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Review article

A critical review of multifunctional titanium surfaces: New frontiers for improving osseointegration and host response, avoiding bacteria contamination

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ABSTRACT

Evolution of metal implants progressively shifted the focus from adequate mechanical strength to improved biocompatibility and absence of toxicity and, finally, to fast osseointegration. Recently, new frontiers and challenges of Ti implants have been addressed to improvement of bioactivity, fighting of bacterial infection and biofilm formation, as well as modulation of inflammation. This is closely related to the clinical demand of multifunctional implants able to simultaneously have a number of specific responses with respect to body fluids, cells (osteoblasts, fibroblasts, macrophages) and pathogenic agents (bacteria, viruses). This complex system of multiple biological stimuli and surface responses is a major arena of the current research on biomaterials and biosurfaces. This review covers the strategies explored to this purpose since 2010 in the case of Ti and Ti alloys, considering that the number of related papers doubled about in the last seven years and no review has comprehensively covered this engaging research area yet. The different approaches followed for producing multifunctional Ti-based surfaces involve the use of thick and thin inorganic coatings, chemical surface treatments, and functionalization strategies coupled with organic coatings.

Statement of Significance

According to the clinical demand of multifunctional implants able to simultaneously have a number of specific responses with respect to body fluids, cells and pathogenic agents, new frontiers of Ti implants have been addressed to improvement of bioactivity, fighting of bacterial infection and biofilm formation, as well as modulation of inflammation. Literature since 2010 is here reviewed. Several strategies for getting bioactive and antibacterial actions on Ti surfaces have been suggested, but they still need to be optimized with respect to several concerns. A further step will be to combine on the same surface a proven ability of modulation of inflammatory response. The achievement of multifunctional surfaces able to modulate inflammation and to promote osteogenesis is a grand challenge.

Contents

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1. Introduction

The demand of clinicians in dentistry and orthopedic medical fields was as first oriented to mechanical properties, durability and biocompatibility. Ti and Ti alloys largely fulfilled these requirements; surface mechanical properties (wear and friction resistance) are still an open issue in the case of artificial joints, but this specific topic will be not discussed in this review that is focused on tissue integration. In a further stage, surface chemical properties and surface-bone interface became the main topics with a clinical demand of faster osseointegration and a focus on the osteoblast response to the implant surface. Even if osseointegration of conventional Ti and Ti alloys is successful in many clinical cases of un-cemented implants, an osseoinductive behavior of the surface is demanded mainly when fast healing is required or quality and/or quantity of bone is poor. Several strategies were explored for this purpose and they can be divided up by those using bioactive approach (*in vivo* induced apatite precipitation) or those based on topographical stimulus of the osteoblasts through tailored roughness. Bioactive behavior can be obtained on Ti and Ti alloys by applying a coating of a foreign material (plasma spray coatings of apatite or bioactive glasses), using electrochemical processes (anodic oxidation or NTs) or chemical surface treatments (in acidic, basic or oxidative environments). A classification of the bioactive surfaces can be done according to the mechanism of bioactivity, which can be related to ion exchange with the body fluids [1] and/or to surface charge effects and micro- or nano-scale topography [2]. Some of these surface treatments are currently used in clinical applications [3,4].

More recently, the clinical demand moved to multifunctional surfaces able to simultaneously give a specific response to colonization by different cells (osteoblasts, fibroblasts, macrophages) and infection agents (bacteria, viruses). This complex system of multiple biological stimuli and surface responses is the main focus of the current research on biomaterials. Antibacterial surfaces able to avoid biofilm formation are highly challenging for bone contact implants. It has been reported that deep infections typically occur in 1–2% of patients with total hip arthroplasties [5] and dental peri-implant disease and infection have become a main focus in terms of prevention and treatment of oral implantology [6]. When deep infection occurs, removal and re-implantation of the implant is often necessary, with additional discomfort of the patients and costs for the health services. The main approaches, nowadays developed and under investigations, focused on simultaneous bioactive and antibacterial actions of Ti surfaces will be here resumed.

A focus of this review is on the inflammatory response elicited by Ti surfaces because it is strictly connected to physiological osseointegration and infections. Fewer macrophages and lower inflammation is reported on the Ti/Ti alloy surfaces than on stainless steel [7] or polyether ether ketone (PEEK) [8]. In general, Ti is well tolerated by the body as long as the implant is in bulk form, mechanically stable and non-infected. If the latter conditions are not met, the implants can be associated with an acute/chronic inflammatory reaction, osteolysis, loosening and failure. Human osteoclasts can corrode stainless steel, cobalt and Ti alloys leading to the production of metal ions responsible for inflammatory reactions. Traces of cellular activities on metal orthopedic explants have recently been reported as inflammatory cell-induced corrosion being the result of the cells sealing on the metal surfaces and releasing reactive oxygen species. The extent and clinical relevance of this phenomenon has yet to be completely understood [9]. A 19-year retrospective study of dental implant failure indicated that 47% of the early implant failures were caused by inflammation [10]. In the case of devices for stabilization of fractures, side effects related to the immune reaction of the body and excessive inflammation are often observed [11]. If we look at the soft tissue-implant interface, Ti showed higher inflammation reaction than ceramics, such as zirconia, even if lower than other metals, such as gold, as shown in the case of abutments [12,13].

This review, prepared by examining the relevant literature published since 2010 to present, aims at providing a picture of current knowledge and challenges concerning multifunctional Ti and Ti alloys surfaces, which can be useful to both experienced scientists and early-stage researchers working in the field.

2. Inflammation response and Foreign Body Reaction as open issues in bone implants: key strategies to control them

Healing reaction of Ti implants can occur through osseointegration (desired outcome) or fibrotic encapsulation through chronic inflammation (failure). The events related to inflammation response to an implant can be summarized in the following eight steps [14–16]: exudation, protein surface adsorption, development of a blood-based transient provisional matrix, recruitment of the cells of the innate immune system (leukocytes, platelets, complement and coagulation systems), migration of neutrophils, substitution by monocytes and differentiation into macrophages, Foreign Body Reaction (FBR), production of Reactive Oxygen Species (ROS), fusion of monocytes/macrophages to form Foreign Body Giant Cells (FBGCs) or apoptosis. The acute inflammation step usually resolves within less than 1 week: persistence of this state beyond 3 weeks is usually related to infection or failure. Osseointegration can be considered as a limited FBR without occurring of chronic inflammation. FBR can be histologically recognized because of a typical aspect with a thin layer of macrophages; type 2 inflammation is early activated and bone resorption is suppressed, suggesting a shift to a more pronounced bone forming environment (till after 4 weeks of implantation) [17]. Subjective host (patient) factors can interfere on this cascade of inflammation events such as age, nutritional status (malnutrition results in increased susceptibility to infections and in changes to the innate immune system), quality of the adjacent tissues, and comorbidities.

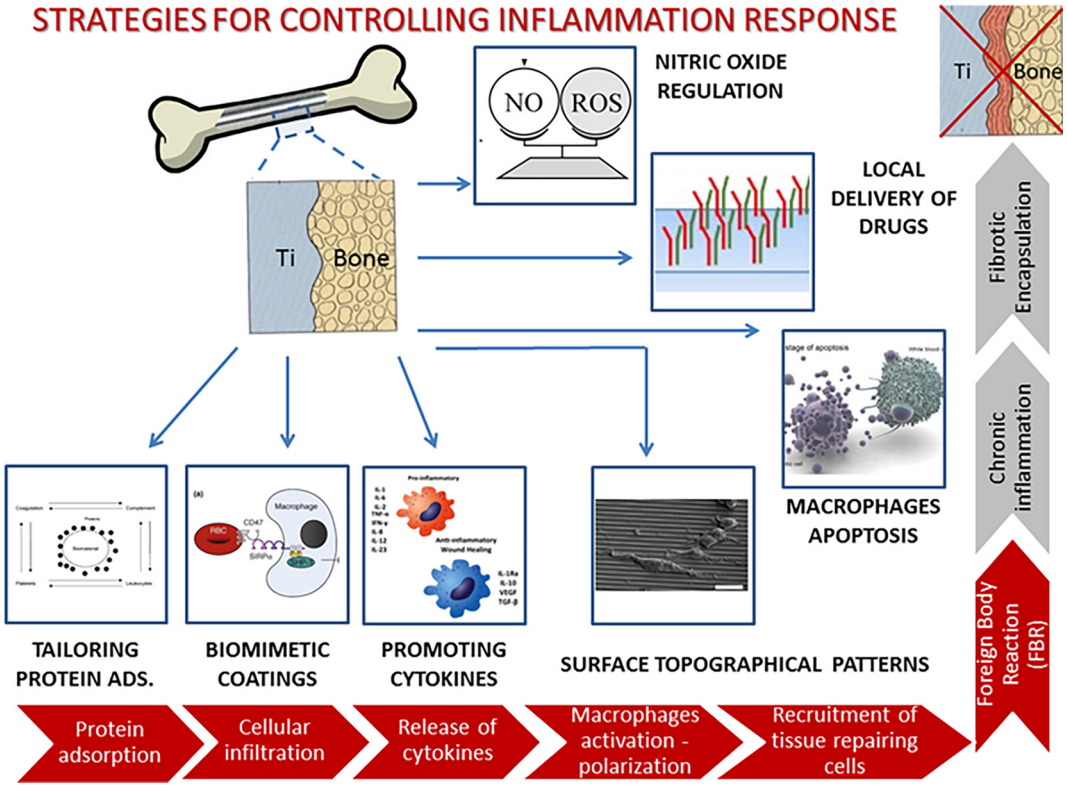

Fig. 1. The inflammatory response pathway and related strategies for controlling inflammation response of implants.

Long-term clinical function of dental implants is dependent on the FBR, that can be disturbed through a breakdown process: macrophages are again activated and may fuse into FBGCs resulting in bone resorption and rupture of mucosal seals, so infection may follow as a secondary event [18]. Ti ions released by corrosion of the implant may be partly responsible for the infiltration of monocytes and osteoclast differentiation involving the deteriorating effects of peri-implant mucositis, which can develop into peri-implantitis accompanied by alveolar bone resorption [19].

In the case of cemented hip joints, the injury at the implant site is due to death and debris of bone through surgical procedure which generally resolves in acute inflammation over a thickness range of 500 μm underneath the surface. After few weeks, fibrocartilage, fibrous tissue and trabecular bone can be observed next to the cement and integration of cement within the trabeculae of the new bone occurs in some areas. On the other side, in case of cement disease, chronic inflammation and related osteolysis can cause implant failure.

In the case of un-cemented hip joints, osseointegration occurs through a moderate FBR, but in some cases chronic inflammation can derive from rough surface topography or highly porous coating with formation of oriented fibrous tissue instead of bone or because of stress shielding effect causing adverse remodeling.

The implant outcome can be altered by Ti surface engineering. Surface engineering is recently moving to modulation of host and inflammation response considering that some advantages derive from a physiologic inflammation response (removing cellular debris, deterring progression of eventual infection), while the risks of a severe inflammation response must be avoided (rejection of implants, early or late failure, negative effects on implant function). It is also speculated that osseointegration of bioactive materials is triggered by a vigorous initial inflammation response [20]: activated polymorphonuclear leukocytes (PMN) adhere on the surface of the bioactive implant releasing prostaglandin E2 (PGE2) and thus recruiting further leukocytes, which become activated and also release PGE2. PGE2 governs also peri-implant vascularization (preventing encapsulation), and promote M2 macrophage differentiation [21]. This first event must be strict time-limited and it must not develop into chronic inflammation. There are a few strategies that can be followed in order to modulate inflammation response of a biomaterial (Fig. 1), as described in the following subsections.

2.1. Tailoring protein adsorption

Many potential ligands for leukocyte receptors derive from adsorbed plasma proteins (IgC, fibrinogen, fibronectin and complement factor C3b) and promoting or preventing adsorption of these proteins can be used as a strategy respectively to promote or to prevent inflammation response. As an evidence of this, grafting heparin on Ti surfaces inhibits inflammation through higher adsorption of albumin and production of anti-inflammatory cytokines (IL-1Ra, IL-10, TGF-β). Going further, BMP-2 can be immobilized on heparin in order to induce specific adsorption of fibronectin: the final aim is to contemporarily induce low-inflammation reaction and higher osteoblast adhesion and mineralization [22]. Considering that acute inflammation has to be resolved in less than 1 week in order to get a positive osseointegration, the best time for anti-inflammation action should be limited to few days and osteogenesis (e.g. BMP release or switching to healing macrophages polarization) should be maximum after 2–3 days. Other authors report that BMP-2 grafted onto micro-textured metal implants may increase inflammation, suggesting that clinical application of BMP-2 may need to be carefully evaluated [23].

As previously described, bioactive coatings result in an enhanced first inflammation reaction that is mediated by protein adsorption; silica hybrid sol-gel coatings induce higher adhesion of complement proteins, expression of osteogenic markers (ALP)

and IL-6 with a clear increase in the inflammatory activity in comparison with sand-blasted and acid-etched titanium surfaces [24].

Wettability has a strong effect on protein adsorption and down-regulation of pro-inflammatory cytokines was registered on hydrophilic-modified Ti surfaces (SLA active surfaces through sandblasting, acid etching and storing in isotonic saline solution) [25,26]. Similarly, a super-hydrophilic Ti surface prepared by ozone gas or oxygen plasma exposition successfully increased MSC proliferation and differentiation, and mitigated pro-inflammatory cytokine production [27,28]. The drawback of this strategy is that the same proteins in most cases act as promoting agents for inflammation and recruitment of bone repairing cells (such as osteoblasts), so prevention of their adsorption can negatively affect osseointegration. Moreover, early inflammation is necessary to healing and it must not be completely prevented. As last, the action of surface chemistry must act on all the whole series of plasma proteins with potential ligands for leukocyte receptors over a long period of time in order to be truly effective, and this is sometimes not proved by simple *in vitro* adsorption tests with a single or few blood proteins.

2.2. Promoting macrophage polarization

Macrophage polarization is a process by which macrophage expresses different functional programs in response to microenvironmental signals; they can be fully polarized and acquire specific phenotype like M1 (classically activated pro-inflammatory macrophages in response of Th1 cell-derived cytokines) or M2 (alternatively activated anti-inflammatory macrophages in response of Th2 cell-derived cytokines). Scar formation is a form of matrix degradation due to un-balancing between M1 (pro-inflammatory) to M2 (pro-wound-healing) macrophage phenotypes. M1 response is required in the first phases of implantation in order to eliminate potential pathogens and to remove dead cells and tissue debris from the wound site [29]. On the other side, excessive pro-wound-healing action (CCL18 strongly up-regulated by IL-4) was shown to be involved in fibrosis [29]. Local exposition of antibodies of pro-inflammatory cytokines can be used in order to neutralize specific cytokines and as a strategy for macrophage polarization in favor of M2. This last strategy was considered in the case of wear-particle induced osteolysis and periodontitis (local administration of IL-4 prevented an inflammatory response and reduced osteolysis in a mouse calvaria model [30]), but clinical tests are not yet available. A bilayer hydrogel coating on titania nanotubes (NTs) was prepared as reservoir to modulate the release of IL-4 and IFN-γ by two hydrogel layers of chitosan/β-glycerophosphate disodium and carboxymethyl chitosan/genipin. The results manifested that IFN-γ released from the system stimulated switching of macrophages to M1 in 3 days, whereas sustained release of IL-4 polarized macrophages to M2 after 4 days [31]. This strategy is of great interest, but kinetic of release is a complex event difficult to be simulated and it must be carefully evaluated in clinical trials.

2.3. Surface topographical patterns

Osteoblasts prefer rough surfaces and full use of roughened surfaces for bone implants (dental and orthopedic) has been made in the past, but it should be critically reviewed considering that fast osseointegration and inflammation are strictly connected [32]. Sandblasted and acid etched Ti surfaces used in dental implants skew macrophages towards M1 activation [33] and are coupled with the ability to release neutrophil extracellular traps [32].

Nano- or micro-oriented patterns (grooves 0.25–2 μm wide) are able to affect macrophage polarization: macrophages elongate on grooved implants on a more M2-like phenotype [34]. Anodized Ti with coatings of titania NTs (50–120 nm in diameter), eventually modified with fluorine [35], results in a reduced density of macrophages, inhibition of nitric oxide production and suppression of MAPK and NF-κB pathways [36–39]. Migration and activation of macrophages were reduced also on randomly nanostructured Ti thin film coatings with inhibition of NO and pro-inflammatory cytokines [40]. The combination of topographical selected roughness/patterns and hydrophilicity may interact synergistically to yield a microenvironment suitable for M2 macrophage polarization, reduced healing times and increased osseointegration [28]. This strategy could be much more developed in the future; a passive strategy has the advantage to be free from any toxicity or contamination risk, which is quite interesting even if it has the drawback of absence of an active and strong action.

2.4. Biomimetic coatings

A strategy is to cover the surface by collagen or transmembrane molecular markers of "self" (such as CD47) or mesenchymal stem cells. As inflammatory cells do not recognize these surfaces as being foreign, inflammatory cell adhesion is reduced with a down-regulation of expressed cytokines. A negative regulation of phagocytosis occurs similar to what occurring for red blood cells: a 'do-not-eat-me signal' is promoted to macrophages. The advantage is that both biomaterial degradation and fibrous capsule formation can be reduced, but the drawback is a complete suppression of defense by acute inflammation response. Another approach is to functionalize the titanium surface with peptides. Antimicrobial peptides, positively charged (cationic peptide CecB) and amphiphilic oligopeptides can be used to functionalize Ti surfaces (through silanization, a film of polydopamine or a PEG spacer) with an influence on infections and innate immunity response. They can have a multifunctional action: down-regulation of pro-inflammatory cytokines, upregulation of the anti-inflammatory cytokines, reduced macrophage activation, inhibition of bacteria adhesion, and higher osteoblast viability than native Ti substrates [41–44]. This approach is of particular interest in view of bio-functionalization of Ti for multifunctional surface properties.

2.5. Local delivery of drugs

Systemic delivery of anti-inflammatory drugs has a number of drawback such as low local concentration and high systemic side effects. Biomaterials can be used to create a local anti-inflammatory microenvironment. Delivery of bisphosponate from hydroxyapatite (HAp) coating on cementless hip prostheses has been tested, but long-term observations are still missing [45]. Electrochemical coating of Ti by titania NTs can be used for loading and release of anti-inflammatory drugs over 30 days: this kind of treatment could be suitable for several orthopaedic applications and bone therapies including treating bone infections, repair and osteomyelitis [46]. Some papers refer about the potential therapeutic applicability of the dietary flavonoid quercetin to reduce pain and inflammatory damages associated with prosthesis wear process and *in vivo* production of titania wear particles [47]. Local delivery of this drug is an interesting approach and a tentative is reported through titania NTs (anodic oxidation) loaded with quercetin and chitosan [48,49]. The local concentration of the drug can be controlled and tuned by controlling the thickness of the chitosan in order to treat postoperative infections, inflammation and for quick healing with better osseointegration of the titanium implants. Titania nanotubes coatings releasing BMP-2 and trehalose have also been tested *in vitro* and *in vivo* showing sustained release over the course of 8 days, significantly promoting osteogenic differentiation of bone marrow stromal cells, but not their proliferation and inhibiting pro-inflammatory factors. This active

strategy is very challenging even if. obviously, it has to face several critical regulatory issues related to incorporation of active biomolecules.

2.6. NO regulation

A surface modification of Ti inspired by the endothelial glycocalyx was explored: titania NTs were coated with heparin–chitosan polyelectrolyte multilayers to provide glycosaminoglycan functionalization and chitosan was modified with a nitric oxide-donor chemistry to provide an antithrombotic small-molecule signal [50]. This is a quite complex approach considering that a lot of biological side effects can be elicited by NO.

2.7. Macrophage apoptosis

A correlation exists between an increase in the fusion of adherent macrophages and a decrease in apoptosis: formation of FBGCs may provide a mechanism of survival for biomaterial-adherent macrophages with a risk of chronic inflammation. A possible strategy is a bioengineered hydrophilic surface able not only to reduce the levels of macrophage adhesion and fusion into FBGCs, but also to increase the levels of apoptosis of adherent cells. Filamentous phages as well as viruses infecting only host strain and nontoxic to human result in a relatively-high initial inflammatory activity (24 h) but, in the later stage (7–10 days), inflammatory response is reduced. Phage films also improved osteoblast adhesion, differentiation, and HAp formation via a combination of topographical and biochemical cues [10]. This is a very intriguing strategy because it simultaneously allows a proper inflammation response on short times and reduces it in the next stages: applying this approach to modulate inflammation over time indeed deserves further investigation in the future.

3. Multifunctional bioactive and antibacterial metal surfaces

Different strategies have been investigated in literature in order to get Ti surfaces simultaneously bioactive and antibacterial (Fig. 2). Recent results are summarized in the following paragraphs, following this rationale for classification. Inorganic antibacterial agents were separately considered (Sections 3.1–3.4) from the organic ones (mainly antibiotics – Section 3.5). As inorganic antibacterial agents, mainly metallic ions and nanoparticles as well as their oxides have been considered, e.g. Ag, Cu, Zn and Ce. Generally speaking, the advantages of inorganic antibacterial agents are a broad spectrum of activity, which allows both treatment of polymicrobial infections and prevention of contamination from unknown bacteria, and low resistance development. In this regard, the problem of resistant bacterial strains is now one of the most critical issues in the use of antibiotics, as discussed in Section 3.5. On the other hand, the main drawbacks are their relative new application which implies difficulties in certification and regulatory aspects and in finding the optimal therapeutic window, with effective antibacterial behavior without cytotoxic effects.

Thick coatings (thicker than 1 μm) were separately considered (Section 3.1) from thin ones (less than 1 μm thick – Section 3.2), as well as surfaces with features on the nanoscale (Section 3.3) and surface modified without a coating of a foreign material (Section 3.4).

3.1. The strategy of inorganic thick coatings

As previously mentioned, the considered inorganic antibacterial agents are mainly Ag [51–64], Cu [65–67], Zn [68–74], and Ce [75] as metallic ions or nanoparticles. A dose dependent antibacterial activity is often reported for inorganic antibacterial agents, but simultaneously a dose dependent cytotoxic effect is almost always present, with particular frequency for Ag-doped surfaces. In order

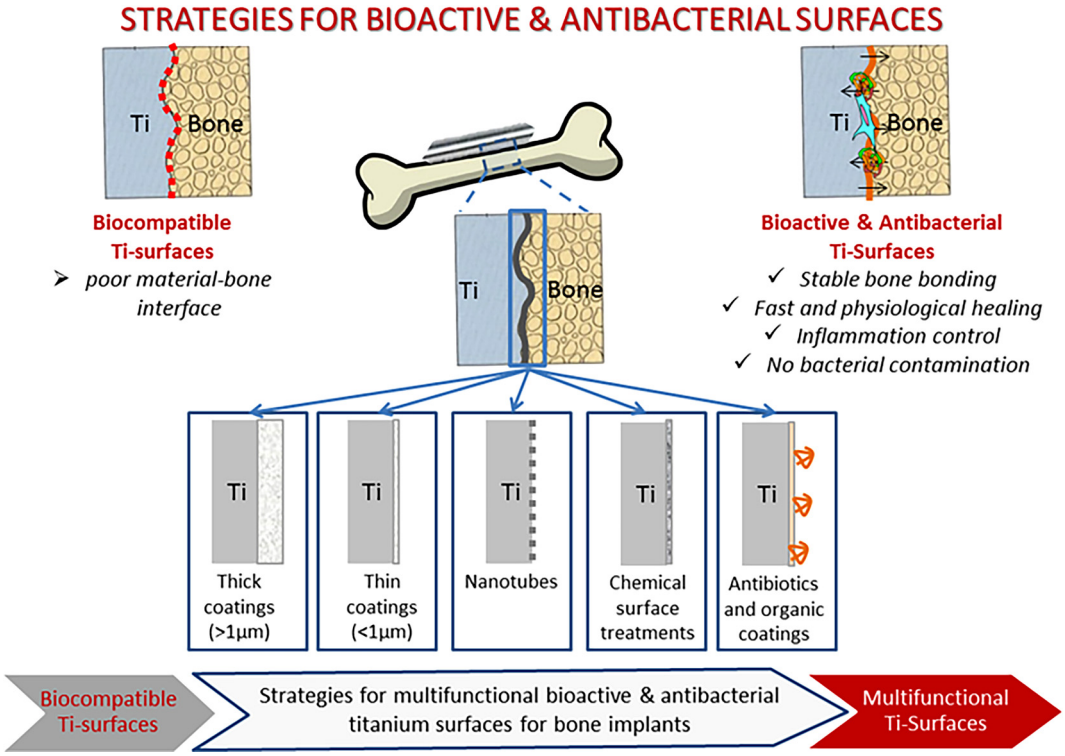

Fig. 2. Strategies for imparting bioactive and antibacterial properties to Ti surfaces through different types of surface coatings or modifications.

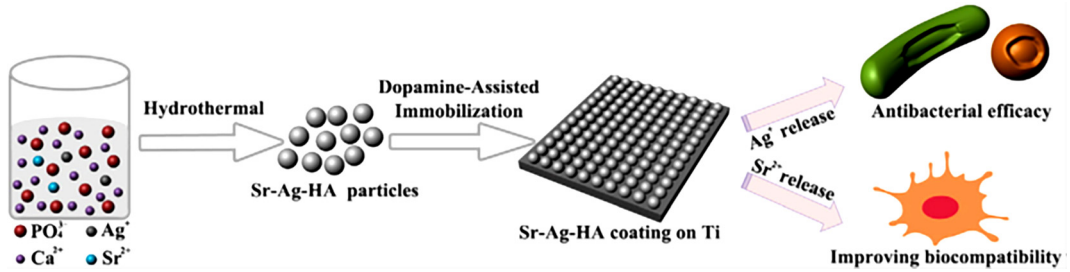

Fig. 3. Fabrication process and biological effects of Sr/Ag-doped hydroxyapatite coatings deposited on Ti implants. reproduced from [60].

to overcome this drawback, the use of a thick HAp coating co-doped with Ag and Sr has been proposed [60,61] with interesting results: the presence of Sr does not inhibit Ag antibacterial action, but significantly improves cell viability for the same Ag content (Fig. 3). Although the viability of osteoblastic cells grown on the Sr/Ag-co-doped coatings was not so as high as that of the cells seeded on bare Ti surfaces, this approach shows great promise and motivates further research work. Furthermore, the controlled release of Sr^{2+} ions could also be a smart mean to limit bone resorption at the implant site and, in general, to locally treat osteoporosis [61].

Poor reproducibility of ion release and antibacterial action have been evidenced in many studies (e.g. ion release and antibacterial tests), especially for Ag-doped surfaces. Zhang et al. recently proposed the thermal treatment of Ag-containing HAp coatings in order to overcome these problems [59] through the oxidation of metallic Ag nanoparticles within the coating, but this issue is up to now highly debated in the scientific community.

As far as a bioactive matrix is concerned, HAp is the most common one for producing thick bioactive coatings as well as for their enrichment with antibacterial agents. HAp bioactivity and its ability to bond to bone are well documented and studied. Moreover, the techniques for its deposition onto metallic substrates (e.g. thermal spray, electrochemical deposition and sol-gel dip coating) are now consolidated and allow the obtainment of reproducible and reliable results with sufficient mechanical adhesion to the substrate [76]. HAp bioactive thick coatings doped with Ag ions [53–57,63,64] or nanoparticles [58,59] are the most widely studied, but also Cu [66], Zn [77], Ce [75] and Sr/Sm [74] doping have been reported.

Bioactive glasses have also been studied for the preparation of thick bioactive coatings with antibacterial properties, in combination with ZnO [68,69] or Ag nanoparticles [52] and organic matrices such as alginate [68,69] or PEEK [52] by means of electrophoretic deposition. Stainless steel (AISI 316L) is often used as a model metallic substrate for optimization of these coatings in view of a further application on Ti and Ti alloys.

Ag-doped β-tricalcium phosphate [62] and Cu-doped $CaSiO_3$ [67] have also been reported as bioactive and antibacterial thick coatings on Ti substrates obtained by electrophoretic deposition.

In addition, bioactive TiO_2 thick layers, obtained by means of Micro Arc Oxidation (MAO) or Plasma Electrolytic oxidation (PEO), have been enriched with Zn [69,71–73] or Cu [65] directly during the electrochemical growth of the coating. Moreover, Ag nanoparticles have been added onto a bioactive MAO TiO_2 layer using poly-dopamine as in situ reducing agent and linker [54]. In this case, the coating is directly grown onto the substrate in order to reduce the potential critical issue related to the presence of a sharp interface of the coating with the substrate. These electrochemical oxides obtained onto Ti and Ti alloys are enriched with calcium and phosphorous ions coming from the electrolytes during the preparation process and are bioactive because of them.

A summary of the scientific papers dealing with this section is reported in Table 1.

One of the major issues related to the industrial development of innovative multifunctional coatings is their effective scalability to industrial production of complex medical devices. In this context, it is extremely important that the proposed techniques can be easily transferred to complex shapes and high production volumes. In this regard, thermal spray equipment is widely diffused at the industrial level and allows coating of complex devices; in a similar way, electrochemical treatments as well as sol-gel dip coatings can adapt to different substrate shapes. As an example, Jia et al. firstly optimized a bioactive and antibacterial coating (TiO_2-polydopamine-Ag) obtained by MAO followed by dopamine dip coating and $AgNO_3$ in situ reduction and then verified its scalability from flat titanium samples to real devices (Ti wires and Ti6Al4 cylindrical scaffolds) [54].

Due to the aggressive nature of physiological fluids, Ti and its alloys have almost completely substituted stainless steel for long-term implants and protection from corrosion remains an important point for implanted metallic surfaces. Anti-corrosion properties of antibacterial and bioactive coatings have been investigated in most of the here reviewed papers [53,54,63,66–72,74,75,78] and an effective barrier effect has been evidenced in all the cases. In this view, the development of multifunctional materials with bioactive and antibacterial behavior, plus corrosion protection ability are of great interest.

A critical role in determining the long-term performance of thick coatings on Ti-based implants is related to adhesion strength in physiological environment. Chemical bonding between the coating and substrate is rare and mechanical anchorage is often the primary mechanism of adhesion, clinically showing both adhesive and cohesive failures. Coatings are usually tested according to the following standards: ISO 13779-2:2008, ASTM F-1147-05, 131 ASTMC633, 69DIN50,161, 132 and ISO 4624. Coatings deposited by MAO process and thermal spraying technique often exhibited higher adhesion strength (around 30–45 MPa), followed by sol-gel and plasma spray (around 25 MPa) [79].

3.2. The strategy of inorganic thin coatings

The use of sputtering-based deposition processes is the most common approach to produce thin sub-micrometric antibacterial and/or bioactive coatings on Ti.

The antimicrobial effect is usually achieved by direct sputter-deposition of a suitable inorganic agent or by co-sputtering with a matrix material; Ag has been often selected as the preferred agent due to its potent antibacterial ability. Kheur et al. [80] sputter-deposited pure Ag thin films on Ti dental implants: drastic reduction of the viable pathogens after 6 h were reported in the case of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Streptococcus mutans* (antibacterial effect) as well as *Candida albicans* (antifungal properties), but the Ag ions released in the culture medium decreased the viability of human gingival fibroblastic cells of about 20% compared to the bare Ti control. The need for a compromise between adequate antimicrobial effect and biocompatibil-

Table 1
Strategies and techniques for the preparation of thick antibacterial, bioactive and biocompatible coatings on different substrates (BG = bioactive glass; HAp = hydroxyapatite; NP = nanoparticles).

Substrate	Coating	Technique	Bioactivity	Antibacterial activity	Biocompatibility	Ref.
AISI 316L Stainless Steel	ZnO-NP + Alg + PVA + (BG or chitosan)	Electrophoretic Deposition	HAp deposition after 2 days in SBF for BG containing coatings	ZnO-NP + Alg + PVA + Chitosan active vs S. aureus/S. enterica	Not tested	[68]
	PEEK + BG+ (Ag-NP) – 100 μm thick		Not tested	PEEK + BG moderately active vs E. coli; activity enhanced by Ag-NP	Not tested	[52]
	Alg + (BG or ZnO-NP) – 3 μm thick		HAp deposition after 7 days in SBF in presence of BG particles.	Alg + ZnO active vs E. coli	Not tested	[69]
Commercially pure Ti	Ag-doped β-$Ca_3(PO_4)_2$ + chitosan – 50 μm thick		Not tested	Active vs S aureus/E coli.	Biocompatible vs MG-63 up to 1.3%mol Ag	[62]
	Cu-doped $CaSiO_3$		Complete HAp coverage after 3 days in SBF	Active vs E coli/S aureus (stronger vs S aureus)	Not tested	[67]
	Ag-doped HAp-lignin		HAp deposition after 7 days in SBF	Active vs S aureus (complete inhibition of planktonic cells) after 24 h	Biocompatible vs peripheral blood mononuclear cells	[64]
Commercially pure Ti	Zn doped TiO_2 – 10 μm thick	Micro Arc Oxydation (MAO)	Not tested	Active (90% reduction of viability) vs E. coli/S. aureus	Not tested	[70]
	Cu-NP enriched TiO_2 – 5–10 μm thick		Not tested	Active vs S aureus (dose dependent on Cu content)	Biocompatible vs pre-osteoblasts up to 0.3 g/l Cu and up to 3 g/l Cu vs endothelial cells	[65]
	Multilayer: Ag doped HAp, $CaTiO_3$, TiO_2-10–14 μm thick		HAp deposition after 1–7 days in 1.5 modified SBF	Active (99%reduction) vs E coli/S aureus	Not tested	[57]
Ti6Al4V	Multilayer: Ag doped HAp, TiO_2 (MAO in $AgNO_3$)-11–19 μm thick		HAp deposition after 72 h soaking in 1.5 modified SBF	Active (99% red.) vs S aureus: 13 mm inhibition halo against E. coli	Not tested	[55]
Commercially pure Ti	TiO_2 + Ag-NP	MAO + dopamine dip coating + $AgNO_3$ reduction	Not tested	Active vs S aureus; bacterial trapping/killing into the NTs	Biocompatible vs MG-63 and in *in vivo* tests	[54]
Ti6Al4V	Zn-doped ZrO_2/TiO_2 – 13 μm thick	Sputtering of Zr + MAO in electrolyte with Zn	Not tested	Zn-doped ZrO_2/TiO_2 active (complete killing) vs S aureus; undoped ZrO_2/TiO_2 is less active	Biocompatible vs MC3T3	[72]
Commercially pure Ti	TiO_2 + ZnO-NP	PEO	Not tested	Active vs E. coli/S. aureus (dose dependent on ZnO content)	Not tested	[71]
	TiO_2 enriched with Ca, P and Zn – 5–10 μm thick		Not tested	Active vs E. coli/S. aureus (Zn dose dependent)	Biocompatible vs rat bone marrow stromal cells; positive action of Zn ions	[73]
Ti, stainless steel	Ag-doped HAp	Electrostatic spraying	Not tested	Active (100% reduction of planktonic cells) vs E coli	Biocompatible vs human osteoblasts	[56]
Ti6Al4V	$Al_2Si_2O_5(OH)_4$ Zn doped + Sr^{2+}/Sm^{2+} doped HAp	Electrodeposition	Not tested	Active vs E coli/S aureus (Inhibition halo)	Biocompatible vs MG63	[74]
	$Al_2Si_2O_5(OH)_4$ Ce-doped + HAp		Not tested	Active vs E coli/S aureus (Inhibition halo vs)	Not tested	[75]
Commercially pure Ti foil	TiO_2 + calcium phosphate	Anodic oxidation(TiO_2) + electrodeposition (Ca-P)	Not tested	Active vs S aureus	Not tested	[78]
Commercially pure Ti	TiO_2 + Ag and Sr co-doped HAp – 25 μm thick	Anodic oxidation (TiO_2 NTs) + Electrodeposition (HAp)	HAp deposition after 5 days in SBF	Active vs S. aureus (Inhibition halo and complete killing of planktonic cells after 24 h)	Biocompatible vs BMSCs	[53]
Ti6Al4V	Ag NP-containing HAp	Painting of the Ag-HAp powder (PVA as binder) + laser surface processing	HAp deposition after 5 days in SBF	Active vs S aureus (Ag dose dependent)	Biocompatible vs mice calvarial bone cells up to 2% Ag	[58]
Commercially pure Ti		Electrochemical crystallization HAp, electrochemical reduction of Ag ions, thermal treatment (8 h@170 °C)	Not tested	Active vs E coli (complete inhibition of planktonic cells after 8 h)	Not tested	[59]
	Ag/Sr-doped HAp	Dopamine-assisted immobilization of Hap prepared by hydrothermal method	Not tested	Active vs E coli and S aureus (Inhibition halo)	Ag dose dependent cytotoxicity for MG63 osteoblast like cells reduced by Sr addition	[60,61]

(*continued on next page*)

Table 1 (*continued*)

Substrate	Coating	Technique	Bioactivity	Antibacterial activity	Biocompatibility	Ref.
	Cu-doped HAp	Electroplating	HAp deposition after 10 days in SBF	Active vs E coli	Biocompatible vs mouse MC3T3 osteoblast like cells	[66]
	Ag-doped HAp + TiO_2	Plasma electrolytic processing	Complete HAp coverage after 10 days in SBF	Active vs E coli (Inhibition halo)	Not tested	[63]
Ti6Al4V	Zn-doped HAp	Flame spray	Not tested	Active vs E coli (inhibition halo of powders and reduction of viable bacteria of coating)	Biocompatible vs fibroblasts	[77]

ity is perhaps the major reason why Ag is usually embedded in a chemically-stable matrix modulating the rate of ions release from the coating.

Ag-incorporating TiO_2 films were sputtered by using a Ag/TiO_2 composite target [81,82] or by co-deposition from two different targets of Ag and TiO_2 [83,84]. These different experimental setups allowed producing single-layer [81,82] or multilayer [83,84] (Ag-doped TiO_2/TiO_x interlayer/pure Ti, see Fig. 4) coatings. The concentration of Ag, embedded as metallic nanoparticles, affected the crystallization of TiO_2 (rutile and anatase) and the hydrophilicity, which increased with the Ag content [81,82]. Multilayered coatings exhibited an excellent antibacterial effect against *Staphylococcus aureus* with a drastic reduction of viable pathogens at 24 h associated with good biocompatibility towards L-929 fibroblastic cells (viability >90%) [83,84].

Akhavan and Gadheri [85] produced multilayered structures comprising a coating of sputtered Ag nano-rods (height 250–500 nm) and an outer capping film of mesoporous TiO_2 (thickness 30 nm). The antibacterial effect of TiO_2-capped Ag nano-rods against *Escherichia coli* was found superior to the biocidal activities of TiO_2-capped Ag "flat" film and TiO_2-capped Ag nanoparticles grown on Ti. The mesoporous TiO_2 cap layer could modulate the release of Ag^+ by inter-diffusion of water molecules and ions through its capillary structure, thus allowing a more prolonged Ag release compared to both the other two TiO_2-capped structures and the uncapped Ag nano-rods, from which a burst release of Ag^+ was observed.

Ag-doped HAp thin films (thickness 600 nm) were sputtered on Ti substrates [86], in the attempt to combine the potent antibacterial effect of Ag with the bone-bonding ability of the bioactive ceramic matrix for obtaining a high-added-value multifunctional system. Although a good antibacterial effect was reported against both Gram-positive (*Staphylococcus epidermidis*) and Gram-negative (*Pseudomonas aeruginosa*) bacteria, there were some concerns about the mechanical integrity of the coating in aqueous media (like biological fluids) as it was prone to delaminate after immersion for 2 weeks in 1 mL of phosphate buffered saline (PBS).

This limitation was successfully overcome by Surmeneva et al. [87] who deposited a three-layer coating made of amorphous calcium phosphate (outer layer, 150 or 1000 nm thick), Ag nanoparticles (1.5 μg/cm^2) and nanocrystalline HAp (interlayer, max. 1000 nm thick) on Ti implants (Fig. 5). The top and bottom calcium phosphate layers were produced by radio-frequency magnetron sputtering, while electrophoretic deposition was used to stack the Ag nanoparticles. Scratching tests demonstrated that this multilayered coating possessed a good resistance to contact damage without undergoing delamination for an applied load of 7 N. About one-third of the original Ag content was released after 3 days in 5 mL of PBS, which supports the suitability of the coating to prevent early bacterial infection – as proved against *Escherichia coli* – and suggests that the overall duration of Ag^+ release could be on the order of weeks. Simultaneously, the osteoconductive properties of the (remaining) calcium phosphate layer are expected to stimulate new bone growth, provided that the concentration of Ag released does not negatively affect cell biocompatibility. This latter goal still remains to be achieved: in fact, *in vitro* tests with MG-63 osteoblasts seeded on sputtered implants showed a decrease of cell mitochondrial activity compared to bare Ti [87].

Apart from Ag, other antibacterial elements were included within sputter-deposited coatings on Ti implants, such as Cu (Cu-Ti layers, 64–122 nm thick [88]), Zn (ZnO layers, <10 nm thick [89]), Zr (ZrO_2 alone or as a matrix incorporating Ag and Cu nanoparticles, thickness 400–500 nm [90]), Au (Au-Ti layers, 120 nm thick [91]) and Ta (Ta_2O_5 layers, thickness 500–700 nm).

A dose-dependent cytotoxic effect on MG-63 cells due to the release of Cu^{2+} from Cu-Ti coatings was observed [88], in a similar

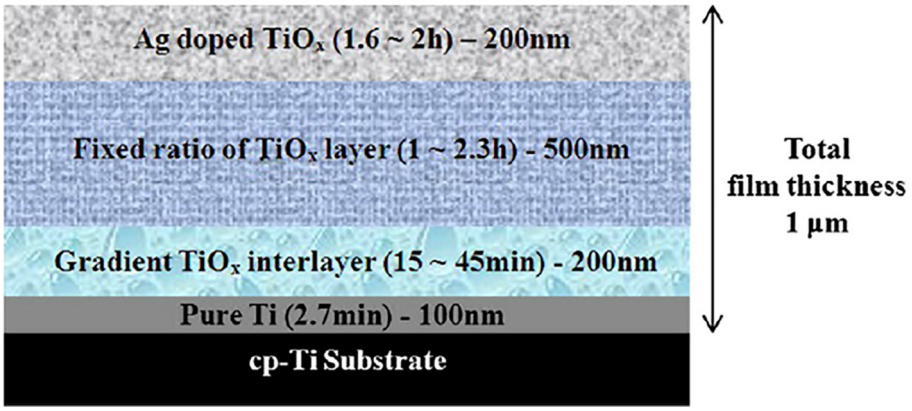

Fig. 4. Structure of the multilayered Ag-doped TiO2 coating produced by multistep co-sputtering from Ti and Ag targets. reproduced from [84].

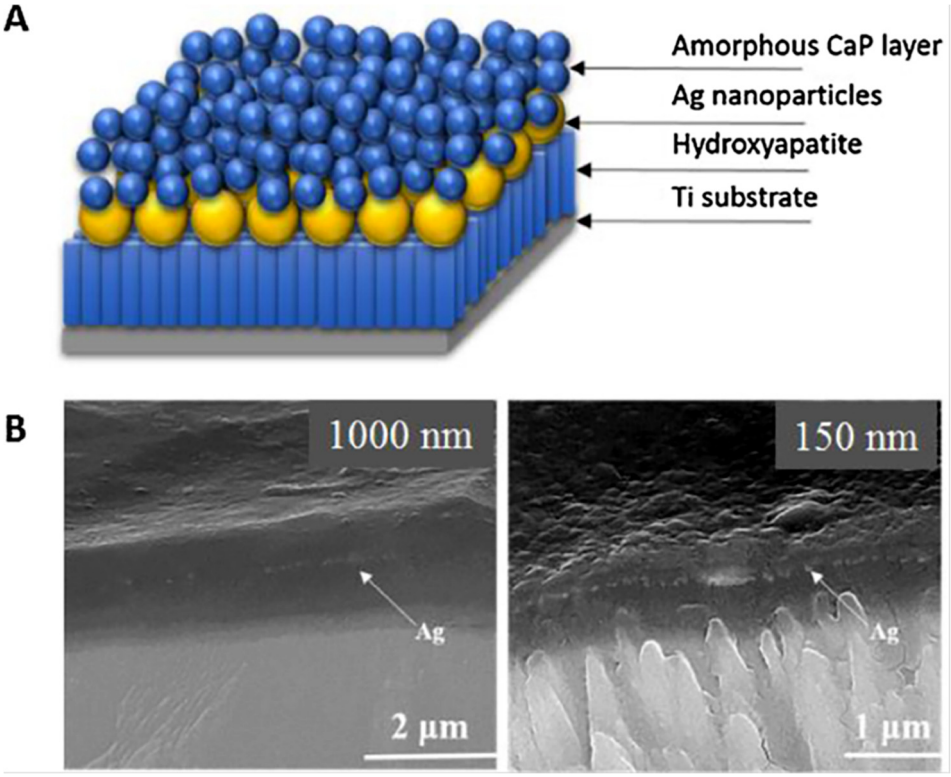

Fig. 5. Three-layer Ag/calcium phosphate coating on titanium: (A) scheme of the coating structure (bottom layer: columnar nano-crystalline hydroxyapatite; top layer: amorphous calcium phosphate); (B) SEM cross-sectional images with different thicknesses of the top layer of calcium phosphate (1000 or 150 nm). reproduced from [87].

way to that mentioned in the case of Ag [80,87], which suggests caution in the use of these coatings and the need for a careful dosage tailoring. A post-sputtering treatment of annealing, which converted Cu-Ti to CuO-TiO_2 coating, was found beneficial to improve biocompatibility (no dead pre-osteoblastic mouse MC3T3-E1 cells were observed after a 5-day *in vitro* incubation) and cell adhesion/spreading [92]. Furthermore, CuO-TiO_2 coatings exhibited improved corrosion resistance and superior antibacterial effect against *Staphylococcus aureus* as compared to pure Ti and TiO_2-coated implants.

Huang et al. [90] reported a superior antimicrobial effect of Ag-doped coatings as compared to those containing Cu and Zr (Ag-doped ZrO_2 > Cu-doped ZrO_2 > ZrO_2 film), which is apparently contradicting the results reported by Wojcieszak et al. [91]; however, different ion contents were used in this couple of studies and, thus, a direct comparison is not possible. Interestingly, Wojcieszak et al. [91] observed a stronger antibacterial and antifungal activity for Cu-Ti film (total destruction of all the pathogens within 4–6 h) compared to Au-Ti and Ag-Ti coatings (total suppression of most microorganism tested after 24 h, partial inhibition of *Enterococcus hirae*), which was attributed to the different mechanisms of action. In fact, atomic absorption spectrometry studies revealed that Au-Ti and Ag-Ti layers had biocidal effect related only to the direct contact of their surface with the pathogens, whereas the antimicrobial activity of Cu-Ti films was associated to both contact-killing and release-killing mode due to efficient migration of Cu^{2+} ions from the coating surface to the surrounding environment.

Chang et al. [93] sputter-deposited Ta and amorphous Ta_2O_5 coatings (500–700 nm) on pure Ti implants; the latter could be converted to crystalline β-Ta_2O_5 via a post-sputtering rapid thermal annealing at 700 °C. It was shown that the microstructure of the coating greatly affected both its biocompatibility and its antibacterial properties. The most potent antibacterial effect against *Staphylococcus aureus* and *Actinobacillus actinomycetemcomitans* was observed for the amorphous Ta_2O_5 coating, whereas the crystalline one was the most favorable to bacterial growth as compared to the other structures. Conversely, however, the amorphous coating was associated to the worst cell viability using human skin fibroblasts, which on the contrary was improved by the highly-hydrophilic surface of the annealed β-Ta_2O_5 film. These results demonstrate that achieving a satisfactory balance between biocompatibility/bioactivity and antiseptic effect is a complex task needing further investigation and optimization. The antibacterial effect of Ta-based sputtered coatings can be further improved by doping (co-sputtering) with Ag [94]. A detailed study by Azadmanjiri at al. [95] also elucidated the reason why Ta-Ag coatings (45 nm thick), which were sputter-deposited on micro- and nano-grained Ti, can significantly improve the hardness and stiffness of the surface compared to the untreated implant (hardness: 9.4 vs. 4.8 GPa; elastic modulus: 154 vs. 130 GPa). The mechanism is based on the negative core charge of dislocations and neighbor space charge accumulation that generate interfacial polarization, thereby improving the interfacial bonding strength of the thin film to the substrate by the formation of mixed covalent-ionic bonds around the dislocation core area and at the interfacial layer. Hence, these multifunctional films show great promise for biomedical applications being biocompatible, biocidal and highly-resistant from a mechanical viewpoint.

Although being highly versatile to produce thin films with tunable thickness at the nanoscale, sputter-based deposition methods require high investment costs for the needed equipment; thus, other less-expensive approaches for fabricating nano-coatings have been developed and reported in the literature. For example, Gasqueres et al. [96] obtained Ag-doped TiO_2 films (100–200 nm thick) on Ti6Al4V alloy by means of anodic spark deposition in an aqueous electrolyte containing Ag nanoparticles. The release rate of Ag^+ ions from the metallic nanoparticles that were embedded within the coating remained constant over immersion for

Table 2
Strategies and techniques for the preparation of thin antibacterial and biocompatible inorganic coatings on different substrates.

Substrate	Coating	Technique	Antimicrobial activity	Biocompatibility	Ref.
C. p. Ti	Ag film	Sputtering	Active vs S. aureus, P. aeruginosa, S. mutans, Candida albicans) at 24 h	Moderate reduction of viability (20%) of human gingival fibroblast at 72 h compared to pure Ti control	[80]
C. p. Ti	TiO_2 + Ag NPs	Sputtering	Active vs S. aureus; significant reduction after 3 h (compared to pure Ti control) and total destruction colonies at 24 h	Minimal reduction of viability (<10%) of L-929 fibroblasts	[83,84]
Si wafer	Ag nanorods/ mesoporous TiO_2	Sputtering	Active vs E. coli; total destruction of colonies in 2–96 h	Not tested	[85]
C. p. Ti	Ag-doped HAp	Sputtering	Active vs S. epidermidis and P. aeruginosa at 8–48 h; coating delamination after 24 h	Not tested	[86]
C. p. Ti	Multilayer Ag NPs/calcium phosphate	Sputtering	Active vs E. coli (turbidity test)	Reduction of mitochondrial activity of MG-63 cells at 1 and 3 days	[87]
Medical grade Ti	ZnO	Sputtering	Active vs S. aureus, S. epidermidis, E. coli, P. aeruginosa; *in vitro* and *in vivo* (mouse subcutaneous infection model)	No cytotoxic effect towards rat bone MSCs	[89]
C. p. Ti	ZrO_2	Sputtering	Active vs S. aureus or A. actinomycetemcomitans; improved action by doping with Ag or Cu	Not tested	[90]
Ti6Al4V alloy	Au/Ag/Cu-doped Ti	Sputtering	Cu-Ti coating active vs E. hirae, S. aureus, E. coli, C. albicans at 24 h	Not tested	[91]
C. p. Ti	Amorphous or crystalline Ta_2O_5	Sputtering	Active vs S. aureus and A. actinomycetemcomitans; improved action of amorphous coating	Better biocompatibility of the crystalline coating towards skin fibroblasts (CCD-966SK)	[93]
Ti6Al4V	Ag-doped TiO_2	Anodic spark deposition	Active vs S. aureus; total destruction of gentamicin-resistant at 12 h	Not tested	[96]

15 days in PBS and allowed complete killing of gentamicin-resistant *Staphylococcus epidermidis* after a 12-h incubation.

A selection of the studies reviewed in this section is provided in Table 2.

3.3. The strategy of NTs and nano-texturing

Textural properties of implant surfaces, such as morphology and roughness, are known to greatly influence cell responses *in vitro* and *in vivo* [97]. Early studies carried out in the 1990s have provided a first evidence that osteoblasts attach and spread preferably on Ti surfaces exhibiting a diffused micrometric roughness [98,99]. From a general viewpoint, it was observed that the micrometric and nanometric peaks and valleys of the implant surface can affect the organization of cell cytoskeleton and, hence, the intracellular transduction signaling pathways [100].

Implant nanotexturing can be obtained by electrochemical oxidation of Ti surfaces in order to produce nanopits [101] and/or NTs [102]. From a technological viewpoint, obtaining TiO_2 NTs with reproducible geometric characteristics is a relatively easy task: the diameter of NTs can be typically tailored within 15–300 nm depending on the potential of anodic oxidation and the tube length is in the range of a few hundreds of nanometers [103]. Some highly interesting studies focused on how the cell fate is dictated by the diameter of TiO_2 NTs [103–106], but the obtained results are controversial and definite conclusions have not been reached yet. A sort of "physically-induced" differentiation could occur on NTs of different sizes with cells stretched under tension; however, it is generally difficult to predict the effect of the NT size only, and also other parameters, such as crystallinity, spatial organization, surface chemistry modification and the specific type of cells considered should be taken into account [103–107]. In fact, it is recognized that not only the surface roughness in itself, but also the morphology of nano-features as well as wettability and surface energy synergistically influence osteoblast responses to Ti implants [108].

An interesting study was reported by Moravec et al. [109] who produced TiO_2 NTs (diameter within 40–60 nm) by electrochemical oxidation of Ti6Al4V alloy in electrolyte solution containing ammonium sulfate and ammonium fluoride. The NTs were further modified either by surface enrichment with calcium and phosphate ions, in the attempt to obtain a biomimetic surface approaching the composition of bone mineral phase, or by heat treatment converting amorphous TiO_2 to anatase. *In vitro* results using Saos-2 cells suggested that surface ion doping can promote cell attachment, which was discouraged on heat-treated NTs; on the contrary, crystallization of anatase seemed to encourage osteogenic cell differentiation compared to amorphous Ca- and P-enriched NTs. Thus, various additional post-treatments of the nanotubular surface could allow modulating the biological response of cells to the implanted biomaterial.

The improvement of bioactivity carried by NT-modified Ti-based surfaces has been well demonstrated by *in vivo* tests. Subcutaneous Ti implants in rats with a nanotubular surface exhibited no significant difference as compared to conventional titanium surfaces in terms of absence of chronic inflammation and fibrous scar tissue formation, but higher concentrations of calcium and phosphorous were found on the NT-modified surface [102]. Similar implants in rabbit tibias showed larger bone-implant contact area and greater healthy bone formation with a significant improvement of the implant-to-bone bonding strength after a 4-week follow-up (10.8 vs. 1.2 N for the untreated Ti control) [110].

In the attempt of mimicking the natural hierarchical structure of alveolar bone, Wang et al. [111] developed a bioinspired surface combining micro-pits and TiO_2 NTs on Ti implants. The tightly arrayed self-assembled TiO_2 NTs, having the diameter in the range of 30–50 nm, aimed to replicate the structure of collagen fibers (60–80 nm) within the mandibular bone. The higher surface energy and hydrophilicity of these bioinspired implants led to enhanced adhesion and growth of osteoblasts as well as better osseointegration in 12 miniature pigs, especially in the early stages of bone healing (3 weeks), as compared to both smooth and just micro-rough counterparts [112]. Clinical trials involving 25 human patients over a 1-year follow-up confirmed the promising results from animal studies [112].

Apart from improving osseointegration, surfaces provided with TiO_2 NTs have been recently recognized able to carry extra-functionalities that can be combined and exploited to develop next-generation multifunctional implants. For example, additional antimicrobial properties can be imparted by applying appropriate

post-treatments to TiO_2 nanotubular surfaces. Liu et al. [113] decorated with TiO_2 nanoparticles (3–8 nm) the outer surface and inner walls of TiO_2 NTs produced on anodized Ti implants. TiO_2 nanoparticles, synthesized through a hydrothermal method followed by UV light irradiation, increased the surface area and photocatalytic ability of the NTs. Over a 7-day testing period, improved antibacterial properties against *Streptococcus mutans* and *Porphyromonas gingivalis* were reported and attributed to a prolonged photo-induced wettability of the implant surface compared to pure Ti and nanoparticle-free nanotubular surfaces. Specifically, a significant down-regulation of glycosyltransferase genes of *Staphylococcus mutans* was observed in the case of nanoparticle-decorated nanotubular surfaces. Early *in vitro* test also suggested that the osteogenic functions of stem cells was stimulated by this two-level surface topography, which could be therefore used for developing multifunctional implants with bioactive and antibacterial properties.

A highly-promising approach to combat bacterial infections is based on the local release of inorganic ionic species that can be effective also against antibiotic-resistant pathogens. Ag, which is known as a potent antibacterial agent, was deposited in the form of nanoparticles on the walls of TiO_2 NTs obtained via anodic oxidation and the resulting implants were capable to elicit an antiseptic effect against *Staphylococcus aureus* [114]. Similar results were reported by Uhm et al. [115] along with a good apatite-forming ability of Ag nanoparticle-coated TiO_2 nanotubular surfaces upon soaking in SBF. Ag/TiO_2 NT layers formed on commercially-pure Ti were proved to be antibacterial and resistant to implantation and explantation procedures in an *ex vivo* animal model (equine cadaver) without undergoing any mechanical damage; furthermore, osteoid formation with early-stage osseointegration was reported to occur around these implants after implantation in rat distal femur after 12 weeks [116].

Yang et al. [117] moved a step forward to bioengineer Ti-based surfaces and built up a thin mussel adhesive protein (Mefp-1)/Ag nanoparticle composite layer on a TiO_2 nanotubular surface by a dip-coating method. Mefp-1 coating was useful to improve the surface hydrophilicity, preosteoblastic cell adhesion and in situ reduction of Ag^+ ions to Ag nanoparticles (size 10 nm) on the NT surface; synergistically, the Ag nano-layer fabricated with the assistance of Mefp-1 was effective for killing both Gram-positive (*Staphylococcus aureus*) and Gram-negative pathogens (*Escherichia coli*). Ideally, this implant has the potential to regulate and direct the competition between cells and bacteria in colonizing the implant surface: in fact, preferential adhesion of pre-osteoblasts was promoted as the surface environment was deadly to bacteria and favorable to bone cells (Fig. 6).

Perhaps the major limitation of these approaches is associated to the postoperative fate of Ag nanoparticles, since many studies have demonstrated the toxicity of metallic nanomaterials to cells and tissues *in vitro* and *in vivo* [118–120]. Furthermore, we should take into account that Ag can exert its antimicrobial effect via contact-killing mode (if bacteria come in direct contact with Ag nanoparticles) and/or release-killing mode due to the release of Ag^+ ions from the nanoparticles (see Fig. 6); in this regard, high cumulative concentration of leached Ag^+ ions from Ag nanoparticles was reported to produce cytotoxic effects, too [121].

Some recent studies have reported that also Cu-bearing Ti alloys have excellent antimicrobial functions [122–124]. Thus, instead of using Ag, Zong et al. [125] incorporated Cu as an antibacterial agent into TiO_2 NT arrays by anodizing magnetron-sputtered TiCu layers previously deposited on pure Ti foils. These Cu-doped nanotubular surfaces exhibited good long-term antibacterial properties (1 month) against *Staphylococcus aureus*, due to the sustained release of Cu^{2+} ions, and up-regulate the secretion of vascular endothelial growth factors (VEGF) by endothelial cells as compared to pure TiO_2 NTs used as a control. This multifunctional implant shows great promise, being simultaneously able to kill bacteria and promote angiogenesis, which is key to accelerate tissue healing processes.

Although being a less potent antibacterial agent compared to Ag and Cu, Zn was also incorporated into TiO_2 nanotubular surfaces by hydrothermal treatment in zinc acetate solutions [126]. The osteogenic differentiation of mesenchymal stem cells could be modulated according to the various Zn amounts and the antibacterial effect against *Staphylococcus aureus* was reported, although this was milder than that obtained with Ag-doped TiO_2 NTs synthesized following a similar approach and tested under analogous experimental conditions by the same research group [121].

Apart from being exploited to elicit an antibacterial effect, inorganic dopants can be useful to stimulate other biological responses: for example, Sr-loaded TiO_2 NTs arrays were shown to inhibit osteoclast differentiation *in vitro* and *in vivo* (rat model) [127], thereby disclosing new strategies for the treatment of osteoporosis, as previously discussed in Section 3.1.

Other added values, such as superhydrophobicity, can be imparted to Ti implants by means of proper post-processing treatments of TiO_2 NTs. Being inspired by the lotus-leaf effect [128],

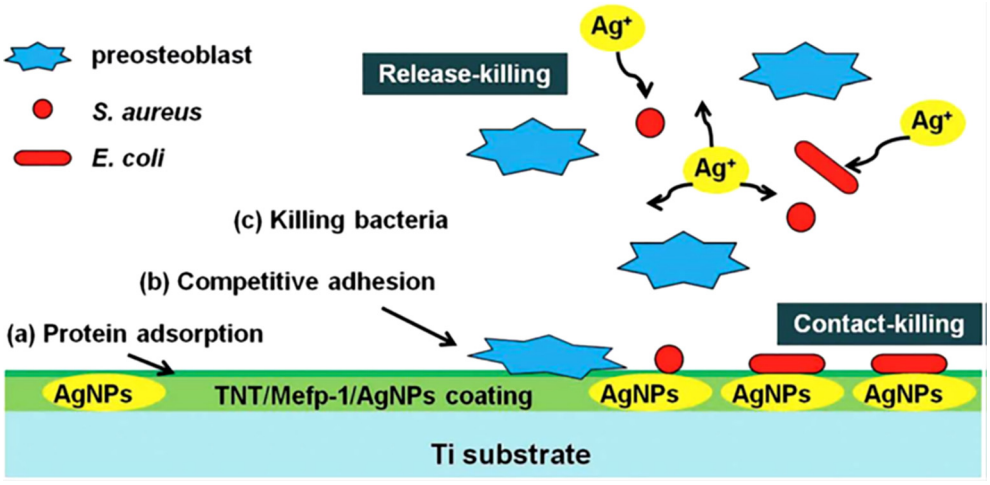

Fig. 6. Scheme illustrating the events occurring at the interface between biological fluids and Mefp- 1/Ag nanoparticle-coated TiO2 nanotubular implant surface: (a) protein adsorption, (b) competitive adhesion of preosteoblastic cells and bacteria, and (c) a combination of release-killing and contactkilling processes. reproduced from [117].

superhydrophobic surfaces, which are of great interest in a number of smart applications including biomedicine, have been obtained to reduce protein adsorption and to suppress platelet adhesion and blood coagulation cascade [129]. Yang et al. fabricated superhydrophobic TiO_2 NTs via electrochemical anodization of Ti implants followed by functionalization with perfluorooctyltriethoxysilane. Improved blood compatibility and anticoagulation property were demonstrated by *in vitro* tests showing that the nanotextured surface actually resisted platelet adhesion and activation [130].

Although Ti-based implants and TiO_2 nanotubular surfaces are mainly thought for use in contact with "hard" tissues (primarily bone) in orthopedics and dentistry, their potential suitability for non-osseous applications has been emerging, too. For example, the study reported above [130] suggests that superhydrophobic TiO_2 nanotubular surfaces could be key for manufacturing next-generation implantable devices in permanent contact with bloodstream. Hence, the meaning of the term "bioactive" should be expanded to include the vast potential of biomaterials "to perform a specific function required to generate the most appropriate beneficial cellular or tissue response in a specific situation" [131]. Peng et al. [132] recently studied the suitability of TiO_2 NTs for cardiovascular applications and found that these nanotextured surfaces can enhance the proliferation and motility of endothelial cells, prevent the proliferation of vascular smooth muscle cells and decrease the gene expression related to coagulation and inflammation in both cell types. Therefore, this study suggests that the presence of a TiO_2 nanotubular surface could potentially provide cardiovascular implants with bioactive and multifunctional characteristics due to the divergent, highly-appropriate responses of endothelial and smooth muscle cells.

3.4. The strategy of chemical surface treatments

Simple chemical treatments are cost-effective and able to form a modified surface layer uniformly even on complex surfaces including inner surfaces of porous bodies. Various types of chemical treatments using various types of solutions have been proposed to confer bioactivity on Ti and its alloys. Acid treatments using HCl, H_2SO_4, HNO_3, HF and their mixed solutions have been applied to Ti dental implants to obtain direct contact of bone to implant without the formation of interfacial fibrous tissue [133]. These treatments result in rough surface with micrometer-scale roughness, which increases total surface area, wettability and surface energy to promote osseointegration *in vivo* as well as osteogenic gene expression, such as BMP-6, BMP-2, and Runx-2, *in vitro* [134–136]. Kawai et al. recently showed that the bone-bonding capacity, osteoconduction and osteoinduction of the HCl/H_2SO_4-treated Ti were further increased when the metal was subsequently heat treated at 600 °C [137–139].

In contrast, it was shown that H_2O_2 treatment using $H_2O_2/TaCl_5$ or H_2O_2/HCl mixed solution formed cracked and chipped titania gel layer on the surface of Ti [140,141], while a crack-free nanostructure was produced on Ti-6Al-4V by HF + H_2O_2 multistep solution treatment or H_2O_2 hydrothermal treatment [142,143]. The treated metals increased wettability, corrosion resistance, cell attachment and cell proliferation [142,143]. An increased capacity of apatite formation was observed when the treated metal was heat treated at 300–600 °C by forming anatase [141,143].

Alkali treatment using NaOH or KOH solution is often used for the surface modification on Ti and its alloys. Nanostructured sodium hydrogen titanate ($Na_xH_{2-x}Ti_yO_{2y+1}$; $0 < x < 2$ and $y = 2$, 3, or 4) with network or flake-like morphology was deposited on Ti, conventional Ti alloys such as Ti-6Al-4V, Ti-15Mo-5Zr-3Al, Ti-6Al-2Nb-Ta having higher mechanical strength than pure Ti, and Ti-Zr-Nb-Ta system new alloys by the 5–10 M NaOH treatment at 60–120 °C [144–148]. The treated metals increased *in vivo* bone-bonding and *in vitro* apatite formation [144–146,148], cell activity such as attachment, proliferation, and upregulated gene expression of Runx-2 in MG63 osteoblast-like cells and rat bone marrow stromal cells [148,150]. However, it also increased pro-inflammation cytokines of MMP9 in macrophages RAW264.7 [149]. A decrease of IL-1β, another pro-inflammation cytokine, was observed without increasing MMP9 and TNF-α when $NaHCO_3$ solution was used instead of NaOH solution [149]. In contrast, Zang et al. recently reported that the mRNA expressions of the NF-κB targets IL-1β, IL-8 and TNF-α in macrophages were suppressed by the NaOH treatment and they were further decreased when the NaOH-treated Ti was silanized by 3-aminopropyl triethoxysilane [150]. Interestingly, a decrease in proliferative osteoprogenitors, such as Msx2 and RP59, and a simultaneous increase in osteoblast differentiation markers such as Runx2, Osx, Dlx5, ALP, BSP, OC and DMP1 were observed when the metal was heat treated at 600 °C after the NaOH treatment, by forming sodium titanate and rutile [151]. Increased bone-bonding as well as apatite formation was reported on the Ti and Ti-6Al-4V, Ti-15Mo-5Zr-3Al, Ti-6Al-2Nb-Ta [145]. The NaOH and heat treatment was successfully applied to plasma-sprayed titanium on a total artificial hip joint and spinal fusion devises of porous Ti that have been clinically used in Japan since 2007 and 2018, respectively.

The sodium hydrogen titanate formed by the NaOH treatment has a great potential to develop multifunctional Ti and Ti alloy implants due to its large capacity of ion exchange. As shown in Fig. 7a, the sodium hydrogen titanate nanocrystals were elongated perpendicular to the substrate, which resulted in large specific surface area. Furthermore, they take layered structure (Fig. 7b) where Na^+ and H^+ ions were intercalated into the TiO_6 octahedral sheets, which enable incorporation of various types of functional metal ions into the sodium hydrogen titanate by ion exchange that is easily achieved by simply soaking in a solution including the metal ions. It is reported that not only univalent ions such as silver (Ag^+) and lithium (Li^+), but also divalent and trivalent ions such as calcium (Ca^{2+}), magnesium (Mg^{2+}), strontium (Sr^{2+}), zinc (Zn^{2+}), and gallium (Ga^{3+}) and more than two types of metal ions could be incorporated simultaneously [152–156]. As a result, large amount and various types of metal ions can be incorporated at desirable combination and ratio by controlling ion concentration and pH of the solution in the second solution treatment [152–156]. When Sr^{2+} and Mg^{2+} ions that are known for their promotion in new bone formation were incorporated in the sodium titanate ($Na_2Ti_6O_{13}$) formed on Ti-6Al-4V by NaOH hydrothermal treatment and subsequent $MgCl_2$ or $SrCl_2$ solution treatment, an increase of ALP and upregulation of osteogenic gene expression of Runx-2, OPN, OCN on MC3T3-E1 preosteoblast cells were observed [156]. Yamaguchi et al. reported that Sr- or Mg-containing calcium hydrogen titanate were formed on Ti by soaking the metal in a mixed solution of 50 mM $CaCl_2$ + 50 mM $SrCl_2$ or 40 mM $CaCl_2$ + 60 mM $MgCl_2$ [154,155]. However, when the Ti with Sr-containing calcium hydrogen titanate was soaked in phosphate buffered saline at 36.5 °C, the metal rapidly released Sr^{2+} ions within 1 h as shown in Fig. 8. Thus, the treated metal was subsequently heat treated 600 °C and finally soaked in 1 M $SrCl_2$ solution. As a result, the treated metal slowly released 0.92 ppm of Sr^{2+} ions within 7 days (Fig. 8). Okuzu et al. reported that Ti with Sr- or Mg-containing calcium titanate upregulated gene expression of integrin β1, β catenin, cyclin D1, ALP and extracellular mineralization on MC3T3-E1 cells, indicating increased proliferation and osteogenic differentiation [157]. They also showed greater biomechanical strength and bone-implant contact than the Ti with calcium titanate, especially at the early stage (4–8 weeks).

In order to obtain antibacterial activity in addition to bioactivity, Inoue et al. [158] first attempted to form an Ag-containing bioactive sodium titanate layer by soaking Ti that had been

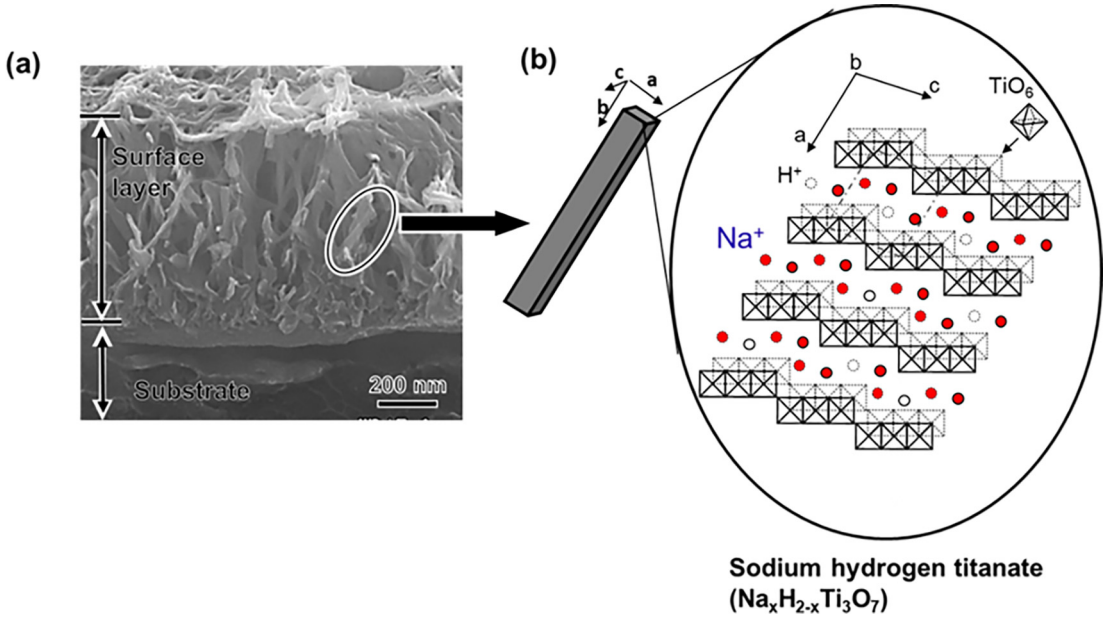

Fig. 7. (a) Cross sectional SEM image of Ti subjected after NaOH treatment. Sodium hydrogen titanate nanocrystals were elongated perpendicular to the substrate. (b) Schematic image of sodium hydrogen titanate taking layered.

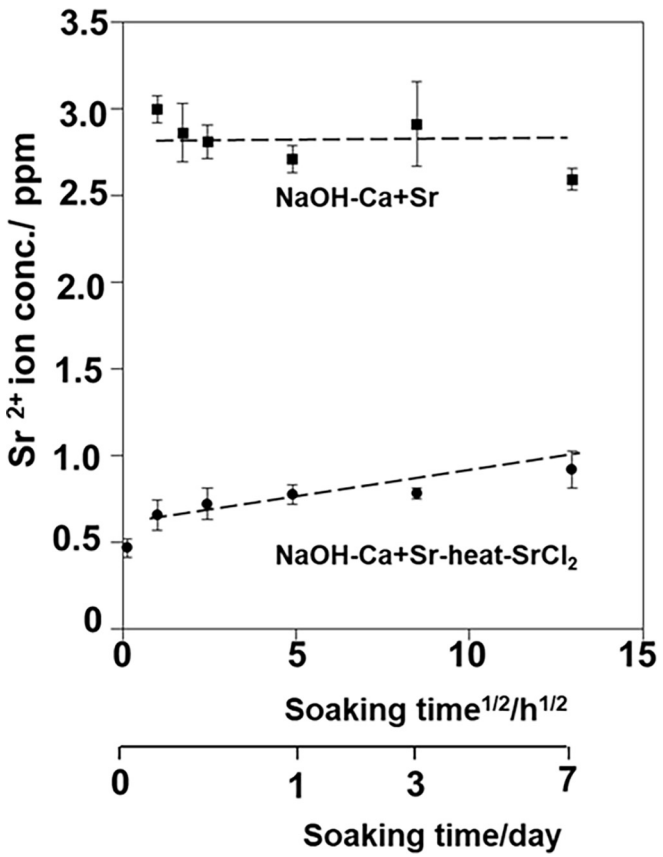

Fig. 8. Release behavior of Sr^{2+} ions from Ti subjected to NaOH-Ca + Sr or NaOH-Ca + Sr-heat-$SrCl_2$ treatment in phosphate buffered saline, measured by ICP. Sr^{2+} ions were rapidly released within 1 h from Ti subjected to NaOH-Ca + Sr treatment, while they are slowly released till 7 days, when the metal was subsequently subjected to heat treatment and $SrCl_2$ treatment.

subjected to NaOH solution treatment or NaOH hydrothermal treatment in 0.05 M of a silver acetate solution. The Ag^+ ions were successfully incorporated into the sodium titanate layer formed on Ti by exchanging Na^+ ions in sodium titanate with Ag^+ ions along with some amounts of precipitated metallic silver particles. Thus, the resultant product successfully exhibited antibacterial activity (more than 99% reduction) on Staphylococcus aureus (S. aureus). It was reported that nano-structured titania with silver nanoparticles was formed on Ti and Ti-6Al-4V by soaking in a mixed solution of H_2O_2 and $AgNO_3$ [159,160]. The treated metals exhibited antibacterial activity (more than 99% reduction) on S. aureus without significant decrease of MG63 cell viability.

Later, Kizuki et al. [161] and Prabu et al. [162] showed that Ag^+ ions can be incorporated into the sodium hydrogen titanate ($Na_{2-x}H_xTi_3O_7$) formed on Ti and Ti-6Al-4V alloy without forming metallic silver particles when the metals were soaked in 0.01–100 mM $Ag(NO)_3$ solution after the NaOH solution treatment. However, the Ag-containing sodium hydrogen titanate formed silver particles again, when subsequently heat treated at 600 °C [161,162]. It was reported that Ag-containing calcium titanate free from silver particles was formed on Ti metal and Ti-15Zr-4Nb-4Ta alloy by soaking in 1 mM $AgNO_3$ solution after the heat treatment at 600 °C following 5 M NaOH and subsequent 100 mM $CaCl_2$ treatment [161]. The treated metals exhibited strong antibacterial activity (more than 99% reduction) on S. aureus, again.

The effect of silver contents in metal surface and concentrations of released silver ions from the metals subjected to chemical treatments on antibacterial activity and cytotoxicity are summarized in Table 3.

These results indicate that bioactive surfaces exhibiting high antibacterial activity without cytotoxicity can be achieved by chemical and heat treatments through selecting moderate Ag content that might be in different ranges according to the contained Ag form, e.g. 0.27–0.67% Ag ions or 0.64–4.9% Ag particles. The release of Ag^+ ions was investigated under different kinds of environment such as ultrapure water (UPW), distilled water (DW), phosphate buffered saline (PBS), and fetal bovine serum (FBS). Since Ag has very low solubility in water, but high affinity to chloride ions and proteins having amino and/or thiol group, the kind of medium used for the test causes significant differences on the amount released Ag. For example, the released amount of Ag^+ ions in FBS was 2.2 times higher than that in UPW [161], and 168–184 times higher than that in PBS [158]. The results of ion release in

Table 3
The effect of silver contents in metal surface and concentrations of released silver ions from the metals subjected to chemical treatments on antibacterial activity and cytotoxicity.

Substrate	Ag surface content	Release of Ag^+ ions	Antibacterial activity on S. aureus	Cytotoxicity on MG63 cell	Apatite formation	Ref.
Ti	0.10–1.61% as ions	1.2 or 2.7 ppm in UPW or FBS (at 0.55% Ag)	$<10^{-7.2}$ CFU ratio (at 0.55% Ag) in film contact	Not tested	<1 day	[161]
Ti	2.6–12.9% as particles	0.25–0.38 ppm in DW	>1 mm halo in disk diffusion	No cytotoxicity at 2.6% Ag	<14 days	[159]
Ti	0.15 or 0.67 in Ag/Ti as ions and particles	0.38 and 0.44 ppm in PBS, 64 and 82 ppm in FBS	$10^{-7.8}$ CFU ratio (at 0.15 Ag/Ti) in film contact, $10^{-6.3}$ CFU ratio (at 0.67 Ag/Ti) in film contact	Not tested	Not tested	[158]
Ti	0.64–3.77% as particles	1.0–4.5 ppm in SBF (at 0.64 or 2.3% Ag)	$10^{-1.0}$ (at 0.64% Ag), $10^{-2.5}$ (at 2.3% Ag) CFU ratio in film contact, Visible halo (at >0.64% Ag) in disk diffusion	No cytotoxicity till 2.3% Ag	<1 day	[160]
Ti6Al4V	0.6–2.2% as ions	4.2 ppm in SBF (at 0.6% Ag)	Not tested	No cytotoxicity till 0.6% Ag, cytotoxicity for 1.3% Ag	<7 days	[162]
Ti6Al4V	4.9–14.4% as particles	0.25–0.38 ppm in DW	>1 mm halo in disk diffusion	little cytotoxicity at 4.9% Ag,	<14 days	[159]
Ti15Zr4Nb4Ta	0.27% as ions	1.5 ppm in FBS	$<10^{-8.1}$ CFU ratio in film contact	Not tested	<1 day	[161]

protein-containing solutions such as FBS seem to simulate ion release behavior *in vivo* more precisely than other acellular media. The released Ag^+ ions seem to exhibit sufficiently high antibacterial activity when their concentration is more than 1.5 ppm in FBS. The number of relevant reports are, however, still scarce and further investigations are necessary for establishing a clear quantitative correlation between amount of Ag^+ ions released and antibacterial activity. It was reported that 10 ppm of Ag^+ ions is acceptable to human body and, above that, Ag may cause severe health effects including neuropathy [163,164]. Although *in vivo* bone-bonding and *in vitro* cell studies investigating osteoblast differentiation markers were not reported in these papers, apatite formation in a simulated body fluid [165] was confirmed on the treated metals except for Ref. [158], indicating that these metals have the potential to bond to living bone. However, sodium hydrogen titanate layer as-formed by the NaOH treatment is mechanically weak, unstable and prone to lose its apatite formation ability in a storage period before implantation or during implantation [166], thus it is recommended that heat treatment should be always added for practical use [166]. It was reported that NaOH-heat-treated Ti exhibited fracture strength of 2.71 ± 1.47 kgf at 8 weeks and 4.13 ± 1.25 kgf at 16 weeks in detaching test whereas NaOH-treated Ti shows 0.51 ± 0.52 kgf at 8 weeks and 0.49 ± 0.38 kfg at 16 weeks, when they were implanted into rabbit tibia [166]. It was shown that the heat treatment increases the scratch resistance and even the apatite-forming ability of the sodium hydrogen titanate and hydrogen titanate that are formed on Ti by the NaOH treatment and NaOH-HCl treatment, respectively [167].

In addition to Ag, some other metallic ions such as Hg, As, Cu, Zn, Ga, and I are known for their antibacterial activity [168–171]. Among them, zinc and gallium ions have been incorporated into the Ti surface by simple chemical and heat treatments. For Zn incorporation, TiO_2 modified with Zn-OH was formed on Ti by alkali treatment using NaOH solution containing $[Zn(OH)_4]^{2-}$ complex [172] while Zn-containing calcium titanate was formed on Ti by soaking in a solution containing Ca^{2+} and Zn^{2+} after NaOH treatment, followed by heat and hot water treatment [152]. The former released 0.0132 ppm of Zn^{2+} ions, while the latter released 0.03 ppm, although antibacterial activity was not evaluated in both reports. For Ga incorporation, gallium titanate was formed on Ti by soaking in a $GaCl_3$ solution after NaOH treatment, followed by heat and hot water treatment [153]. The resultant metal slowly released 3.75 ppm and exhibited high antibacterial activity against multidrug resistant Acinetobacter baumannii (MRAB12) [153].

In terms of clinical use, micro-scale roughness surface produced by acid treatment durable for shear strength is useful for screw-type implants such as dental implants. Although the bone bonding and apatite formation on the acid-treated Ti surface is significantly increased by the subsequent heat treatment, they tend to be affected by the existence of alloying elements [173], and therefore there is a limitation of the acid-heat treatment on the application to Ti alloys. On the contrary, nano-scale roughness surface produced by H_2O_2 or alkali treatment is weak for shear strength, but suitable for inner surface of porous body such as plasma-splayed porous Ti on a total artificial hip joint and porous Ti body of spinal fusion devise. The NaOH treatment selectively removes alloying elements from the alloy surfaces, and is applicable to conventional Ti alloys such as Ti-6Al-4V, Ti-15Mo-5Zr-3Al, and even to newly developed Ti-Zr-Nb-Ta system alloys when Ca was incorporated into the alloy surfaces [174]. The heat treatment increases bone-bonding as well as apatite formation, again, and also improves mechanical scratch resistance of the surface layer. The multifunctional surface releasing functional ions such as Sr, Mg, Ag, Ga and so on can be achieved by modifying the NaOH-heat treatment, and should be promising material in future. However, there are still scarce information on the relationship between release amount of ions and cytotoxicity, antibacterial activity, and cell activity.

3.5. From the strategy of coupling antibiotics and organic coating to bioactive materials to the development of hybrids and smart coatings

Antibiotics are widely employed for the prevention and treatment of peri-prosthetic infections: in fact, despite of a wide research on several antibacterial surfaces, the antibiotic-loaded implant materials (e.g. bone cement, temporary cement spacer, temporary fixation devices) are almost the only active antibacterial biomaterials that have reached the market up to now [175,176].

One of the main advantages in the use of antibiotic, for the control of implant-associated infections, is that they have an effective, widely recognized and accepted antibacterial activity. In addition, their use for local delivery reduces their potential toxicity compared to systemic administration.

On the other hand, the main drawbacks of antimicrobial strategies based on antibiotics are the increasing risk of bacterial resistance, an initial burst release followed by prolonged release below the minimum inhibitory concentration (MIC) – which increases the risk of resistance development –, and a potential adverse effect for osseointegration [177,178]. In addition, their narrow spectrum activity (antibacterial action limited to specific strains for each drug) must be taken into account and can limit their effectiveness in the treatment of polymicrobial infections and in the prevention of bacterial contamination from a priori unknown strains.

As far as the problem of a non-optimal release profile of antibiotics is concerned, some authors proposed the realization of a pH-sensitive bonding between the drug (gentamicin) and the carrier (polynorborene spherical nanoparticles) which can allow the controlled release of gentamicin at acidic pH (which is typical of infection) [179]. This approach seems promising, but at the moment these nanoparticles have been grafted to Ti6Al4V substrates which are not bioactive and a completely multifunctional surface has not been obtained yet by this strategy.

The increasing development of antibiotic resistance together with the potential hypersensitivity, allergy and toxicity associated to antibiotic therapies are at the basis of the European strategy aimed at their reduction, which stimulates the development of alternative solutions [180–182].

In this context, despite of a certain caution in antibiotic use compared to the unlimited application diffused till few years ago, the research on antibiotic-loaded surfaces for bone contact application is still active and antibiotic loading onto biomaterials has been discussed in several reviews in the last years [176,177,183–185]. The cited papers report antibiotic introduction into PMMA bone cements, biodegradable polymers, calcium phosphates and HAp, Ti NTs as well as covalent grafting to metallic surfaces.

The present section considers antibiotics, as antibacterial agents, coupled to bioactive surfaces aimed at the improvement of bone bonding/regeneration for the development of multifunctional bone implant surfaces. Research papers published from 2010 to present have been considered, discussed and summarized in Table 4.

Antibiotics are somehow loaded onto bioactive surfaces, able to induce apatite precipitation *in vitro* and, consequently, to support the biochemical bond with bone *in vivo*. This property has been conferred to Ti surfaces as a first step in the preparation of multifunctional surfaces (bioactive and antibacterial) by different ways such as HAp obtained by cold spraying [186] and dip coating [187], calcium phosphates as cement introduced into reservoirs obtained on metallic implants [188] or as composite coatings with chitosan obtained by electrochemical methods [189], bioactive glasses co-deposited with chitosan by means of electrophoretic deposition [190,191] and bioactive Ti layers obtained by sputtering [192] or chemical oxidation [193]. Various antibiotics, such as gentamicin (the most investigated one), vancomycin, tobramycin, rifampicin, co-amoxiclav and tetracycline hydrochloride have been loaded onto the above cited bioactive surfaces in different ways. Drugs were loaded by solvent evaporation, spreading or dipping on the prepared bioactive coatings [192,193,189], co-deposition in the electrophoretic process [190], loading in additional carriers such as hallosyte NTs [189], cyclodextrin [187] or PLGA [194], or inclusion in the bioactive precursor of the coating [186,188]. In some cases [190,191], bioactivity was reported in the research on antibiotic loading, while in the other ones [186–189] the works simply refer to bioactive substrate.

Other strategies involve the incorporation of antibacterial agents into the previously-obtained nanotubular coating. TiO_2 NTs can act as carriers for a number of anti-infective and anti-inflammatory organic biomolecules, such as penicillin [195], gentamicin [196], indomethacin [197,198], and ibuprofen [199], which can be locally released according to controlled rates. The use of TiO_2 NTs as platforms for smart drug delivery has been comprehensively reviewed by Wang et al. in a recent paper [200].

A significant antibacterial behavior has been generally demonstrated for all the above cited surfaces (as detailed in Table 4); however a direct comparison is not so easy because of the high variability in the techniques used for antibacterial activity evaluation.

In addition to the inorganic bioactivity described above, the opportunity to combine a high osteointegrative potential, obtained by grafting bone morphogenetic proteins (BMP), with the antibacterial behavior associated to antibacterial agents or antibiotics (carboxymethyl Chitosan or vancomycin) has been explored for Ti implants [201–203]. The first solution has been obtained by surface functionalization of titanium with chitosan and BMP using dopamine or polydopamine as linking agents [201], while layer by layer deposition of chitosan coated bovine serum albumin (BSA) nanoparticles loaded with BMP-2 or dexamethasone, and oxidized sodium alginate loaded with vancomycin have been proposed in [202,203]. Layer by layer deposition of chitosan, BMP-2 loaded BSA nanoparticles and oxidized sodium alginate [204] or chitosan and BMP-2 loaded polydopamine microspheres [205] have also been proposed for the obtainment of bioactive titanium surfaces with multiple biological functionalities, but no antibacterial activities are mentioned in these works. In these cases, HAp precipitation is not obtained because no bioactive compounds are introduced but the bone bonding ability is improved by the addition of a biological molecules (BMPs, dexamethasone). The final surface can be considered as a multifunctional one in which bioactivity is elicited by biomolecules instead of a newly formed apatite layer. Coatings loaded with BMPs [201,202] as well as dexamethasone [203] improve adhesion, proliferation and ALP production of osteoblasts and bone marrow stromal cells, demonstrating their ability to add high bone bonding ability to titanium surfaces. No adverse effects on bone stimulating ability of BMP-2 has been evidenced by the combination with vancomycin [202]. The biological stimulus of BMP has been coupled with the inorganic bioactivity of hydroxyapatite nanoparticles by means of pulse electrochemical driven rapid layer by layer deposition of nano-coatings onto titanium substrates [206]. In this case a multifunctional bioactive behavior has been obtained but not antibacterial agents have been introduced, so complete multi-functionality of the surfaces has not yet been reached.

In order to overcome the above cited drawbacks of antibiotics, other organic coatings/molecules have been proposed for the reduction of bacterial contamination onto bioactive surfaces. Collagen-mimetic proteins [207], antimicrobial peptides [208] and chitosan [209,210] have been coupled with metallic substrates for this purpose. Bioactive behavior is obtained by the addition of a bioactive glass in the chitosan coating [202] or by coupling an antimicrobial peptide to a bioactive metallic substrate (chemically modified Ti) [208] or by the addition of a biological stimulus (BMP-2 for instance), as described before [210]. In the case of collagen-mimetic proteins [201] the material does not possess inorganic bioactivity intended as the ability to induce apatite precipitation, but is able to improve the surface osseointegrative potential by osteoblast stimulation with the grafted biomolecule, as previously discussed for BMP grafting.

Hybrid coatings containing hydroxyapatite nanoparticles (as bioactive phase), silver nanoparticles (as antibacterial agent) and polypyrrole [211] or chitosan [212] were obtained by electrochemical deposition on titanium substrates. BMP-2 loading was also considered [212]. The above cited coatings resulted biocompatible for osteoblasts and bone marrow stromal cells, able to stimulate cell adhesion, proliferation and differentiation (ALP production) and effectively antibacterial against E coli and S epidermidis. The combination of chitosan with silver evidenced the possibility to increase biocompatibility without reducing antibacterial activity [212].

Finally stimuli responsive coatings of polydopamine/polypyrrole microcapsule loaded with dexamethasone were obtained onto titanium substrates by electrochemical deposition [213]. These coatings are able to release dexamethasone under electrical stimuli and improve bone marrow stromal cells adhesion, proliferation and ALP production, and resulted biocompatible and stable *in vivo*. This solution seems promising for the development of

Table 4
Strategies and techniques for coupling of antibiotics or organic coatings to obtain bioactive, antibacterial and biocompatible materials (BG = Bioactive Glass, HAp = Hydroxyapatite).

Substrate	Coating	Technique	Biomolecule loaded	Bioactivity	Antibacterial activity	Biocompatibility	Ref.
AISI 316L	Chitosan + BG + $Al_2Si_2O_5(OH)_4$ NT	Electrophoretic Deposition	Tetracycline hydrochloride	HAp deposition after 3 days in SBF	Active vs E. coli and S. aureus up to 24 h	Not tested	[191]
Ti	Chitosan + BG	Electrophoretic deposition	Vancomycin	HAp deposition after 28 days in SBF	Active vs S aureus (100% killing with addition of vancomycin >0.5 g/l)	Not tested	[190]
Ti-gr. 4	TiCaPCON $TiC_{0.5}Ca_3(PO_4)_2$	Selective Laser Sintering Co-sputtering	Augmentin® (amoxicillin and clavulanic acid) or Ag	Not tested	Active vs S. epidermidis, S. aureus, K. pneumo ozaenae	Not tested	[192]
Ti6Al4V	Bioactive macroporous Ti oxide layer	Plasma chemical oxidation (PCO)	Gentamicin-Na dodecylsulphate Gentamicin-tannic acid	Not tested	Active in *in vivo* model (rat osteomyelitis)	Not tested	[193]
Ti6Al4V	HAp	Vacuum cold spraying	Gentamicin sulphate	Not tested	Active vs E coli (99% killing)	Not tested	[186]
Ti6Al4V	$CaPO_4$ cement	Selective Laser Melting	Gentamicin sulphate	Not tested	Active vs S. aureus and S. epidermidis (inhibition halo)	Not tested	[188]
Ti15Mo	Bioactive Ti oxide layer coated with PLGA	Plasma electrolytic oxidation + Dip coating	Gentamicin	Not tested	No adhesion of S aureus up to 4 h	Biocompatible for MG-63) up to 7 days	[194]
Ti	Chitosan/$CaPO_4$e	Electrochemical deposition	Gentamicin	Not tested	Not tested	Not tested	[189]
Ti	Carboxymethyl chitosan (CMCS)	Grafting	Human BMP-2	Not tested	Active vs S epidermidis (85% reduction)	Biocompatible for osteoblasts	[201]
Ti	HAp + cyclodextrin	Plasma spray + Dip coating + thermal treatment	Tobramycin and rifampicin	Not tested	Active vs S aureus and E cloacae	Not tested	[187]
Ti	Collagen from bacteria	Surface grafting		Not tested	Active vs S aureus and S epidermidis (more than 2 order of magnitude of reduction)	Biocompatible for primary osteoblasts, MG-63, Saos-2	[207]
Ti		Surface grafting by silanization or absorption	Human lactoferrin-derived peptide (hLf1-11)	Not tested	Significant reduction of adhesion and biofilm formation for S sanguis and L salivarius	Biocompatible for human foreskin fibroblasts	[208]
AISI 316L, tested	Ti6Al4V	Chitosan + BG	Electrophoretic deposition		Hap deposition after 21d in SBF	Active vs E coli adhesion	Not [209]
Ti	Ti nanotubes	Anodization	Penicillin/ Streptomycin or Dexamethasone	Not tested	Not tested	Improved osteoblast adhesion	[195]
Ti	Ti nanotubes	anodization	gentamicin	Not tested	70% reduction S epidermidis adhesion after 4 h	Biocompatibility analogous to nanotubes without antibiotic, 65% increase in osteoblast adhesion compared to flat Ti	[196]
Ti	Ti nanotubes	Two step electrochemical anodization	Indomethacin in TPGS polymeric micelles	Not tested	Not tested	Not tested	[197]
Ti	Periodically modulated Ti nanotubes	Three steps anodization	Indomethacin	Not tested	Not tested	Not tested	[198]
Ti	Ti nanotubes + PLGA coating	Anodization	Ibuprofen	Not tested	Not tested	Improved adhesion of MC3T3-E1	[199]
Ti	BSA NPs stabilized with chitosan + BSA NPs stabilized with chitosan and oxidized alginate	NaOH treatment + polydopamine coating + Layer by layer deposition	BMP-2, Vancomycin loaded onto BSA NPs	Not tested	90% reduction S epidermidis	Improved adhesion, proliferation and ALP production for BMSC	[202]
Ti	Oxidized sodium alginate + chitosan coated BSA NPs	NaOH treatment + polydopamine coating + Layer by layer deposition	Vancomycin, dexamethasone	Not tested	Antibacterial for S epidermidis	Improved adhesion, proliferation and ALP production for BMSC	[203]
Ti	Chitosan + oxidized sodium alginate + BSA nanoparticles	Polydopamine coating + layer by layer deposition	BMP-2	Not tested	Not tested	Improved adhesion, proliferation and ALP production for BMSC. In vivo (rabbit) new bone formation	[204]
Ti	Chitosan + PDA microspheres	NaOH treatment + Layla by layer deposition	BMP-2	Not tested	Not tested	Improved adhesion, focal contacts development, proliferation and ALP production for BMSC	[205]

Table 4 (*continued*)

Substrate	Coating	Technique	Biomolecule loaded	Bioactivity	Antibacterial activity	Biocompatibility	Ref.
Ti	Polydopamine + HAp NPs	Electrochemical driven rapid layer by layer	BMP-2	Not tested	Not tested	Improved adhesion, proliferation and ALP production for BMSC. In vivo (rats) new bone formation.	[206]
Ti	Chitosan	NaOH treatment, silanization (APTES) glutharaldheide activation	BMP-2	Not tested	Not tested	Increased ALP activity and Ca deposition for BMSC. In vivo (rabbits) osteoconduction	[210]
Ti	Polypyrrole + HAp NPs + AgNPs	Pulse electrochemical syntesis	–	Not tested	Antibacterial for E coli and S Epidermidis (>90% bactericidal ratio)	Improved ALP activity for BMSC	[211]
Ti	HAp + Chitosan + AgNPs	Electrochemical deposition	BMP-2	Not tested	Antibacterial for E coli and S Epidermidis (>90% bactericidal ratio)	Improved adhesion, proliferation and ALP production for osteoblasts and BMSC	[212]
Ti	Polydopamine/ polypyrrole microcapsules	Electrochemical deposition	dexamethasone	Not tested	Not tested	Improved adhesion, proliferation and ALP production for BMSC. In vivo (rats) biocompatible and stable	[213]

electrical therapy in tissue regeneration, but no antibacterial functionalities have been introduced on these surfaces up to now, so this strategy is not yet fully multifunctional.

4. Discussion and conclusions

Surface engineering is recently moving to multifunctional properties and modulation of host response, bioactivity and antibacterial behavior are the functionalities required for physiologic osseointegration of Ti implants, avoiding the risks of chronic inflammation or infections. The following strategies can be pursued in order to modulate inflammation response: tailoring protein adsorption, promoting macrophage polarization, surface topographical patterns, biomimetic coatings, local delivery of drugs, NO regulation, macrophage apoptosis. Some attempts to combine these promising strategies to surfaces with bioactive and antibacterial behavior were carried out (coatings with heparin and BMP-2 [22] or antimicrobic peptides [41–44] or filamentous phages [10]), superhydrophilic surfaces [25,26]) but the achievement of Ti surfaces having these three functionalities actually proven (bioactivity, anti-inflammatory/antibacterial action) is still poorly explored and open to future developments. This issue is of a great relevance because, in usual bioactive coatings and rough surfaces of Ti bone implants, osseointegration is triggered by a vigorous initial inflammation response that could evolve to chronic inflammation.

The combination of osteogenesis to promote osseointegration and antimicrobial action discouraging bacterial colonization is much more explored on Ti surfaces and can be achieved by depositing inorganic bioactive layers of variable thickness (from few tens of nanometers to some tens of micrometers) that incorporate an appropriate inorganic agent, e.g. Ag, Cu or Zn, eliciting an antimicrobial effect via contact-killing or release-killing mode, or both. Osseointegration is significantly enhanced if the matrix material of the coating has osteoconductive properties, such as HAp, bioactive glasses or electrochemically grown oxide layers. However, there still are some drawbacks to this approach. Albeit most of the processing techniques experimented to produce these coatings are well consolidated, they may require high investment costs for the equipment – like in the case of sputtering, which is the preferred option to deposit sub-micrometric thin coatings, or plasma spray, which is widely employed to deposit thick coatings. Furthermore, the metallic ions released from the coating may have a toxic effect on the surrounding cells and tissues; thus a careful selection of the coating composition should be performed to determine the acceptable threshold allowing cells to survive and bacteria to be killed. Besides exerting an antimicrobial effect, some ions can also elicit additional therapeutic effects, like angiogenesis (e.g. Cu^{2+}), which is key to accelerate the processes of tissue healing and self-repair. Moreover, co-doping with different ions (e.g. Ag and Sr) can limit the cytotoxic effect of Ag and add to the surface the bone regenerative potential of Sr.

Obtaining nanotubular surfaces by electrochemical oxidation is another valuable approach to fabricate multifunctional Ti or Ti alloy implants: TiO_2 NTs were shown useful both to promote osseointegration and to elicit an antimicrobial effect through photocatalytic activity and/or release of metallic ions/nanoparticles incorporated in their structure. Interestingly, TiO_2 nanotubular surfaces show also promise in other fields than bone-contact implants, such as cardiovascular applications to prevent blood coagulation due to their superhydrophobic properties. Furthermore, there is early evidence that TiO_2 NTs are able to modulate the expression of molecules involved in inflammation of endothelial and smooth muscle cells; future research is expected to assess whether analogous effects may occur to bone cells, too, thus providing researchers with new tools to modulate host response to bone implants.

Alternatively, micro- and/or nano-scale roughness surfaces equipped with functional metal ions such as Ca, Sr and Ag can be achieved on Ti and Ti alloy implants by surface chemical treatments using acid and alkali solutions. Sodium hydrogen titanate formed by NaOH treatment showed high ion exchange capacities with univalent, divalent and trivalent ions as well as multiple ions that could be incorporated simultaneously. Slow release of these functional ions is achieved by combining heat treatment at moderate temperature. The bioactive and antibacterial Ti and Ti alloy implant produced in this manner is useful to promote osseointegration and to elicit an antimicrobial effect due to the release of metallic ions and micro- and/or nano-topology that enhances cell response and upregulate gene expressions.

The use of inorganic antibacterial agents (introduced into bioactive thin or thick coatings, as well as in modified surface layers) have the advantages of a broad spectrum of activity (which allows the treatment of polymicrobial infections and the prevention of contamination from unknown pathogens) and low resistance development. On the other hand, it is a quite innovative approach with some concerns related to certification and regulation, and finding the proper therapeutic window of these antibacterial agents may be a complex task.

Antibiotics, on the opposite, have a widely recognized and accepted antibacterial activity (antibiotic-loaded implants/cements are almost the only commercialized antibacterial devices

up to now), but the problem of increasing resistance development cannot be ignored. Local delivery of antibiotics from the surface of implants can limit their potential toxicity associated with systemic administration, but the optimal release profile and some concerns related to the risk of reduced osseointegration of antibiotic-loaded implants are still open questions. In order to overcome the main issues of antibiotics, alternative organic antibacterial agents (e.g. chitosan, antimicrobial peptides) have been proposed and are currently under investigation.

It must be underlined that both *in vitro* and *in vivo* evaluation of antibacterial properties of biomaterials have not been standardized yet and it is often difficult to compare results coming from research works based on different protocols. Some industrial standards are often applied for this purpose, especially on *in vitro* evaluation of antibacterial properties. However, their relevance to *in vivo* results is often not discussed well. As to *in vivo* tests, animals of different species and ages have been used for the evaluations of types of implants with different design and shapes. There are no unified quantification criterion, and thus strategies used in various studies may be not compatible with one another. At present, there is a regulatory gap that should be remedied in future.

In conclusion, to the best of our knowledge, several strategies for getting bioactive and antibacterial actions on Ti surfaces have been suggested with promising results, but they still need to be optimized with respect to several concerns. A further step will be to combine on the same surface a proven ability of modulation of inflammatory response. The achievement of multifunctional surfaces able to modulate inflammation and to promote osteogenesis for a physiologic osseointegration, as well as to fight infections and to avoid chronic inflammation, is one of the grand challenges for the 21st-century biomaterials scientists.

Development of multifunctional surfaces is both a great scientific and technological challenge which should be able to overcome the laboratories boundaries to reach the market and clinical applications. As discussed above and in the whole review, some coating/surface modification techniques are still used for the industrial production of medical devices (e.g. sputtering, plasma spray deposition and chemical treatments), but introduction of therapeutic ions, biomolecules or drugs, in order to produce a fully multifunctional surface, unavoidably increases the complexity of materials and obstacles entry into the market. In fact, presence of functional elements changes classification of the final device increasing complexity and costs of certification.

The combination of high innovation (which is not always positively accepted by market and clinicians), high complexity and high production/certification costs is one of the main critical issues to be overcome for a wide diffusion of multifunctional solutions. In fact, as critically discussed in [214], the main "valleys of death" where new technologies can be lost, moving from the bench to the beside, is technological transfer to industries and clinical implementation.

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