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Functionalization of titanium based metallic biomaterials for implant applications

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Abstract Surface immobilization with active functional molecules (AFMs) on a nano-scale is a main field in the current biomaterial research. The functionalization of a vast number of substances and molecules, ranging from inorganic calcium phosphates, peptides and proteins, has been investigated throughout recent decades. However, in vitro and in vivo results are heterogeneous. This may be attributed partially to the limits of the applied immobilization methods. Therefore, this paper highlights the advantages and limitations of the currently applied methods for the biological nano-functionalization of titanium-based biomaterial surfaces. The second part describes a newer immobilization system, using the nanomechanical fixation of at least partially single-stranded nucleic acids (NAs) into an anodic titanium oxide layer as an immobilization principle and their hybridization ability for the functionalization of the surface with active functional molecules conjugated to the respective complementary NA strands.

1 Introduction

Titanium and its alloys have been widely used as metallic materials of choice in orthopedics and oral/maxillofacial surgery throughout recent decades because of their excellent biocompatibility, which is mainly attributed to two facts. Firstly, the mechanical properties (especially modulus of elasticity) are better adapted to those of bone when compared with other metallic implant materials, thus considerably reducing stress shielding. Secondly, the surface is always covered by a passive oxide layer with a thickness of a few nanometers, which is responsible for the materials corrosion resistance and biologically inert response in vivo [1, 2]. Such a behavior results in a very good osseointegration of the material, especially for healthy patients. Nevertheless, early implant failure and problems during healing may occur for patients with certain predisposing factors such as smoking or systemic diseases such as diabetes, osteoporosis or chronic inflammation [3–5]. Moreover, the increasing age of the population adds to several factors: the increasing number of patients with poor bone quality and the increasing lifespan after primary surgery increases the probability for the exigency of revision [6]. These present and upcoming challenges thus require an osseoconductive surface on the implant. Therefore, direct surface manipulation is a main field of interest in current biomedical materials research.

The starting point for all attempts to influence the osseointegration process is the interaction between the surface and the tissue. Upon implantation, a complex, uncontrolled adsorption cascade develops at the implant surface [7, 8]. Within the first few seconds, the surface is covered by water and ions, followed by the unspecific adsorption of plasma proteins that reach equilibrium between desorbing and adsorbing proteins at longer time scales [9]. This process is influenced by the composition, energy, charge and the charge-transfer capabilities of the implant surface. Depending on these surface properties, which are determined by the implants pre-treatment, adsorbing proteins can change their conformation during the interaction process. Consequently within a short time, the surface is covered by a protein layer with

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Table 1 Surface modification techniques for titanium and its alloys based implants

Surface modification treatment	Modified surface	Objective
Mechanical methods		
Machining Grinding Polishing Blasting	Rough or smooth surface formed by subtraction process	Produces specific surface topographies, cleans & roughens the surface; improves adhesion in bonding
Chemical methods		
Chemical treatment		
Acidic treatment	<10 nm of surface oxide	Removes oxide scale & contaminants
Alkaline treatment	~1 μm of sodium titanate gel	Improves biocompatibility, bioactivity & bone conductivity
Hydrogen peroxide treatment	~5 nm of denser inner oxide with outer porous oxide	Improves biocompatibility, bioactivity & bone conductivity
Sol–gel treatment	~10 μm of thin film such as calcium phosphate, TiO_2 and SiO_2	Improves biocompatibility, bioactivity & bone conductivity
Anodic treatment	~10 nm to 40 μm of TiO_2 layer with adsorption and incorporation of electrolyte ions	Produce specific surface topographies, increased corrosion resistance & increased biocompatibility, bioactivity & bone conductivity
Chemical vapor deposition (CVD)	~1 μm of TiN, TiC, TiCN, diamond and diamond like C thin films	Improves wear resistance, corrosion resistance and blood compatibility
Biochemical methods	Modifications through sialinized titania, photochemistry, self-assembled mono layers, protein resistance	Induces specific cell and tissue response by means of surface immobilized peptides, proteins and growth factors
Physical methods		
Thermal spray Flame spray Plasma spray HVOF DGUN	~30–200 μm of coatings such as titania, HA, calcium silicate, zirconia, silica, alumina	Improves wear resistance, corrosion resistance and biological properties
Physical vapor deposition (PVD) Evaporation Ion plating Sputtering	~1 μm of TiN, TiC, TiCN, diamond and diamond like C thin films	Improves wear resistance, corrosion resistance and blood compatibility
Ion implantation & deposition Beam line ion implantation PIII	~10 nm of surface modified layer and ~ μm of thin films	Modifies surface composition: improves wear resistance, corrosion resistance and bio-compatibility
Glow discharge plasma treatment	~1–100 nm of the surface modified layer	Cleans, sterilizes, removes native oxide layer

conformations ranging from native to completely denatured [10]. Cells will recognize this more or less denatured protein layer and will be influenced on their adhesion, proliferation, differentiation and active matrix remodeling behaviour.

Excellent osseointegration (eliminating fibrointegration/encapsulation) and long term stability are mandatory for permanent implants such as dental implants or endoprostheses. Therefore, surface modifications needs to fulfill three primary tasks:

1. to prevent unspecific adsorption of potentially denatured proteins at the surface,
2. to attract cells of the native tissue or progenitor cells able to differentiate into the appropriate cell type, and
3. to provide biochemical signals to induce native healing mechanisms.

To achieve these goals, different attempts have been made to modify the surface characteristics of titanium-based implant materials using different methods and have been summarized in Table 1 [11–51].

Increasing the surface roughness achieved by grit-blasting or titanium plasma spraying [52] results in an improved mechanical interlocking of the implant owing to bone ingrowth into the cavities [53–55].

Surface chemistry and morphology can be altered by acidic treatment (etching) [56], alkaline treatment [57] or deposition of different phases of calcium phosphate [58–61]. However, there is a consensus in the research community that the biochemical properties of titanium surfaces have to be modified by immobilization of biologically active functional molecules (AFMs) such as peptides, proteins and others to deal with the mentioned challenges [62]. Most of these attempts are aimed at the stimulation of the specific host tissue cells and promote tissue healing and remodeling (in accordance with tasks 2 & 3 above and may also influence the unspecific adsorption of plasma proteins task 1). This has successfully been targeted by coating the surface with hydrophilic polymers such as polyethylene glycol (PEG) [63–66], which also exhibits good anti-bacterial properties [67, 68].

In general, two approaches are taken in the current biomaterials research. One is to mimic the native environment of the host tissue by immobilizing whole components of the host environment. In the case of bone this includes hydroxyapatite (HA), which represents approximately 70% of total bone mass, as well as the proteins of the extracellular matrix (ECM; approx. 20%). Type I Collagen is the main structural protein of the organic bone matrix and together with fibronectin and other adhesion proteins, it mediates cell–matrix interactions. Growth factors are important signaling molecules, triggering cell–cell and cell–matrix interactions [69]. Bone sialoprotein and osteopontin are involved in cell binding to mineralized bone [70]. And, finally, proteoglycans and their sugar components should be mentioned as compounds involved in interactions between collagen, growth factors and the cells [71].

The second approach uses small molecules that are often functional parts of larger molecules. They are immobilized to recruit appropriate cells of the host tissue, which then produce their own ECM and actively remodel the host environment. Examples for such molecules are peptides such as the sequence arginine-glycine-aspartate (RGD) [72], laminin sequences [73] or the collagen-derived P-15 peptide [74], peptidomimetics [75] or the aptamers [76, 77].

A comprehensive review of all possibly applicable bioactive substances would be far beyond the scope of this article and has been described in detail by other researchers [62, 78–82].

In the first section this manuscript will outline the currently used immobilization methods for the binding of AFMs to titanium-based biomaterials with their advantages and limitations. In the latter section, a newly developed modular immobilization system for AFMs is presented as an approach to overcome those limitations. The new approach immobilizes at least partially single-stranded nucleic acids into an anodic titanium oxide layer and uses their hybridization ability for loading the surface with AFMs, which are conjugated to the respective complementary NA strands, thus making the surface more biologically controllable and active.

2 Immobilization of functionally active molecules

Generally immobilization methods for AFMs must be evaluated with respect to several properties that are, in part, contradictory. In all cases, immobilized AFMs must display their bioactive domain to the cells of the host tissue in a native conformation and must be accessible to the cells, i.e. require a certain distance from the surface. The integrity of the AFMs to be immobilized must not be affected and no harmful substances involved in the immobilization process should remain at the surface to be accidently released in vivo.

Some molecules (e.g. peptides) must be immobilized irreversibly; others (e.g. growth factors or antibiotics) have to be released in a specific concentration or time-dependent manner to be effective. Realizing a defined release behavior of surface-bound molecules from the implant surface is the most critical step. In the past, functional ligands have been immobilized at implant surfaces, adsorptively, covalently, via electrochemical techniques (anodization and electrorefining), or using self-assembled layers. As will be shown below, all methods have their own advantages and limitations with respect to their feasibility, impact on the integrity and activity of the bioactive molecules as well as on the binding stability and release characteristics of the immobilized molecules.

2.1 Adsorption (physical immobilization)

Adsorption is the simplest method for immobilizing biologically functional molecules and may be carried out by just dipping the material into the appropriate solution. However, it is based on comparatively weak interactions, comprising of electrostatic and Vander Waals forces, hydrogen bonds or hydrophobic interactions.

Electrostatic interactions, for example, rely on the attraction of oppositely charged species and are therefore determined by the ratio between the isoelectric point (IEP) of the surface and the pKa-values and valence state of adsorbing species in a liquid environment. For the air-formed passive layer and anodic oxide layers on titanium alloy surfaces the IEP can be expected to be at a pH value of approximately 4.3, suggesting that the titanium surfaces should be charged negatively under in vivo conditions [83]. This negativity over the surface film has been used by several scientists [84] to immobilize RGD-modified PEG grafted to poly-L-lysine (PLL), where positively charged PLL acted as a backbone with multiple anchor points.

Fibrillar collagen is often adsorptively bound to titanium surfaces [85–87] and proved to be stable against competitive adsorption of serum proteins in vitro [88]. Weak physical interaction forces may be compensated to a certain degree by increasing the number of interacting sites. Auernheimer et al. [89] used a branched anchor with four phosphonic acid groups for their c-(RGDfK)-peptide to coat titanium surfaces adsorptively. Though they did not evaluate the binding stability of their peptide under the influence of protein containing media, they could show that their coatings withstood dry heat of 70°C for up to 8 days as well as a re-passivation treatment in nitric acid followed by ultrasonic agitation in water and detergent.

Binding stability of adsorbed species over the surface is also controlled by several environmental variables such as pH, ionic strength and protein concentration. If any of these change, adsorbed molecules may desorb in an uncontrolled manner. Therefore, the results of in vivo experiments are heterogeneous. Wikesjo et al. [90] tested bone morphogenic protein 2 (BMP-2) adsorbed onto anodic, porous titanium oxide layers of a commercial dental implant surface in a defect model in adult dogs for up to 8 weeks and observed an increased local bone formation compared with implants without growth factor. Several scientists conducted various studies involving coatings of titanium implants with calcium phosphates (CP) and/or BMP-2 [91–93]. They used a biomimetic process to co-precipitate CP and BMP-2 and compared this type of coating with CP coated and adsorbed BMP-2, CP coated as well as uncoated samples in an ectopic rat model. They observed osteogenic activity for the group with incorporated BMP-2 but not for adsorbed BMP-2. Unfortunately, they did not include data concerning the release kinetics of the growth factor but claimed that adsorbed BMP-2 was released more rapidly than incorporated BMP-2 [92, 93].

Similar findings were obtained by Schliephake et al. [94] who investigated a multi-component system on etched titanium implant surfaces, comprising adsorbed type I collagen, chondroitin sulphate (ChS) and BMP-2, in a dog model. ChS was incorporated into the collagen fibrils during fibrilogenesis. BMP-2 was adsorbed on the collagen/ChS surfaces. In this study, no augmentation of the peri-implant bone formation or bone to implant contact could be attributed to the growth factor coating. The researchers concluded that the binding stability for BMP-2 was not sufficient, because of the quick release of BMP-2 within 120 h with an initial burst during the first 24 h.

2.2 Covalent (chemical immobilization)

Covalent attachment of bioactive molecules to surfaces has the advantage of stable immobilization and is therefore widely used [95–97]. However, especially for metal oxides it requires multiple steps and involves the use of problematic substances from the physiological point of view, which have to be removed during the cleaning steps. Xiao et al. [96] illustrated such a procedure of silanization with 3-aminopropyltriethoxylsilane (APTES), for coupling a RGD sequence on Ti-coated glass, and by Martin et al. [98] for attachment of chitosan as an antibacterial agent. A prerequisite for such a technique is the existence of free surface hydroxyl groups, which have to be generated by treatment with HNO_3 or piranha solution. APTES, dissolved in water or toluene, may then react with these groups to form Ti–O–Si bonds. In a second step, the free terminal amino groups of APTES are activated with a cross-linker such as glutaraldehyde, which can further react with amino groups of the protein or peptide. Besides the tedious procedure, the strong covalent bond to the surface noted as an advantage above may turn to a drawback because of its irreversible nature, which makes this technique unsuitable for molecules requiring controlled release.

3 Self-assembled monolayers

Self-assembly of monolayers is a principle often used for immobilization of functional molecular chains at surfaces. It is based on the interaction between anchor groups of the molecules and specific interaction sites on the modified surface. Immobilization of thiol-modified molecules on gold surfaces used in sensor applications have been described by Huang et al. [99]. The technique has been adapted for titanium-based biomaterials by pre-coating their surface with gold. However, this approach may be questionable for clinical use, because a new metallic component is inserted in the biomaterial/tissue interface, which may result in enhanced local redox reactions with their possible adverse effects. Fortunately, there exist other possible anchor groups for binding organic molecules to titanium surfaces, amongst which are the molecules with high affinity towards metal oxides are phosphates (especially phosphonates). A number of studies deal with the adsorption of phosphates or phosphonates alone. For alkyl phosphates formation of self-assembled monolayers (SAMs) was observed by several researchers [100–102]. Spori et al. [103] found that alkyl phosphates with a chain length between 10 and 18 C atoms adsorbed at TiO_2 layers on silicon display a higher degree of ordering with longer chain length. Furthermore, they suggested a stronger bidentate binding mode bridging between two Ti atoms, though no stability tests with the adsorbed molecules were performed.

Philippin et al. [104] compared the formation of monolayers of alkyl phosphonic acid with that of alkyl trichlorosilanes, and suggested that the latter form better ordered monolayers in accordance to electrochemical

impedance spectroscopic measurements. Gao et al. [105] investigated alkylphosphonic acids bound to anatase, zirconia and alumina with nuclear magnetic resonance and found only weak interaction between Ti and P–O owing to not completely deprotonated hydroxyl groups. Conversely, Viornery et al. [106] confirmed through the XPS and SIMS measurements of three different phosphonic acid molecules adsorbed to commercially pure Ti as formation of covalent bonds of the kind Ti–O–P. Other groups use subsequent heat treatment to increase the covalent character of the bond between the metal substrate and the phosphate or phosphonate anchor groups [107–109].

Currently, there are few studies dealing with the stability of self assembled monolayers on titanium. Silverman et al. [110] compared phosphonate-anchored self assembled monolayers after heat treatment with siloxane-anchored molecules and found the former were more stable against hydrolysis in water at pH 7.5 and exhibited higher shear strength. They suggested a bidentate binding of the phosphonate groups and concluded that binding of phosphonates is not limited by the amount of surface hydroxyl groups, because the estimated surface density of the alkyl chains exceeded that of the estimated surface hydroxyl groups by a factor of 3. In a more recent study Mani et al. [111] evaluated the stability of adsorbed self assembled monolayers on titanium in TRIS-buffered saline (TBS) and double distilled water at 37°C as well as in air with normal laboratory illumination and UV irradiation. They compared methyl and hydroxyl-terminated dodecyl phosphonic acid (DDPA and OH-DDPA, respectively), dodecyl phosphate ($DDPO_4$) and dodecyl trichlorosilane. As a reference thiol, self-assembled monolayers (SAMs) on Au were tested under the same conditions, representing the current gold standard. In this study all phosphonate and phosphate anchored SAMs desorbed to a large extent during storage in TBS within 1 day. Trichlorosilane SAMs on Ti and thiol SAMs on Au were stable for up to 7 days under the same conditions. Storage under ambient laboratory conditions removed most of the thiol self assembled layers within a day, whereas phosphonic acid self-assembled layers on Ti were stable for up to 14 days. After UV irradiation for 12 h, the alkyl chains of the phosphonic acid monolayers were decomposed and only the phosphonate groups remained on the Ti surface. On gold, decomposition of the chains was followed by the oxidation of thiolates. The authors concluded that deposition of phosphonic or phosphate-anchored SAMs from aqueous solution may not be appropriate for titanium surfaces.

3.1 Electrochemical methods

Because of the amphoteric nature of the titanium oxide, both cathodic and anodic procedures can be used to immobilize active functional molecules and to modify their properties on the implant surface. Because of the principal differences in the underlying mechanisms, both processing routes are applicable for different tasks and have been treated separately.

3.1.1 *Cathodic polarization*

During negative polarization (flow of extra electrons), the pH value near the electrode increases owing to hydrogen evolution and can be represented by the equation shown below;

$$2H^+ + 2e^- \rightarrow H_2$$

This hydrogen evolution can be used to deposit phosphates on titanium surfaces from supersaturated solutions because their solubility decreases with rising pH values [112]. The structure of the deposited phosphates encompasses amorphous phosphates, brushite, octacalcium phosphate and hydroxyl apatite depending on electrolyte composition, temperature and electrochemical parameters. Roßler et al. [113] used improved near-physiological processes to achieve mineralized collagen coatings on titanium surfaces. The process has further been adapted by Scharnweber et al. [114] to co-precipitate an antibacterial agent on Ti6Al4V surfaces using the pH-dependent solubility of chlorhexidine. The process can be universally applied to all substances showing a pH-dependent solubility, and has been successfully applied by Scharnweber et al. for chitosan coatings [115].

3.1.2 *Anodic polarization*

With anodic or positive polarization at low potentials (+0.7 V vs. silver chloride electrode (SCE)), conducting polymers could be deposited on metal substrates. De Giglio et al. [116, 117] used this method to coat titanium surfaces with polypyrrole (PPY) films as an anchor for the coupling of RGD peptides, collagen and HA [116, 117].

At higher anodic potentials, the thickness of the passive oxide can be increased in a controlled manner to thicknesses of up to few nanometers (100 nm). During the oxide growth, it is possible to incorporate molecules or nano-sized particles present at the oxide/electrolyte interface at least partially into the anodic oxide. This fact has been used by Scharnweber et al. [118] to immobilize type I collagen [118] and a cyclic phosphonate-anchored RGD peptide [119] and is the basis of the modular immobilization system presented below.

The basic principles of the formation of anodic titanium oxide layer on titanium has been summarized, though it is well known and has already been reviewed previously [120–124]. The thin passive film on titanium surfaces

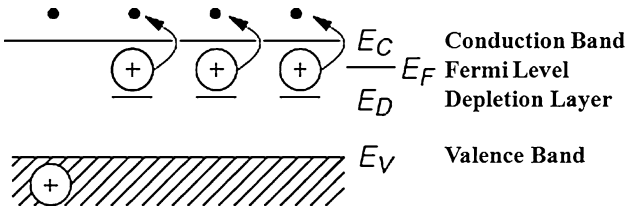

Fig. 1 Schematic diagram of the energy levels of an *n*-type semiconductor titania film

consists mainly of a sub-stoichiometric oxide, exhibiting an *n*-type semiconductor behavior and represented by the general formula $Ti_{1+x}O_2$. Zhang et al. [125] determined the difference in the conduction and valence energies as represented in Fig. 1 to be 3.3 eV for amorphous, sputtered TiO_2 films on glass substrates, which for the native passive film may be in the same range. Local oxygen point defects in the oxide act as electron donors [123, 124, 126]. Scharnweber et al. [127] investigated the semiconducting properties of the three alloys commercially pure Ti, Ti–Al–V and Ti–Al–Nb in phosphate buffer at pH values between 4.4 and 9.2 and determined comparable donor densities for commercially pure Ti and Ti–Al–V and almost 50% lower values for Ti–Al–Nb alloy all of the order of 10^{20} cm^{-3}.

Because of these properties, charge transfer during anodic polarization occurs in the first instance by migration of Ti^{2+} and O^{2-} through the oxide. This results in the formation of new oxide at both the metal/oxide and oxide/electrolyte interfaces, thus forming a bilayer system and has been shown in Fig. 2.

The total oxide layer thickness depends linearly on the applied potential, with a growth parameter of 1.4–2.9 nm/V and has been shown for several titanium alloys in Fig. 3 [124, 126].

The extent of oxide formation at the two interfaces is determined by the transfer numbers of the migrating species, which in turn are dependent on the strength of the electric field [128]. Besides oxide formation, oxygen evolution has to be considered as a parallel reaction at the oxide/electrolyte interface owing to the already mentioned

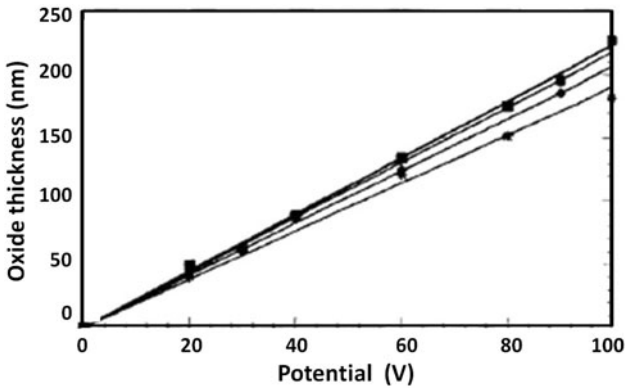

Fig. 3 Oxide thicknesses as a function of potential during anodization

possibility for electron transfer processes. Thus, the possible reaction pathways at that interface can be summarized according to reactions given below. The dissociation of water generates oxygen ions, which are able to migrate through the oxide or react with molecular oxygen via intermediate oxygen radicals.

$$3H_2O \rightarrow O^{2-} + 2H_3O^+$$

$$6H_2O \rightarrow 2O^{\bullet} + 4H_3O^+ + 4e^- \rightarrow O_2$$

Titanium ions approaching the interface are oxidized via the intermediates titanyl ions and hydroxylated titanium oxide according to several mechanism reported in the literature and has been summarized below:

$$Ti^{4+} + 3H_2O \rightarrow TiO^{2+}2H_3O^+$$

$$TiO^{2+} + 4H_2O \rightarrow TiO(OH)_2 + 2H_3O^+$$

$$TiO(OH)_2 \rightarrow TiO_2H_2O$$

A number of side effects may be caused by these reaction pathways in general, and particularly *n* regard to the immobilization of functional biological molecules

1. Generation of hydronium ions may result in a decrease in the pH value near the electrode, which may have a direct impact on the surface bound molecules and their

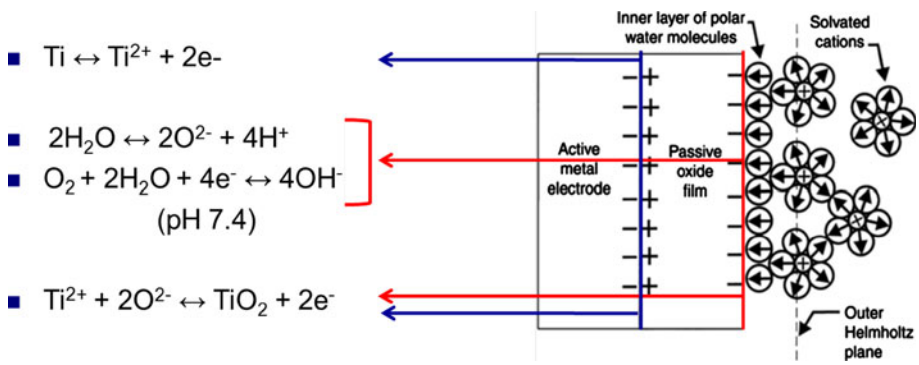

Fig. 2 Bilayer oxide structure over the titanium implant surface

immobilization behaviour and should be compensated by an appropriate buffer capacity of the electrolyte,
2. Generated titanyl ions, whose solubility increases with decreasing pH value, may bind to the immobilized biomolecules, thus rendering them inactive. This may be prevented by applying additives serving as capture molecules or by choosing a design in which the active groups have a sufficient distance from the surface, because the titanyl ions are expected to react near their point of origin,
3. Generated oxygen radicals can cause direct damage to bound active functional groups/molecules. The extent of oxygen evolution strongly depends on the surface properties of the material (donor density) and the polarization parameters (potentiostatic or galvanostatic mode, current density, polarization time). Because the material is mostly determined for a given application, there exists only a limited choice of alternatives.

4 Self-organization of functional molecules

Self-organization is ubiquitous in nature and the call for self-organization is based on the potential to switch from a 'top down/high energy' process to a 'bottom up/low energy' approach if complex systems or structured surfaces must be created in miniature.

A typical example for a 'top down' process is the production of semiconductor elements such as processors or memory devices, where several steps, e.g. coating, light exposure and etching, must be done. Owing to physical barriers, 'top down' approaches have nearly reached their limits. The 'bottom up' procedure, however, aims to build complex structures from selected molecules using their ability to self-organize. Such molecules will form the intended structures without further need of external influence if the appropriate molecules and conditions are chosen. This may allow for creating smaller and more defined structures, using molecules as building blocks.

Among the biomolecules usable for self-assembly, nucleic acids are probably those with the highest potential for forming a large variety of structures. This theory is based on the molecular recognition between complementary sequences and the ability to generate double and triple helices, G-quartets, Hoogsteen and wobble pairings, and mismatched structures.

Recent achievements in the field of DNA nanotechnology can be used as the basis for modular biosurface engineering, since it is possible to create two-dimensional (DNA-origami) and three-dimensional patterns (poly-hedra) [129–135]. Such defined structures can be used to bind other materials or molecules like gold nanoparticles [136] or proteins in a defined regular pattern and to grow silver nanowires [137]. So-called DNA nanomachines and nanomotors often rely on switching from a quadruplex to a duplex structure and back [138, 139]. Most of them are fuelled by added nucleic acids. Nucleic acid self-organization is therefore seen as a powerful tool for surface structuring and texturing, controlling processes and drug delivery that could be applied to engineer titanium implant materials.

5 Functionalization of the biomaterial/tissue interface

As discussed above, several methods currently exist for immobilization of active functional molecules onto titanium-based implant materials. Among these, covalent coupling results in stable immobilization at the expense of a complex procedure and bigger hurdles for approval by the authorities owing to the involvement of several potentially toxic substances in the preparation process (irritant and reactive amino or mercaptoalkyl alkoxysilanes or linkers such as the reactive and carcinogenic glutaraldehyde). Moreover, the method is irreversible, which renders it inapplicable for growth factors and other molecules which must be released to be effective.

On the other hand, physical affinity or adsorption as the simplest coating method does not offer appropriate binding stability. Though release is favoured for some species, the release behaviour of adsorbed species is of a spontaneous nature and hardly controllable. Consequently, multi-component systems have been developed, where a base coating (fibrillar collagen) with sufficient stability is combined with other components (growth factors), which may be released. However, this approach may also not result in defined release behavior.

The newer method which uses anodic polarization to immobilize AFMs by their partial entrapment into the thickened oxide layer thus appears promising, because the process is simple, can be carried out under near physiological conditions, and results in stably bound molecules comparable to covalent coupling.

In summary, bio-functionalization of titanium-based materials can be achieved by various methods, but only a limited number of AFMs can be immobilized simultaneously. Additionally, not all immobilization procedures can be applied to all AFMs. This impedes a concomitant immobilization of several functional molecules in designated mixtures, which may be beneficial for tailoring implant surfaces for the needs of specific patient groups. Furthermore, release behaviour of bound AFMs cannot be controlled satisfactorily with the current methods.

To overcome these drawbacks, several researchers have combined the electrochemical immobilization as a fundamental method with the huge possibilities offered by the

self-organization potential of nucleic acids and has been described below [140–142]. This technique can be considered as a convenient system for surface bio-functionalization based the immobilization technique that allows the immobilization of a higher number of different AFMs. The principle of the immobilization system is depicted in Fig. 4.

In a first step nucleic acid single strands, referred to as anchor strands (ASs), are regioselectively adsorbed via 5′-terminally phosphorylated sites (P-ASs) at the air-formed passive layer of the titanium-based alloy. Adsorption is followed by anodic polarization, during which the adsorbed P-ASs are fixed by partial incorporation into the anodic oxide layer during oxide growth. Adsorption and fixation are considered as one step because they are carried out successively in the same electrolyte vessel. In an another step, the immobilized ASs are hybridized with their complementary strands (CSs) conjugated to biologically active molecules, enabling a prearranged functionality. In some cases (RGD peptides) a stable fixation is intended, while other AFMs (e.g. antiphlogistics, antibiotics) have to be released. This can be controlled via the stability of the chosen nucleic acid sequences. All the nucleic acids applied have to be checked for undesired biological activity prior to use by sequence comparison with known functional nucleic acids such as aptamers, (deoxy)ribozymes, aptazymes, siRNA, miRNA, etc. Compared with the well-established methods of adsorption and covalent bonding, this immobilization method offers a number of advantages.

1. It is a convenient and toxicologically harmless method for surface modification with anchor strands.
2. It allows for immobilization of different functional groups in one step using hybridization. The functionalization can be tailored to the specific needs of different indications if different mixtures of conjugates with adjusted molar ratios are applied.
3. The release behavior of the functional groups can be tailored, from nearly irreversible immobilization up to an early release by adjusting the hybrid stability. This can be achieved by varying the hybrid length, the G-C contents or the number of mismatches and by predestined restriction sites for nucleases.
4. The specific functionalization, i.e. the hybridization of mixtures of complementary strand conjugates adapted to certain medical indications with the fixed anchor strands, may be carried out immediately prior to implantation, which enables a higher flexibility of the medical treatment.

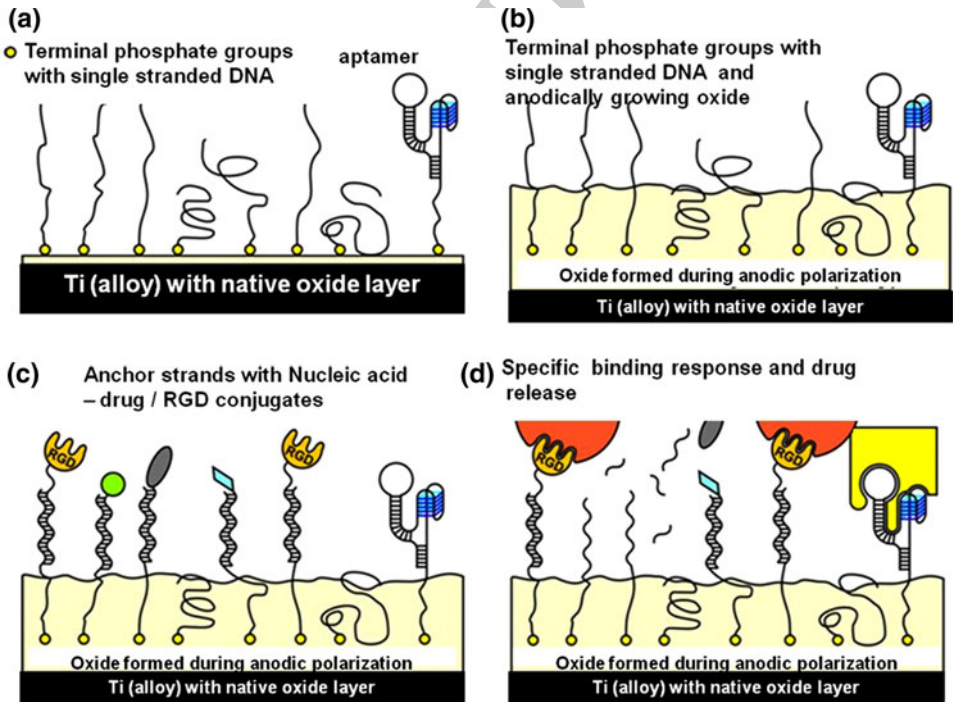

Fig. 4 Bio-functionalization of active functional molecules using anodic oxidation. **a** Single-stranded anchors or functional nucleic acids (aptamers) are adsorbed via their terminal phosphate groups on the native oxide layer of titanium materials. **b** Anodic polarization leads to a partial entrapment of adsorbed nucleic acids in the thickened oxide layer. **c** Nucleic acid conjugates of bioactive molecules are hybridized to the single-stranded anchor strands fixed on the surface. **d** In the mammalian organism, osteoblasts can bind to RGD peptides on the surface. Aptamers can also bind cells or certain molecules. Both lead to faster integration into the surrounding bone. Released drugs from the surface-bound conjugates may be antibiotics, antiphlogistics, or growth factors

6 Conclusions

Immobilization of extracellular matrix proteins and their peptide derivatives to generate bioactive behavior of titanium-based implant materials in order to enhance specific host response is the prime area in the current biomedical materials research.

The use of newer approach to overcome current limitations with various immobilization processes is a new modular immobilization concept for co immobilizing various biologically active functional molecules onto the implant surface at the same time. The technique uses the nanomechanical fixation of single-stranded nucleic acids into anodic titanium oxide layers and their hybridization ability for loading the surface with functional ligands conjugated to respective complementary strands. The feasibility of self-organization based on hybridization of nucleic acid conjugates to anodically immobilized nucleic acids has recently been established successfully using an RGD-peptide as a first AFM molecule.

Future development for the immobilization system requires not only its adaptation to surface conditions of real implants but also all the major titanium-based implant materials need to be tested. Also hybridization of other functional molecules (growth factors), complementary strands and conditions allowing defined release behaviour of the conjugates needs to be investigated. Beyond hybridization of nucleic acid conjugates on biomaterial surfaces, self-organization still offers great opportunities. Two-dimensional structures in various patterns and shapes could possibly be used for controlled surface patterning of implants. DNA dendrimers may be used to heighten the number of hybridizable anchor sequences at the surface, or form drug containers for transport and delayed release.

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