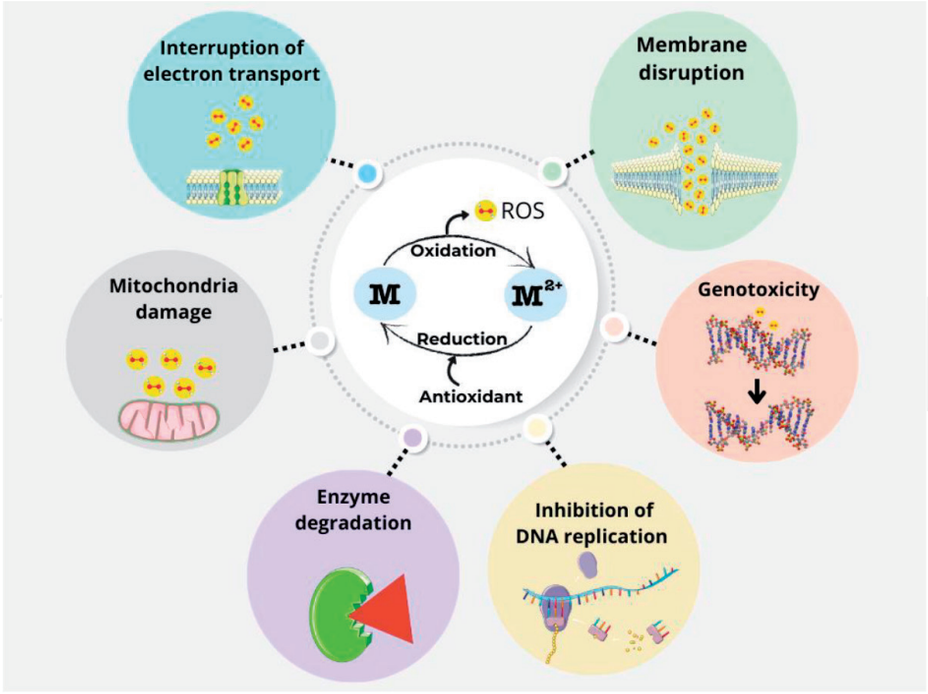

Figure 2.
Mechanisms of ROS toxicity. The Figure created by author using parts of figures from Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

compositional adjustment is an interesting strategy, combining different solubility rates and releasing kinetics to obtain antibacterial properties consistently during a long period of time [59]. **Figure 3** presents a schematic representation of Projection of Ag-substituted HA according to planes 110, 001, and 111.

3.2 Antibacterial functionalization of HA

Functionalization can be defined as a modification of the material surface by incorporating functional groups, biomolecules, nanoparticles, and other components with the objective to modify or enhance properties. The surface functionalization is mainly used to increase bioactivity, osseointegration, and angiogenesis but also can be used to produce antibacterial coatings on HA. Functionalization can be achieved by linking biomolecules by a covalent bond to the HA surface, by using physical adsorption to generate a covering layer, or by creating a hybrid coating.

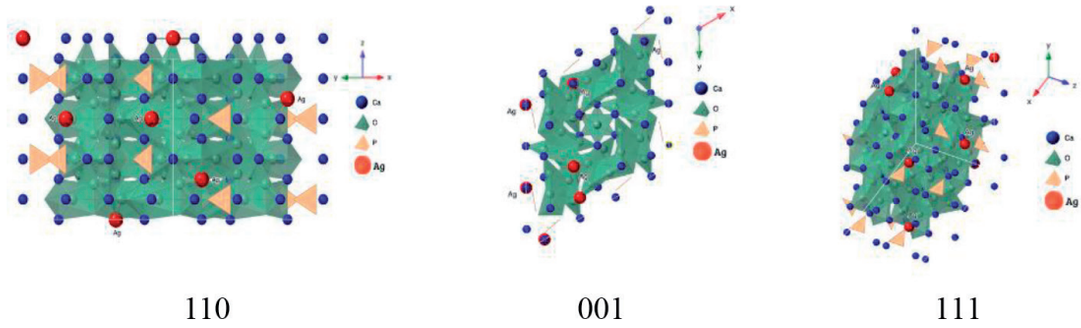

Figure 3.
Projection of Ag-substituted HA according to planes 110, 001, and 111. Figure created by author.

3.2.1 Functionalization by covalent bonding

A covalent bond can be created because the HA surface presents approximately 2.6 P-OH groups per nm^2 that can be used as an anchoring point to tether a molecule to the surface of HA via hydrogen bonding [62]. The target molecule can be covalently anchored if containing a functional group as amines, carboxylates, and thiols that can react with the hydroxyl moieties [63–65].

Another approach is to use a molecular adhesive as a spacer between the HA surface and the antibacterial compound. Molecular adhesives act like a bridge, creating a covalent bond between the hydroxyapatite surface and the biomolecule of interest [66, 67]. Many molecular adhesives can produce a covalent link with HA, but the most used are silane coupling agents that contain a reactive silanol group at one end and at the other end a hydrolysable group, typically alkoxy, halogen, or amine [68]. CEPTES and APTES are examples of silane molecules that contain a reactive group of carboxyl and amino, respectively, that can react with a functional group of a target biomolecule [65, 69, 70].

The main drawback of the use of silane agents as adhesive molecules is their high reactivity. Silanes interact through hydrogen bonding with the hydroxyl groups of the surface, but lateral polymerization may occur, generating multiple siloxane layers. The creation of a monolayer of silane molecules at the HA surface normally requires anhydrous conditions, extended reaction times, elevated temperatures (50–120°C), and rigorous control of the reagent concentration [68].

Formulated bio-adhesives using mussel adhesive proteins (MAPs) are a good alternative for silane coupling agents [71, 72]. Many MAPs have been isolated containing L-3,4-dihydroxyphenylalanine (Dopa) residues. The functional part of Dopa residues necessary to create covalent bonds is the catechol group.

A catechol group is composed of a benzene ring and two hydroxyls in the ortho position. Catechol groups can be oxidated into quinones under alkaline and neutral conditions, creating a stable coating with controllable film thickness [73, 74]. Dopamine, caffeic acid, and L-3,4-dihydroxyphenylalanine have been used to produce stable coatings containing the molecule of interest [75–77].

3.2.2 Functionalization by physical adsorption

Physical adsorption is a straightforward method to generate antibacterial functionalization because it can be achieved by soaking HA powders or substrates into a solution containing the antibacterial molecule. The main drawback is the weak bonds or interactions resulting in high release kinetics of the adsorbed molecule.

The nature and chemistry of the HA control the interaction with biomolecules, and the surface chemistry and porosity are fundamental variables. The interaction between adsorbed molecules and hydroxyapatite surfaces is predominantly Van der Waals forces, hydrogen, and weak coordination bonds [78].

The adsorption capacity is dependent on the surface area available. When there is no internal porosity, the biomolecules are mostly adsorbed onto the surface, limiting the loading capacity. Mesoporous hydroxyapatites are excellent structures for biomolecule release using physical adsorption mainly by their high surface area, but also because the pore size and interconnection can be customized [65, 79].

The chemical composition of the HA surface can be functionalized to improve adsorption and reduce the releasing kinetics prolonging the duration and efficiency of the antibacterial properties (**Figure 4**) [80, 81].

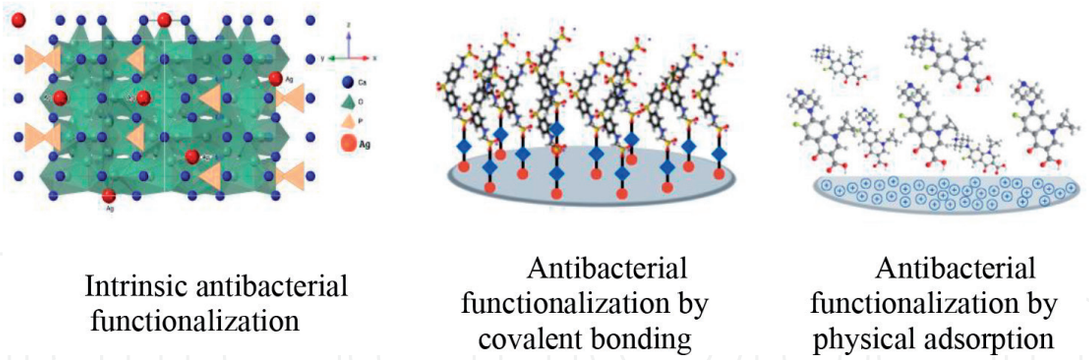

Figure 4.
Strategies to produce antibacterial properties on HA. Figure created by author.

3.2.3 Functionalization by antibacterial composites

The production of antibacterial composites is a valuable approach to produce antibacterial functionalization of hydroxyapatite. The combination of polymers, nanoparticles, antibiotics, and different structures of HA have unlimited possibilities for the obtention of medical devices with antibacterial properties.

Among the methods used to prepare composite, the incorporation of antibacterial compounds into the precursor solution is highly employed in co-precipitation, sol-gel, and hydrothermal methods to produce antibacterial HA powders with high homogeneity [42, 82, 83]. Additionally, the solution containing the antibacterial compound can be applied to HA substrates by dip coating and spin coating [84–86]. Another approach is to use electrochemical methods, such as electrodeposition [34, 87–89], micro-arc oxidation [90], and electrophoretic deposition [91], to generate HA coatings containing antimicrobial compounds. Electrospinning can be used for the creation of composite nanofibers and coatings with a combination of functionalized-HA nanoparticles and antimicrobial compounds [92–94]. Other methodologies include the spraying of a solution containing the precursors of HA mixed with the antibacterial compound [95–97].

Different compounds can be used to produce antibacterial functionalization of HA including antibacterial polymers, nanoparticles, antibiotics, and proteins. Among them, antibacterial polymers, such as chitosan, and polycaprolactone (PCL), are frequently preferred for the obtention of HA composites with antibacterial properties [87, 90, 91, 98–102]. Metallic nanoparticles, such as silver, zinc dioxide, titanium dioxide, and niobium pentoxide, have been combined with graphene and hydroxyapatite to produce composites with antibacterial properties [64, 80, 94, 103–105].

Many antibiotics have been used to produce antibacterial functionalization. Among them, β-lactam antibiotics, such as amoxicillin, can be adsorbed onto HA nanocrystals to be used alone or in composites fabricated by electrospinning [92, 106].

Fluoroquinolones, such as ciprofloxacin, have been used to functionalize nano-HA crystals and composites of chitosan, poly(vinyl alcohol), and HA sponges [107, 108]. Other fluoroquinolones, such as enoxacin, have been employed together with nano-HA/Polyurethane-cement to enhance the antibacterial properties of bone cement [109]. Adsorption has been used to introduce broad-spectrum antibiotics of the tetracycline class and cyclic oligosaccharides, such

as cyclodextrin, onto HA surfaces [78, 88, 95]. Moreover, antibiotics from the glycopeptides class as vancomycin has been loaded into porous substrates to obtain a controlled release [84, 91]. Taha et al. [95] prepared a cyclodextrin polymer loaded with rifampicin, an antimycobacterial antibiotic, to prepare a coating onto titanium-coated hydroxyapatite surfaces. Covalent immobilization of antibiotics like doxorubicin can be achieved using HA nanoparticles functionalized with amino groups or by more complicated methods, including the fabrication of polymer brushes, to anchor aminoglycoside antibiotics, such as gentamycin, to HA substrates [110, 111]. Furthermore, aminoglycosides antibiotics, such as streptomycin, have been encapsulated in HA nanoparticles [93].

Proteins that present a broad spectrum of antibacterial activity can be used for the antibacterial functionalization of HA surfaces. One example is protamine, a cationic protein rich in arginine residues, used for Koizumi et al. [112] to functionalize different calcium phosphates by adsorption. Peptides with between 10 and 15 amino acids are preferable for the antibacterial functionalization of surfaces because of the lower production cost compared to proteins [113]. These peptides so-called antibacterial peptides (AMPs) are very interesting due to their selectivity and high antibacterial efficiency at low concentrations. Their efficacy is based on the adoption of amphipathic structures and their cationic character. So far, more than 700 types of AMP have been isolated from different organisms. AMPs can be used to functionalize HA surfaces by electrostatic and covalent attachment [114].

4. Characterization methods

4.1 Bioactivity

One of the most important factors affecting the bioactivity and biocompatibility of HA implants is the release of substances that can cause toxicity, hypersensitivity, allergies, or even osteolysis depending on the released product, their concentration, and the exposure time [115]. Ensuring a controlled release of substances after the implantation is one of the key strategies to improve the implant performance, as it can affect osseointegration and implant long-term viability. The ISO 10993-17 is the standard that establishes the limits for leachable substances in medical devices.

Immersion tests are used to quantify the products released at body temperature (37 ± 1°C) under static or dynamic conditions. The level of substance released is mainly dependent on the implant's surface area and the composition of both implant and the body fluid in contact. Therefore, the released products should be determined using a solution with the closest composition to the body fluid in contact with the implant under working conditions. Complex biofluids can be replicated with phosphate buffer saline (PBS), Hank's solution, simulated body fluid (SBF), Ringer's solution, artificial saliva, and eagle's minimum essential medium (EMEM), as well as other fluids [116, 117].

The ability of an orthopedical implant to induce the formation of biological apatite on its surface is one of the requirements to determine correct osseointegration. The precipitation of apatite can be replicated *in vitro* by the immersion of the sample in simulated body fluid, a saturated solution with a composition comparable to the human blood plasma [118].

The cytotoxicity of an antibacterial functionalization is determined by cell viability and cell proliferation. Cell viability refers to the number of live, healthy cells in a

sample and cell proliferation is defined by the valance between cell divisions and cell loss through cell death or differentiation. Cell viability assays can be used to evaluate cell health and can be assessed by culturing the chosen cells over either the sample or an extraction vehicle. The cell viability can be quantified using redox indicators that interact with metabolites produced by healthy cells. Another method is the use of dyes that react only with healthy cells like methylene blue, triptan blue, neural red, or by live/dead assays [119–121].

Cell proliferation is defined as the increase of cell number after the final step of the cell cycle due to cytokinesis or cell division. Many strategies can be used to assess cellular proliferation as the use of nucleoside-analogs incorporated during DNA synthesis, the quantification of cell cycle-associated proteins, and the use of cytoplasmatic proliferation dyes [122–125].

All methods are valid to compare proliferation but it is important to consider their strength and limitations and, to improve the accuracy of the results, multiple assays should be performed [126]. The standard procedure of in vitro cell viability and proliferation assays is exposed in ISO 10993-5: Biological evaluation of medical devices-tests for in vitro cytotoxicity.

4.2 Antibacterial properties

The antibacterial properties of a biomaterial can be tested by studying antimicrobial susceptibility *in vitro*. Multiple methods can be used to evaluate, either quantitatively or quantitatively, the antibacterial activity of HA coatings and powders.

Qualitative measurements may not provide quantifiable results but offer valuable information regarding the bacteria's sensitivity to antimicrobial functionalization of materials.

- The Kirby–Bauer disk diffusion susceptibility test, also known as the agar disk diffusion method, is a standardized procedure to qualitatively determine the sensitivity or resistance of bacteria to antimicrobial compounds [127]. The presence or absence of growth around the disk is an indirect measure of the bacterial inhibition by the antimicrobial compound. The Kirby–Bauer test was designed to test antibiotic-impregnated disks, but many authors have also used it to test antibacterial substrates. This method cannot be used to determine the minimum inhibitory concentration (MIC) but can be approximate for some microorganisms and antibiotics by comparing the inhibition zone using systems that can read and interpret the results [128]. The main advantages of this method are its simplicity and low cost.

Quantitative tests provide more accurate information about bacterial growth in presence of an antimicrobial compound. These methods are normally based on the measurement of the turbidity of a bacterial solution to indirectly assess the bacteria's sensitivity to an antibacterial compound.

- Among the quantitative test used, the broth dilution test can be used to test both coatings and powders that release the antibacterial compound. This method is based on the preparation of dilutions of the antibiotic or the extract in a liquid growth medium. The dilutions are inoculated with a previously known concentration of bacterial suspension. After overnight incubation, the turbidity is measured, and the MIC is defined as the lowest concentration that prevented the growth of the microorganism.

- Once the MIC is determined, it may be useful to determine the interaction of the antibacterial compound depending on the time. The time-kill assay is based on the preparation of antimicrobial extracts with dilutions lower than MIC, and up to 16 x MIC that is inoculated with the same concentration of bacterial suspension, and their growth is measured during different intervals of time [129].

Likewise, in any biological test, the results obtained from a bacterial sensitivity test are dependent on variables, such as the inoculum size, the type of growth medium, and the incubation time [130]. Updated standards should be used to obtain reliable results.

Among the standardized methods to quantitatively evaluate antibacterial activity, there is the ASTM E2149-standard test method for determining the antimicrobial activity of antimicrobial agents under dynamic contact conditions and ASTM E2180-standard test method for determining the activity of incorporated antimicrobial agent in polymeric or hydrophobic materials.

5. Conclusions

This book chapter presented the mechanisms that bacteria use to attach and proliferate on implants. Moreover, the main strategies used to provide antibacterial properties to hydroxyapatite powders and substrates were exposed.

The obtention of medical devices with suitable antibacterial properties must be complemented by excellent biocompatibility and adequate mechanical properties. Novel strategies include the combination of different methodologies and the use of different compounds to improve the properties.

The main difficulty in developing antibacterial functionalization of implants is the lack of homogeneity in the in vitro assays, which limits the comparison of the strategies employed. Additionally, many factors can affect the results from in vitro assays, such as the type of cell, their origin, incubation time, and the compound used to quantify the proliferation.

To accelerate the development of suitable antibacterial functionalization, more efforts must be made to use standardized protocols for bioactivity and antibacterial in vitro assays. The homogenization of the assays is necessary for an accurate comparison of the release of substances and bioactivity. Furthermore, through an in-depth study of the antibacterial properties during long periods of time, a selection of the suitable strategy for each application can be made.

Even though many attempts have been made to produce antibacterial functionalization of HA, none have been used industrially. Considerably work still needs to manufacture a cost-effective implant's antibacterial functionalization.

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