Chemical and biological functionalization of titanium for dental implants

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Dental implant materials serve a variety of purposes. The majority of them are used as intraosseous appliances in the jaw bones for permanent anchorage of tissue integrated prostheses. Successful clinical use of these materials is based on the integration into the adjacent bone tissue. Compromised bone conditions and periimplant bone defects can impair this interaction and require enhancement of osteogenesis to accomplish the desired level of bone implant contact. Established techniques use modifications of surface morphology and inorganic surface chemistry. Advanced strategies focus on the anchorage of bone matrix components to the material surface and on the delivery of osteogenic signaling molecules to enhance periimplant bone regeneration. Biologically active components are immobilized through a variety of procedures such as adsorption, covalent coupling, electrochemical surface modifications and self organized organic layers on the implant surface.

Introduction

Dental implant materials encompass a variety of materials with different degrees of interaction with the human body. In general, they can be divided into those that are in direct contact with oral tissues, in particular the jaw bones, and those that are part of the dental prosthesis inside the oral cavity fixed to the intraosseous components underneath. While the latter group is designed for a mechanically stable function with minimum biological interaction with the oral milieu, the former ones are supposed to establish rapid contact with the adjacent bone tissue in order to provide anchorage to the intraoral prosthetic appliances as early as possible.

The most important factor for dental implant success is the direct contact between the material surface and the periimplant bone. Immediately after insertion of the implant, this contact provides primary stability through friction and mechanical interlocking between the implant thread surface and the bone trabeculae. During the following weeks, the periimplant bone is remodelled and replaced by newly formed bone.[1,2] The major part of the final bone implant contact is thus based on newly formed bone that originates from the adjacent periimplant bone and is laid down on the implant surface in an osteoconductive manner.[3,4] Osteoconductive bone formation requires recruitment of osteogenic cells to the implant surface. This recruitment is based on an adequate biological signal level that orchestrates the process of migration of undifferentiated mesenchymal cells and their differentiation into bone forming cells. A crucial step to start this cascade of events is the rapid adsorption of biologically active molecules on the implant surface immediately after insertion (flash spread).

Under healthy conditions, this biological process of periimplant bone healing provides reliable results with implant success rates of more than 90% after 10 to 15 years.[5,6] Compromised bone conditions that result from trauma, infection or systemic diseases, can be associated with inferior biological quality or loss of bone tissue volume and thereby jeopardize the clinical outcome.[7,8]

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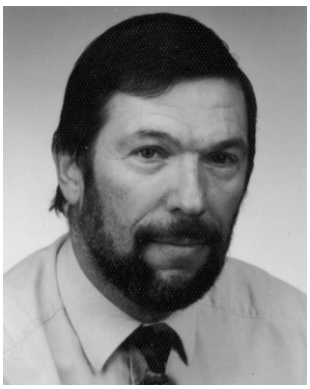

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Table 1 Classification of biofunctionalization methodology

		Chemistry		
Morphology	Physico-chemistry	Inorganic	Organic	Inorganic/organic coatings
Micromorphology to improve • Adhesion • Proliferation • Differentiation of cells Mechanical interaction with surrounding tissue – interlocking	Adaptation of • Surface energy • Isoelectric point • Ionic/electronic conductivity	Plasma oxidation F-Treatment CaP Nanocrystals Calcium phosphate phases • Thermal • Exposure to SBF[a] • ECAD • Sol–gel	• Adsorption • Covalent coupling • Nanomechanical fixation • Self organisation principles • Drug delivery coating *e.g.* antibiotics/ growth factors	Compound coatings from CPP with • collagen • CHDx • Chitosan • Growth factors

[a] SBF = simulated body fluid.

Biofunctionalization of dental implant materials in intraosseous applications can help to overcome these limitations by fostering the regenerative capacity of the adjacent bone and providing biological stimuli at the implant interface that compensate for the reduced biological quality of the host bone tissue.

Under physiological *in vivo* conditions, titanium implants are covered with an amorphous Ti-oxide layer of few μm thickness. Modifications of this surface in order to enhance the level of peri-implant bone regeneration that can be grouped into two major avenues: (1) modifications of surface morphology, and (2) modifications of surface chemistry by either (i) physico-chemical surface modifications, or (ii) chemical surface modifications. The latter ones can be grouped into those that use inorganic components and those using organic molecules (see Table 1). Some of these approaches are already in clinical use while others are about to work their way from bench to bedside.

1 Modification of surface morphology

The vast majority of dental implants today are made of commercially pure (cp) titanium and are designed as root form screws or cylinders. Early on, there have been attempts to increase the interface area and to improve the ability of mechanical interlocking with adjacent bone through titanium plasma flame spray coatings (TPS).[9–11] During this process, titanium powder is partially melted by blasting into a high temperature plasma flame and sprayed onto the titanium implant. This creates a rough implant surface that provides increased mechanical retention in bone tissue when compared to implants with a smooth, machined surface.[12] However, difficulties in eliminating adverse by-products that occur during the high-temperature process and concerns about delamination of titanium particles during implant insertion and functional loading[13] have led to a gradual replacement of TPS coatings by other types of surface modifications in implant dentistry, that provide a microtextured surface characteristic.

Modifications of surface morphology of titanium implants are currently accomplished by removing material from the implant surface through etching (sulfuric acid–hydrochloric acid) (Osseotite),[14] grit-blasting with either Al_2O_3 or TiO_2 (FriOss, TiOblast)[15] or a combination of both.[16] Grit-blasting creates a surface texture reaching from submillimeter to micrometer level, whereas etched surfaces are characterized by pits and groves down to the nanometer level. Several factors are considered to be contributing to the improved performance of these surface modifications when compared to smooth machined implant surfaces. One positive effect on cellular behaviour is mediated through the microtexture itself. Different degrees of surface roughness have shown to modify cellular production of receptors that mediate the adhesion to titanium surfaces.[17] Moreover, increased secretion of molecules indicating osteogenic differentiation of cells in contact with the microtextured surface and enhanced production of cytokines involved in bone formation have been reported *in vitro*.[18,19] Roughened (grit-blasted) titanium surfaces have shown enhanced mineralization and transcription of osteogenic markers in osteoblasts cultures when compared to grooved (acid-etched) surfaces.[20]

Another positive effect comes through the increase in surface energy that is associated with increased surface roughness.[21] In general, interactions of a biomaterial with the surrounding biological system are strongly dependent on its surface charge and surface energy. An increase in surface energy improves the wettability of the implant surface facilitating the adsorption of serum proteins and other biomolecules such as fibronectin.[22,23] As hydrophilic surfaces are associated with less conformational changes of the adsorbed proteins this can support the cascade of cellular events involved in the attachment, migration and differentiation of bone forming cells.

However, for titanium and titanium based alloys there are additional effects that need to be considered. Semiconductor-properties,[24] crystal structure,[25] and the thickness of the oxide layer that is always present on the surface additionally play an important role for the interaction with biomolecules. The effect of the semiconductor-properties themselves is influenced by the flatband-potential and the band gap.[26] Further, the defect density in the oxide layer determines electronic conductivity especially at potentials anodic from the flatband potential.[27] A high electronic conductivity increases the likelihood of redox reactions between adsorbed biomolecules and the underlying metal[28] which may have adverse effects in that it causes deformational changes of adsorbed biomolecules. Deformed molecules may not be recognized properly by their receptors on the cell surface, which may be detrimental for their biological activity.

Finally, microstructured implant surfaces are considered to provide microanchorage for the blood clot and the transitory fibrin matrix that is initially formed at the bone implant

interface. Microretentions allow for fixation of the organic matrix when wound contraction commences. The retention of the fibrin matrix is supposed to enhance periimplant tissue formation when compared to smooth machined surfaces from which the matrix is partially detached during wound contraction.[29]

The optimum surface roughness (S_a) for both *in vitro* differentiation of osteoblasts and intraosseous fixation *in vivo* has been identified to be in the range between 3 and 3.90 μm.[30,31] Sandblasted surfaces yielded better results for 3 μm when compared to 0.5 μm,[30] sandblasted and acid etched surfaces show a decrease in functional attachment above roughness values of S_a: 3.90, S_t: 56.78 and S_{dr}: 2.15.[31] In preclinical applications, implants with microstructured surfaces have been reported to show earlier and more intensive bone implant contact than implants with smooth machined surfaces.[15,32–35] A number of recent clinical studies indicate that implants with these surface modifications are successful also in compromised bone situations.[36–38]

2 Physico-chemical surface modifications

As titanium oxide is very reactive, it is immediately covered with a thin coating of hydrocarbons (a few nanometers thick) when exposed to air. This process is associated with a decrease in surface energy leading to an increase in the water wetting angle and a more hydrophobic surface.[39] Hydrophobic interactions between this material surface and the protein itself go along with stronger interacting forces between the implant surface and the adsorbed protein facilitating critical conformational changes of the adsorbed molecule. This may result in immunological reactions or decreased functional activity of the respective protein. Recent modifications of microstructured surfaces, thus, have been directed towards further enhancement of early bone reactions by avoiding surface contamination with hydrocarbons and have prompted the development of a modified sandblasted and acid etched surface. Immediately after grit blasting and etching the implants are rinsed under N_2 protection and stored continuously in isotonic saline solution. This has resulted in substantially enhanced wettability with significantly reduced contact angles.[40] *In vitro*, this "ultra-hydrophilic" surface has been associated with increased adsorption of fibronectin and elevated levels of osteocalcin production when seeded with osteoblasts.[41] Qu *et al*. have found enhanced cluster formation and increased expression of key osteogenic regulatory genes in osteoblasts.[42] Preclinical testing of this type of surface modification has resulted in accelerated bone formation in early stages of periimplant bone regeneration and enhanced bone implant contact in areas of implant surfaces previously not covered by bone.[43–45] This surface modification has been introduced into clinical treatment as SLActive®.

3 Chemical surface modifications

Chemical surface modifications encompass a large variety of approaches some of which are already used in clinical practice while others are currently being evaluated on a preclinical level. Roughly, there are three groups of chemical surface modifications: (a) modifications or coatings that use inorganic chemistry, (b) modifications using organic molecules and (c) those that use combinations of both (see Table 1).

(a) Surface modifications and coatings using inorganic chemistry

Inorganic components are looked at as being particularly interesting for various reasons. Calcium is considered to be involved in the binding process of biologically active proteins from the periimplant milieu in that calcium ions adsorb to the TiO_2 surface and further to macromolecules with high affinity for Ca^{2+}.[46,47] Calcium and phosphate are also considered as valuable reservoirs for the process of mineralisation during bone formation. Approaches to incorporate calcium and phosphates on to implant surfaces have a rather long history of calcium phosphate coatings. The excellent integration of calcium phosphate ceramics into bone tissue has brought about the idea to increase the biocompatibility of metallic implants in intraosseous applications and enhance periimplant bone formation by coating the implant surface with a layer of calcium phosphates, as reported in the 1980s.[48,49] A large variety of coating techniques such as sputter coating (for review see ref. 50) and plasma flame spray coating have been applied,[51] that resulted in increased bone-implant contact or an improved ability to bridge periimplant bone gaps.[52–57] However, the rather thick calcium phosphate layers that were composed of different calcium phosphate phases (CPP) with a high content of amorphous calcium phosphate frequently were lacking long term stability due to phase transition within the coating layer resulting in tensile strains and fragmentation. Subsequent biologic attack *in-vivo*[56,58–63] after mechanical failure of the coatings has been associated with adverse tissue reactions during degradation of separated coating fragments.[64,65]

Failure of thick calcium phosphate coatings to take part in the periimplant bone remodelling or become an integral part of the adjacent mineralized bone tissue has changed the concept of using inorganic chemistry for surface treatment. Novel technologies have been developed that produce thin continuous calcium phosphate coatings or surface modifications that incorporate inorganic components with incomplete coverage of the titanium surface.[66] There are sliding transitions between these surface modifications and the thin continuous calcium phosphate coatings but for didactic reasons the two groups are considered separately.

Inorganic surface modifications. Currently, there are three approaches of surface modifications using inorganic chemistry which are established in clinical use. One of them is anodic plasma oxidation that is used to produce a structured titanium oxide layer on the implant surface. During this procedure, implants are immersed in an electrolyte solution and are subject to high voltage anodic polarization. Under these conditions, multiple local micro arcs occur at the interface anode/electrolyte. Due to ionisation of oxygen these arcs result in local plasma-like conditions that cause evaporation of the electrolyte as well as melting and oxidation of the anode surface. The thermal effect activates neighbouring areas and results in successive plasma formation which finally forms an oxide layer on the whole anode surface. The melting of the superficial oxide layer in conjunction with the evaporation of the electrolyte creates a textured surface by formation of multiple micro craters (Fig. 1(a)). Following this treatment, the structured oxide layer thickness amounts up to a few μm whereas the untreated machined smooth surface exhibits an oxide layer thickness of several nm.[67,68] Moreover, due to

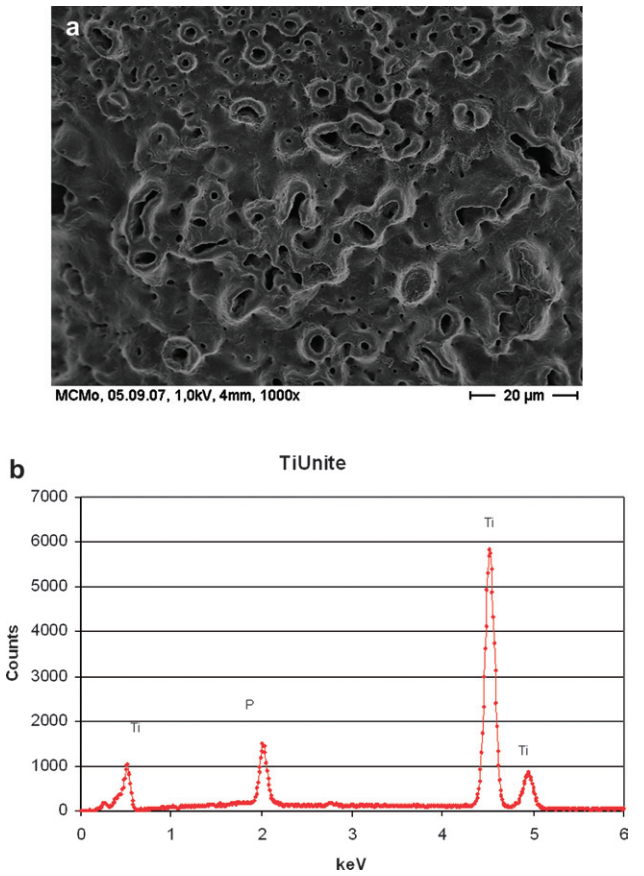

Fig. 1 (a) SEM micrograph of a Ti implant surface after anodic plasma oxidation (TiUnite®). (b) EDX spectrum of the implant surface showing a peak for phosphorus.

evaporation of the electrolyte with inclusion of the electrolyte ions into the plasma, these ions are incorporated into the microtextured oxide layers during melting of the surface[69–71] (Fig. 1(b)).

There are three advantages associated with this surface modification. The increased surface roughness enhances surface energy which improves protein adsorption. The increased thickness of the oxide layer helps to preserve the biological effect of protein adsorption to the implant surface as redox reactions of the adsorbed protein with the underlying metal, which can result in conformational changes and deterioration of its biological quality[72] are less likely to happen. Finally, incorporation of inorganic components such as Ca, Mg and phosphate from the electrolyte into the oxide layer during anodic plasma oxidation positively altered implant surface chemistry. These chemically modified microstructured oxide surfaces exhibited increased bone to implant contact and removal torque values when compared to microstructured surfaces without incorporation of inorganic components.[69–71,73] The structured oxide surfaces with and without incorporation of inorganic components have been introduced into clinical use as Ticer® (ZL Mikrodent) and TiUnite® (Nobel Biocare) surface on titanium implants.

There are two other clinically established techniques of inorganic chemical surface modifications that are based on existing modifications of surface morphology. One of them employs fluoride treatment of TiO_2 grit-blasted implant surfaces (Osseospeed®, Astra) while the other approach uses discrete crystalline deposition (DCD) of calcium phosphate crystals on dual acid etched (HCl/H_2SO_4) surfaces (Nanotite®, Biomet 3i). The rationale for the fluoride treatment of implant surfaces is based on the fact that fluorides are known to activate alkaline phosphatase, a bone specific enzyme, and to stimulate osteoprogenitor cells *in vitro*[74] as well as to enhance bone matrix formation and mineralization through increased seeding of apatite crystals.[75]

Fluoride treatment is performed through immersion of implants in hydrofluoric acid resulting in an increased fluoride ion content of up to 9.0%.[76] A proposed mechanism of fluoride action *in vivo* is that the oxygen of available phosphates in the body milieu replaces the titanium bound fluoride forming a covalent bonding between titanium and phosphate in bone.[77] Fluoride ion-modified TiO_2 grit-blasted c.p. titanium surfaces have shown to enhance early expression of osteoblastic cell differentiation markers[76] *in vitro* and the implants were associated with an increased bone to implant contact and improved mechanical retention in bone four weeks after insertion.[78] The enhancement of the bone implant contact rate was particularly appreciable at early periods of periimplant bone formation.[79] Clinical trials have shown stable osseointegration of implants under functional loading in an early loading protocol.[80]

Surface modification of dual acid etched surfaces by discrete deposition of calcium phosphate nanocrystals (DCD) is accomplished by immersing the implants into an alcohol-based dispersion of nano-sized calcium phosphate crystals of 20–100 nm nominal size range. This results in 50% coverage of the implant surface by calcium phosphates.[66] Deposition of calcium phosphates is probably supported by the ability of titanium surfaces to bind polyvalent cations. This binding is based on electrostatic interactions between titanium-linked O^- on the implant surface and the cations.[47] The calcium phosphate depositions on the implant surface enhance the adsorption of calcium-binding macromolecules. Implants with DCD surfaces have shown to resist significantly higher tensile forces than implants without DCD treatment in preclinical tests.[66]

There are only few studies which compare the effect of different surface modifications and the effects of the individual chemical modifications that go along with them are difficult to distinguish as basic surface morphology differs slightly between the individual approaches or is even altered during the procedure. Using preosteoblast cell lines, Masaki *et al.* have shown that alkaline phosphatase as an early osteoblastic marker is significantly increased on "ultrahydophilic" SLA surfaces compared to unmodified and fluoride modified TiO_2 grit-blasted ones, whereas these surfaces enhanced expression of a late marker of osteogenic differentiation Cbfa1/RUNX-2.[81]

Inorganic surface coatings. Novel concepts of inorganic coating of implant surfaces use the enhancing effect of calcium phosphates on periimplant bone formation but at the same time take into account that coatings are subject to remodelling and degradation *in vivo*. This requires the production of much thinner coatings in order not to overburden the remodeling and resorption capacity of periimplant tissues. Hence, new coating strategies such as electrostatic spray deposition, supersonic particle acceleration or magnetron sputtering were developed and experimentally tested.[82–86] The thinner coating layers produced by these novel technologies have shown to be equally effective as thicker coatings with respect to enhancement of early

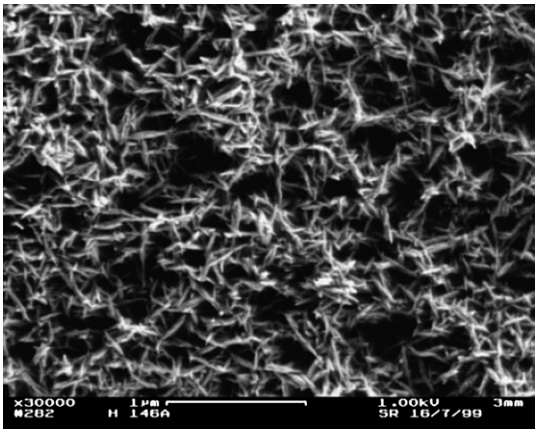

Fig. 2 SEM micrograph showing nano hydroxyapatite (HA) needles deposited through electrochemically assisted deposition (ECAD). Reproduced with permission from ref. 91, Copyright 2003, Wiley-Interscience.

bone response.[83,87,88] Degradation under in vivo conditions has not only shown to be non-detrimental to periimplant bone reactions[89] but was even supportive of osteoconduction with enhanced bone-implant contact.[86]

Among the newly developed biomimetic coating methods which recently have been reviewed in this journal[90] the electrochemically assisted deposition (ECAD) of calcium phosphate phases (CPP) offers a number a promising advantages. An important feature of ECAD of CPP is an efficient biomimetic low-temperature process resulting in the generation of very thin coatings with a high specific surface area and with excellent homogeneity of the coating also for irregularly shaped and porous structures. The method is based on the cathodic polarization of an electronically conducting substrate in an aqueous solution containing calcium and phosphate ions. Under these conditions, the formation of OH^- ions at the substrate surface occurs, causing a local increase in pH of the electrolyte at the implant/electrolyte interface. As the solubility of CPP is pH dependent, this rise in pH leads to an increase in the relative supersaturation σ of the electrolyte with respect to CPP, resulting in the formation of a CPP coating on the substrate surface. In this way a thin hydroxyapatite (HA) layer can be formed on the titanium surfaces under near physiological conditions (Fig. 2).[91] Based on early works in this field[49,92,93] current efforts are focussed on the deposition of nano-crystalline CPP[91] and the realization of the process under *in vivo*-like conditions to allow the incorporation of organic components of interest such as proteins,[94–96] chitosan,[97–99] and chlorhexidine (CHDx).[100]

The adhesion strength of the calcium phosphate layer is increased by partially integrating the hydroxyapatite crystallites into an oxide layer that is grown under subsequent anodization. When these coatings were tested on titanium alloy surfaces (Ti6Al4V) and commercially pure titanium implants with machined surfaces in dog mandibles, the bone implant contact on both surface coatings was significantly increased when compared to uncoated controls.[101,102]

(b) Surface modifications and coatings using organic chemistry

The knowledge that adsorption of biomolecules to the implant surface is a key process for cell adhesion and growth on biomaterials on the one hand and the ability to produce these molecules for pharmaceutic applications on the other suggest the use of organic coatings of titanium surfaces to enhance periimplant bone healing by attaching these molecules to the implant surface. In this way the critical process of adsorption of biomolecules could be short cut or even tailored to specific patient needs to make surfaces more attractive for cells with osteogenic potential. Moreover, by placing a selected pattern of signals on the surface, the process of tissue integration could be considerably accelerated. For *in vivo* applications, a number of molecules appear particularly attractive from the perspective of cell adhesion and growth. These are proteins or peptides that are involved in cell communication, tissue repair and bone repair in particular.

Among these, RGD motifs (a sequence of three amino acids Arg-Lys-Asp) mediate cell attachment and migration of cells through binding to integrin receptors on the cell surface.[103,104] Different biomolecules presenting these sequences bind to different integrin subunits. Collagen e.g. binds to $alpha_2beta_1$ units while RGD motifs of bone specific proteins such as vitronectin, osteopontin and osteoprotegerin bind to $alpha_vbeta_3$ and $alpha_vbeta_5$ receptors.[105,106] Binding of RGD peptides to the integrin receptors activates a cytoplasmatic signalling pathway to the nucleus that can alter cellular architecture or initiate cell migration and proliferation. In order to address the integrin receptors in a specific manner, more complex peptide sequences (RGDXYZ motifs) than the short RGD sequence are required.[107–111] The attachment of RGDXYZ motifs specific for selected components of the native extracellular matrix (ECM) of bone on an implant surface could thus selectively direct cells with osteogenic potential to the implant surface.

Another important contribution to bone healing and new bone formation comes from the organic bone matrix itself. Besides collagen I, which makes up approximately 90% of the bone matrix proteins and is crucial for cellular adhesion of bone cells as well as coordination of mineralization, the group of non-collagenous bone matrix proteins contains a number of growth factors that are important for the recruitment and final osteogenic differentiation of undifferentiated mesenchymal cells. In particular, bone morphogenic proteins (BMPs) and transforming growth factor beta (TGFβ) are involved in this process. Glycosaminoglycans play an important role in the storage and release of growth factors from the bone matrix. The use of an organic coating of titanium implants using collagen and/or non-collagenous bone matrix proteins, therefore, could considerably enhance the formation of new periimplant bone, as the implant surface presents a bone-like appearance mimicking a target for bone forming cells.

During recent years, new techniques have been developed to coat titanium dental implants with organic molecules that are considered to play a pivotal role in the mediation of implant tissue contact. Currently two attempts are followed in this area, one using biomimetic ECM-like synthetic polymers (see review in ref. 112) and a second based on the use of components of the native ECM.

Methods to immobilize biologically active molecules (BAMs) onto biomaterial surfaces depend on the stability of the BAMs themselves, as well as their molecular weight and structure. Furthermore, the desired stability of immobilization of BAMs determines the technology used for binding. Additional factors

that may be influential for the selection of the immobilization method are concepts to immobilize more than one BAM on the implant surface and to tailor surface properties for specific patient groups immediately before surgery.

In general, biologically active molecules can be attached to the surface of titanium implants through adsorption, covalent binding, nanomechanical incorporation and self-organizing organic layers. Choosing adsorption as the basic process encompasses a spectrum reaching from physisorption to chemisorption. According to Roessler *et al.*[113] oxide layers on titanium based materials show isoelectric points around 4.1 indicating that these oxide layers carry a negative charge under *in vivo* conditions. Thus, macromolecules positively charged under these conditions should adsorb with great stability due to electrostatic interactions. This forms the basic idea for a methodology realized by the group of M. Textor.[114–116] based on previous work by Ruiz-Taylor *et al.*[117] for immobilization of poly(L-lysine) on titanium surfaces. In this approach, poly (L-lysine) does not form the BAM itself but acts as an anchor molecule for covalently coupled poly(ethylene glycol) (PEG) chains and RGD groups. The relatively complex architecture of these layers resulted in coating systems highly resistant against protein adsorption[115,116] on the one hand but promotive for cell adhesion due to the incorporated RGD-motifs on the other.[114]

Preformed adsorptive coatings of fibrillar proteins such as collagens I, II and III have shown high stability when exposed to competitive adsorption of serum proteins *in vitro*.[118–120] This behaviour is considered to be based on weak hydrophobic interactions due to the high ratio of surface area/molecular weight of the fibrilar protein compared to globular serum proteins. Collagens I and III based matrices have also been attached by physisorption to form more complex artificial ECM (aECM) on titanium surfaces additionally containing proteoglycans[121] and/or glycosaminoglycans.[122,123]

Finally, chemisorptive interactions can be used to immobilize molecules onto titanium surfaces. These interactions have been shown to exist *e.g.* between phosphonate groups and a titanium oxide surface.[124,125] However, in order to create sufficiently stable fixation of large molecules more than one phosphonate/phosphate groups appear to be necessary.[126]

In summary, adsorptive binding methods combine the advantage of being simple and applicable to a nearly unlimited extent. They allow for sterilization of the implant surface with subsequent formation of multi-component-layers. Drawbacks are the rather low stability of BAM fixation, a non-defined release behaviour of BAMs and possible conformational changes of the directly adsorbed molecules.

An alternative approach to choosing adsorption for immobilization is the method of covalently coupling BAM to biomaterials surfaces. For titanium based surfaces aminosilanes are widely used to bind to the oxide surface with the free terminal NH_2 groups used for coupling of BAMs. Nanci *et al.*[127] were among the first to introduce the method for titanium surfaces by showing that covalently coupled alkaline phosphatase (ALP) was still enzymatically active. In the meantime many other biologically active molecules have been tested for biofunctionalization of implants. ECM components like laminin,[128] fibronectin and heparin,[129] and collagen[130] as well as antibiotics[131] have been successfully covalently bound to titanium surfaces. The methodology has also been applied to growth factors[128,132] indicating remaining biological activity in an animal model.

The advantage of covalent binding is the stable fixation of the BAM, combined with the chance to preserve biological activity to some degree if the molecule is combined with linkers/spacers of sufficient length.[129] However, there are a number of disadvantages that relate to the efficacy and clinical applicability of the complex multi-step procedure. If more than one BAM is considered for immobilization, it is difficult to combine different molecules in a defined way on the surface. Moreover, if a controlled release from the implant surface is intended, *e.g.* for the generation of concentration gradients,[100] the covalent stable fixation may be disadvantageous. Finally, the need to sterilize the BAMs after immobilization can be a major drawback if sensile proteins such as growth factors are considered because their biological function would be severely compromised. Conformationally more stable molecules such as structural proteins and peptides therefore are more suitable for this type of immobilization.

A third approach to binding of biologically active molecules is particularly suitable for so called valve metals (especially titanium, niobium, tantalum) which are widely used as biomaterials either pure or as alloys. For these materials the anodic growth of the air-formed oxide layer can be used for nanomechanical incorporation and immobilization of BAMs. The method is based on the growth mechanism of anodic oxide layers on this materials that in general results in a two sublayer system with the inner sublayer formed at the metal/oxide interface and the outer at the interface between oxide and electrolyte. Oxide formation in the second interface is about 50%[133] of the total oxide growth corresponding to about 2 nm V^{-1} for titanium.[39] It occurs *via* reaction of metal ions with water and electrolyte components. Thus ions, molecules and even nanoparticles that are preliminarily stabilized by adsorption/fixation on the initial oxide/electrolyte interface can be incorporated into the growing oxide layer as has been shown by a number of electrochemical investigations.[39,134] The concept of nanomechanical fixation has already been applied for the incorporation of phosphate ions.[135] Also partial incorporation of a cyclic RGD-peptide conjugated to phosphonate anchors[136] and fixation of adsorptively immobilized fibrillar collagen[137] have been accomplished by this technology. Recently, this technique has even been applied successfully to the regioselective incorporation of terminally functionalized oligonucleotides (Fig. 3). The immobilized

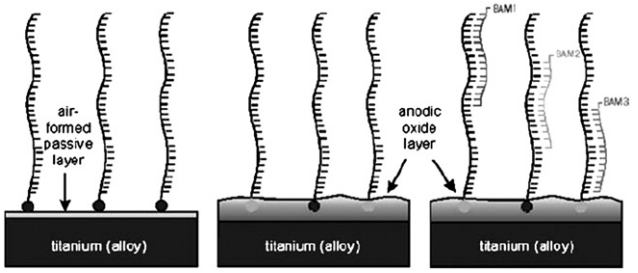

Fig. 3 Schematic drawing illustrating regioselective incorporation of terminally functionalized nucleotides and their hybridization with complementary strand conjugates with bioactive molecules. Reproduced with permission from ref. 126, Copyright 2007, Wiley-Interscience.

nucleotides employed for biofunctionalization of biomaterial surfaces can be used in two different ways. If the molecules provide aptamer properties they can act directly as BAMs.[138] They can, however, as well be used for hybridization with complementary strands conjugated with BAMs[126] following the self organization principle.

The self-organization principle is an entirely different mode of immobilization based on a two-step procedure. The primary step is used to coat the surface with one component of the self-organizing system using one of the techniques described above. The immobilized substance can *e.g.* be a component of the native ECM or molecules derived thereof. If one or more functionally active ECM molecules are brought in, their immobilization results from the specific interactions between them in the native environment. Thus these biologically adapted interactions will in fact stabilize the native conformation of the immobilized component and combine this with an *in vivo* like presentation and release behaviour. This opens up a highly interesting field of matrix engineering on the implant surface with a great potential for periimplant bone regeneration. This strategy has been used to bind relatively simple molecules like fibronectin to collageneous matrices[118,139] and is currently increasingly applied for immobilization of growth factors. In this way, heparin has been used for biomimetic immobilization of rhVEGF[140] and bFGF.[140,141] Similar matrices of collagen type I and chondroitinsulfate (CS), have been used to bind biomimetically rhBMP-4 in sub-μg amounts in a self organizing manner (Fig. 4).[142,143] Due to their interactive potential with respect to bone matrix assembly and binding of growth factors, proteoglycans and glycosaminoglycans in general are most interesting candidates is this area. Even designed components derived thereof through methods of functional glycomics have been in more advanced approaches of matrix engineering.[144–147] Self-organization has been realized also by introducing other molecular systems for functionalization. One approach is based on the previously mentioned poly(L-lysine)-PEG system by Ruiz-Taylor *et al.*[117] They combined a biotin-streptavidin system with the basic poly(L-lysine)-PEG coating and could show, that streptavidin-conjugated proteins were selectively deposited on these surfaces (Fig. 5).

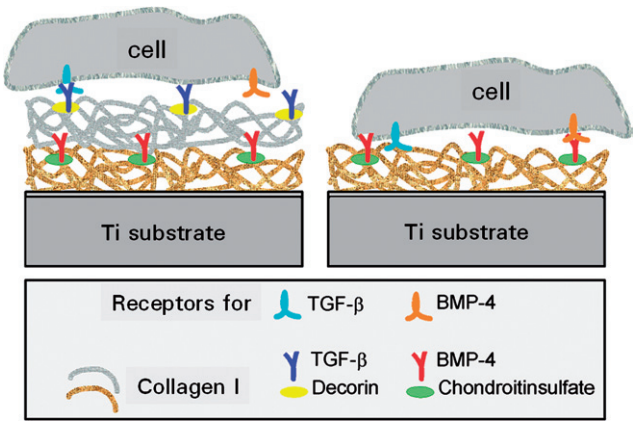

Fig. 4 Schematic drawing showing an artificial ECM-based principle of self organization. Growth factors are bound to the assembled artificial ECM containing proteoglycans and/or glycosaminoglycans and allow for selective activation of attaching cells.

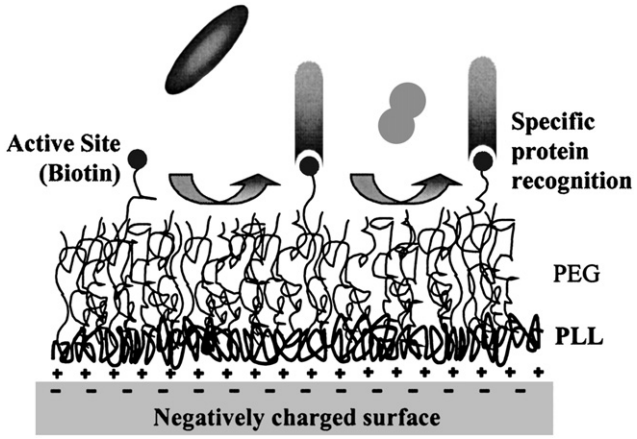

Fig. 5 Schematic drawing of the organization of a poly(L-lysine)-grafted poly(ethylene glycol) (PLL-g-[$(PEGm)_{12.x}$(PEG-site)$_x$]) interface. Hydrophilic PEG chains prevent unspecific protein adsorption, whereas biotin coupled to PEG chains allows for selective binding of proteins coupled to streptavidin. Reproduced with permission from ref. 117, Copyright 2001, National Academy of Sciences.

Surface modifications and coatings using organic chemistry are currently being evaluated *in vitro* and tested preclinically *in vivo*. The *in vitro* and *in vivo* results vary considerably according to the method of immobilization. RGD peptides have been widely tested with different modes of anchorage to biomaterial surfaces. When anchoring molecules were used, the length of the spacer molecule between the surface and the RGD motif was important for the effect on cell adhesion and proliferation.[148,149] Coating of inorganic implant surfaces using acrylamide and thiol anchors as well as a combination of aminosilanes and a heterobifunctional crosslinking agent to bind RGD motifs has resulted in significantly higher numbers of adhering osteoblasts.[105,149,150] Phosphonate and thiol anchors have been employed for binding of RGD peptides to the surface of metal implants.[151–153] Both approaches have been associated with enhanced periimplant bone formation *in vivo* by increased ingrowth into fibre meshes in rabbit calvarial defects[154] and improved bone regeneration around cylindrical implants in canine and goat models.[136,152,153]

Nanomechanical anchorage of collagen I fibres on the surface of titanium implants has been recently evaluated *in vitro* and *in vivo*.[155–157] This surface modification has shown to enhance adhesion and differentiation of osteoblasts *in vitro*[155,156] and to promote periimplant bone formation after three months in dog mandibles when compared to smooth uncoated titanium surfaces.[157] Preliminary approaches of matrix engineering have been employed by a combination of RGD peptides using acrylate anchors and the nanomechanical anchorage of collagen I fibres[157] resulting in increased bone implant contact and bone density during early stages of periimplant bone formation already after one month (Fig. 6(a) and (b)). Additionally, collagen I coating layers have been used as a basis for more complex coatings that have also incorporated non-collagenous bone matrix components in a self-organizing manner. In this way, collagen I and chondroitin sulfate (CS) were employed together for adsorptive coating of titanium surfaces.[122] *In vitro* evaluation of these coatings exhibited significantly enhanced

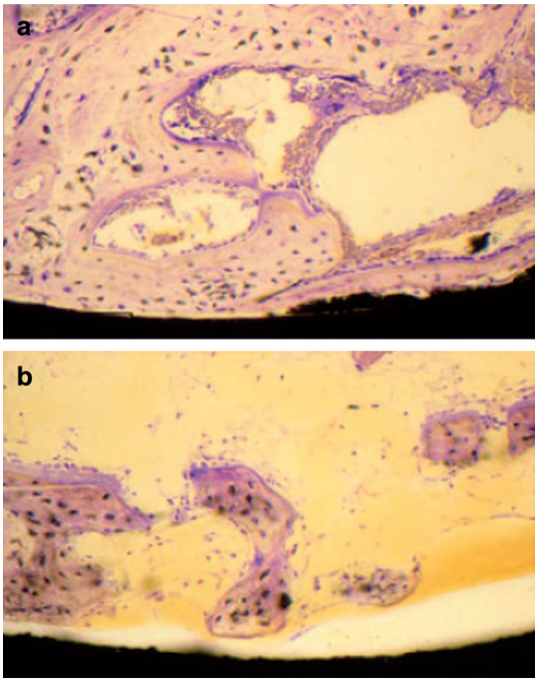

Fig. 6 (a) Bone implant interface of a machined Ti implant with Coll I/RGD coating. A thick and continuous layer of bone is visible on the implant surface (black). (b) Bone implant interface of an uncoated machined Ti implant. Sparse and thin bone regenerates are visible with only minor contact to the implant surface (black). Reproduced with permission from ref. 157. Copyright 2005, Wiley-Interscience.

osteogenic differentiation for CS containing modifications indicating a specific potential also for other bone matrix components such as glycosaminoglycans.[122]

Finally, growth factors that are stored in the native bone matrix, have been added to the self organized matrix layers of collagen I and CS. Recombinant human bone morphogenic proteins 2 and 4 (rhBMP2 and 4) had been adsorbed to this layer by immersion into of a solution of rhBMP and have been evaluated *in vivo* in dog and minipig models.[142,143,158] The surprising result was that coatings of collagen and CS without the growth factor achieved a higher osteogenic potential compared to aECM loaded with rhBMP-4[142,143] and that a significant enhancement of bone formation *in vivo* by the addition of rhBMP2 could not be clearly shown.[142,143] Differences in adsorption and presentation of native growth factors that probably become assembled *in vivo* in a more favourable way compared to the aECM *in vitro* may account for these results.

(c) Inorganic/organic surface modifications

For dental implant surfaces designed for bone contact, a compound material of CPP and organic components could offer a number of advantages through additive effects of both phases by creating a structure and surface that even better resembles that of natural bone and therefore may be even more conducive to osteogenic cells. As this topic has already been reviewed in a former review in this journal,[90] only specific aspects with respect to dental implants and new developments will be discussed here.

One approach in this direction is to combine CPP with collagen type I, the organic main component of bone. First experiments to form such composite coatings, using the ECAD process already mentioned, can be traced back to a paper of Okamura *et al.*[159] In more detailed experiments Roessler *et al.*[95] applied a multi-step process. To avoid any non-biological interaction between the protein and its environment they adsorbed fibrils of collagen I onto a base layer from hydroxyapatite (HA). Subsequently, the collagen was mineralized again using ECAD at a working temperature of 36 °C. The resulting thickness of this layer was app. 600 nm.[160] Because of the short polarization time chosen, the collagen structures remained visible after the mineralization in SEM investigations showing the characteristic native banding pattern of collagen fibrils (63–67 nm). However no periodic correlation of HA crystals with the banding pattern could be detected. Other than the morphology of the pure hydroxyapatite layer that is characterized by crystallites with typical length of 300 nm and diameters of 60 nm, this layer provided a surface layer that is formed by a network of completely mineralized collagen fibrils.

FTIR investigations of the HA/collagen coating showed in general a superposition of pure HA and collagen[95] (Fig. 7). However, for the amide-I band a broadening (ranching from 1638 to 1655 cm) and shift to lower wavenumbers was detected in the mineralized coating corresponding well to the broad amide-I band of native bone. This has been discussed as indicating an interaction between the two components in the coating in analogy to the *in vivo* situation. When tested *in vivo* these coatings have resulted in significantly increased bone implant contact compared to uncoated smooth titanium surfaces already after one month and significantly increased periimplant bone density when compared even to an non-mineralized collagen I coating.[102]

Only recently, Fan *et al.*[94] developed a simpler procedure by adding acid soluble collagen type I to the deposition electrolyte of the ECAD process, intending to combine fibrillogenesis of collagen with deposition of CPP. The final CPP deposit consisted of flake-like crystals from octacalcium phosphate (OCP) in a fibrous collageneous network, but no banding of the collagen fibres could be detected. This indicated that the self assembly of tropocollagen to supramolecular structures did not result in the formation of fibrils with native substructure under the chosen conditions.

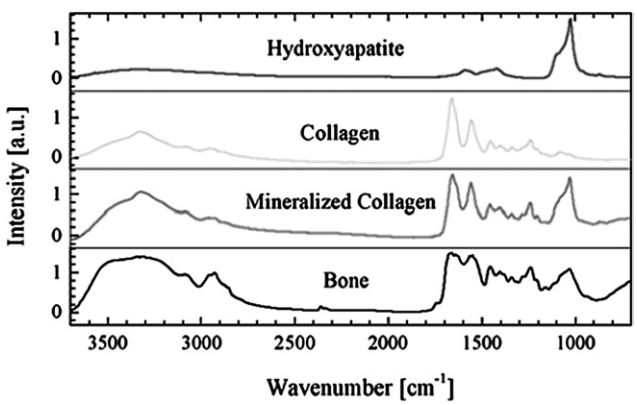

Fig. 7 FTIR spectrum of a mineralized collagen coating prepared by the ECAD process in comparison to spectra of pure HA, collagen, and native bone. Reproduced with permission from ref. 95. Copyright 2001, Springer.

In a second approach, aiming at the inclusion of potential therapeutic agents into CPP layers for a sustained release, similar to Fan, Cheng *et al.*[96] performed the ECAD process in the presence of bovine serum albumin (BSA) as a model protein. They designed the process similar to Roessler *et al.*,[95] by performing a co-deposition of the protein with a CPP on preformed HA layers. Of the protein added, at least 85% were stably incorporated into the resulting layer, leading to a surprisingly high protein content of 500 μg cm^{-2} in layers with a total mass of 3.3 mg cm^{-2}. The incorporated protein mass was about 70 times higher compared to the 7 μg BSA cm^{-2} that could be brought to the surface by pure adsorption. When exposed to a phosphate buffer of pH 7.4, the combined layers exhibited a release of only 15% of the incorporated BSA within a period of 70 h, whereas purely adsorbed BSA was completely desorbed within about 2 h. These experimental findings show that the combination of organic and inorganic components may have the potential to design controlled release systems provided that the protein to be immobilized shows sufficient conformational stability at alkaline pH-values, which occur during the ECAD process at the interface.

Summary and outlook

Biofunctionalization of materials for dental implants involves a wide field of techniques and components ranging from defined morphologies down to the nm-range up to complex artificial extracellular matrices.

Clinical application is currently limited to the design of surface morphology which, in a number of cases, is combined with inorganic components such as fluoride, calcium and CPP. The inclusion of organic components such as adhesion peptide sequences, structural proteins and growth factors is actually intensively researched on the *in vitro* level and in preclinical animal models. A topic of high interest in this area are sophisticated immobilization methods that allow for patient specific functionalization of implant surfaces with multiple BAMs in combination with a defined release behaviour. Here, the oligonucleotide system appears to be very promising. The additional advantage of this system is the fact that the oligonucleotide coated surface can be easily sterilized after manufacturing and loaded with the desired numbers of BAMs immediately before surgery. Modifying the length and sequence of the complementary strands furthermore offers the opportunity to tailor the release profile of the specific BAM in a wide range. Based on a more refined understanding of growth factor binding and release in native ECM of bone this would allow the design of "smart" surfaces.

A second promising way for the design of tailored implant surface properties is based on the work performed in the field of matrix engineering producing artificial extracellular matrices combining structural proteins with glycosaminoglycan derivatives. Recent innovations and findings provide an advanced understanding of interactions between sugars and proteins from the glycomics area. They would allow for the creation of derivatives that are able to bind, accumulate or stabilize various *systemic* mediators (growth factors, interleukines) and thereby influence their bioactivity. Surfaces modified with such derivates are expected to modulate the healing processes in various tissues, not only for healthy patients but even more for those with compromised healing conditions. Glycan-modified surfaces may also play a role in tissue-engineering and regenerative medicine, being able to steer stem cells in desired directions due to their interactions with specific growth factors.

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