Biological nano-functionalization of titanium-based biomaterial surfaces: a flexible toolbox

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Surface functionalization with bioactive molecules (BAMs) on a nanometre scale is a main field in current biomaterial research. The immobilization of a vast number of substances and molecules, ranging from inorganic calcium phosphate phases up to peptides and proteins, has been investigated throughout recent decades. However, in vitro and in vivo results are heterogeneous. This may be at least partially attributed to the limits of the applied immobilization methods. Therefore, this paper highlights, in the first part, advantages and limits of the currently applied methods for the biological nano-functionalization of titanium-based biomaterial surfaces. The second part describes a new immobilization system recently developed in our groups. It uses 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 BAMs conjugated to the respective complementary NA strands.

Keywords: immobilization; implant; electrochemistry; bioactive molecules; nucleic acid; conjugate

1. INTRODUCTION

Titanium and its alloys have been extensively used as biomaterials in bone surgery throughout recent decades because of their generally good biocompatibility, which is mainly attributed to two facts. Firstly, the mechanical properties are better adapted to those of bone when compared with other metallic implant materials. Secondly, the surface is always covered by a passive layer with a thickness of a few nanometres, which is responsible for the material’s corrosion resistance and bioinert behaviour in vivo (Schenk 2001; Bozzini et al. 2008; Popa et al. 2008). Generally this behaviour results in a very good osseointegration of the material, especially for otherwise healthy patients. Nevertheless, early implant failure and problems during healing may occur for patient groups with certain risk factors such as smoking or systemic diseases such as diabetes, osteoporosis or chronic inflammation (Kamkin et al. 1996; Esposito et al. 1998; van Steenbergh et al. 2002, 2003). Furthermore, the increasing age of the population adds two more factors. Firstly, the number of patients with poor bone quality is constantly rising. Secondly, the increasing lifespan after primary surgery increases the probability for the exigency of revision (Johnsen et al. 2006). These present and upcoming challenges require an osseoconductive surface on the implant. Therefore, direct surface manipulation is a main field of interest in current biomaterials research.

The starting point of 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 (Kasemo & Lausmaa 1986; Kasemo & Gold 1999). Generally this behaviour results in a very good osseointegration of the material, especially for otherwise healthy patients. Nevertheless, early implant failure and problems during healing may occur for patient groups with certain risk factors such as smoking or systemic diseases such as diabetes, osteoporosis or chronic inflammation (Kamkin et al. 1996; Esposito et al. 1998; van Steenbergh et al. 2002, 2003). Furthermore, the increasing age of the population adds two more factors. Firstly, the number of patients with poor bone quality is constantly rising. Secondly, the increasing lifespan after primary surgery increases the probability for the exigency of revision (Johnsen et al. 2006). These present and upcoming challenges require an osseoconductive surface on the implant. Therefore, direct surface manipulation is a main field of interest in current biomaterials research.

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the surface is covered by a protein layer with conformational changes ranging from native to completely denatured (Sadana 1992; Ratner 2001). Cells will recognize this more or less denatured protein layer and will be influenced on their adhesion, proliferation, differentiation and active matrix remodelling behaviour.

Fast and tight osseointegration as well as excellent long-term stability are mandatory for permanent implants such as dental implants or endoprostheses. Therefore, surface modifications have to fulfill three main tasks: (i) to prevent unspecific adsorption of potentially denatured proteins at the surface; (ii) to attract cells of the native tissue or progenitor cells able to differentiate into the appropriate type; (iii) to provide biochemical signals to induce native healing mechanisms.

To achieve these goals, different attempts have been made to alter the abovementioned surface properties of titanium-based implant materials. Increasing surface roughness achieved by grit-blasting or titanium plasma spraying (Le Guehenec et al. 2007) results in an improved mechanical interlock of the implant owing to bone ingrowth into the cavities (Wennemerg et al. 1998; Ogawa & Nishimura 2003; Szmukler-Moucler et al. 2004; Schwartz et al. 2008). Surface chemistry and morphology can be altered by acid-etching (Pebé et al. 1997; Rupp et al. 2006), alkaline treatment (Krupa et al. 2005) or deposition of calcium phosphate phases (CPP) (Hayashi et al. 1994; Rößler et al. 2003; Feng et al. 2004; Gan et al. 2004; Borsari et al. 2009). However, there is a consensus in the research community that the biochemical properties of titanium surfaces have to be modified by immobilization of bioactive molecules (BAMs) such as peptides, proteins and others to deal with the abovementioned tasks (ii) and (iii) above, and may also influence the unspecific adsorption of plasma proteins (task (i)). This has successfully been targeted by coating the surface with hydrophilic polymers such as poly(ethylene glycol) (PEG; Dalsin et al. 2005; Schuler et al. 2006; Zoulalian et al. 2006; Wach et al. 2008), which also exhibits antibacterial properties (Maddikeri et al. 2008; Zhao et al. 2009).

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 this environment. In the case of bone this includes hydroxyapatite (HAP), which represents approximately 70 per cent of total bone mass, as well as the proteins of the extracellular matrix (ECM; approx. 20 mass-%). Collagen I is the main structural protein of the organic bone matrix. Together with fibronectin and other adhesion proteins it mediates cell–matrix interactions. Growth factors are important signalling molecules, triggering cell–cell and cell–matrix interactions (Schliephake 2002). Bone sialoprotein and osteopontin are involved in cell binding to mineralized bone (Sodek et al. 1992). And, finally, proteoglycans and their sugar components should be mentioned as compounds involved in interactions between collagen, growth factors and cells (Klinger et al. 1998).

The other 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 environment. Examples for such molecules are peptides such as the sequence arginine—glycine—aspartate (RGD) (Ruoslahti 2003), laminin sequences (Nomizu et al. 1995) or the collagen-derived P-15 peptide (Valentini & Weber 2004). Other molecules are peptidomimetics (Ahn et al. 2002) or aptamers (Guo et al. 2007a, b).

A comprehensive review of all possibly applicable bioactive substances would be far beyond the scope of this article and has been carried out in detail for certain aspects of that field by others (Waddington & Embery 2001; Lappalainen & Santavirta 2005; Ellingsen et al. 2006; Morra 2006; de Jonge et al. 2008; Kim et al. 2008; Schliephake & Scharnweber 2008; Junker et al. 2009; Raynor et al. 2009).

In the first part this paper we will draw attention to the currently used immobilization methods for the binding of BAMs to titanium-based biomaterials with their advantages and limitations. In the second part we newly developed modular immobilization system for BAMs is presented as an approach to overcome those limitations. This new method immobilizes at least partially single-stranded nucleic acids (NAs) into an anodic titanium oxide layer and uses their hybridization ability for loading the surface with BAMs, which are conjugated to the respective complementary NA strands.

2. IMMOBILIZATION METHODS

Generally immobilization methods for BAMs must be evaluated with respect to several properties that are, in part, contradictory. In all cases, immobilized BAMs must display their bioactive domain(s) 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 BAMs 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. RGD peptides) must be immobilized irreversibly; others (e.g. growth factors or antibiotics) have to be released in a specific concentration–time profile to be effective. Realizing a defined release behaviour of surface-bound molecules is probably the most critical issue in this field. In the past BAMs have been immobilized at titanium surfaces adsorptively, covalently, via electrochemical techniques, or using self-assembled layers. As will be shown below, all methods have their own advantages and limits with respect to their feasibility, their impact on the integrity and activity of the BAMs as well as on binding stability and release characteristics of the immobilized molecules.

2.1. Adsorption

Adsorption is the simplest immobilization method, as it may be carried out by just dipping the material into the appropriate solution. However, it is based on
comparatively weak interactions, comprising electrostatic and van der 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 surfaces the IEP can be expected to be at a pH value of approximately 4.3 according to Rößler et al. (2002). Therefore, titanium surfaces should be charged negatively under in vivo conditions. This has been used by Tosatti et al. (2003) 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 (Nagai et al. 2002; Kim et al. 2005; Teng et al. 2008) and proved to be stable against competitive adsorption of serum proteins in vitro (Roehlecke et al. 2001). Weak interaction forces may be compensated to a certain degree by increasing the number of interacting sites. For that reason Auernheimer et al. (2005) 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 HNO₃ followed by ultrasonic agitation in H₂O and ultrasonic detergent cleaning.

Binding stability of adsorbed species is controlled by environmental conditions (pH, ionic strength, protein concentration). If they change, adsorbed molecules may desorb in an uncontrolled way. Therefore, the results of in vivo experiments are heterogeneous. Wikesjö et al. (2008) tested bone morphogenetic protein 2 (BMP-2) adsorbed onto/into anodic, porous titanium oxide layers of a commercial dental implant surface (TiUnitet, Nobel Biocare AB, Göteborg, Sweden) in a defect model in dogs for up to 8 weeks and observed an increased local bone formation compared with implants without growth factor. Hunziker and co-workers conducted several studies involving coatings of titanium implants with CPP and/or BMP-2 (Liu et al. 2005, 2007a,b). They used a biomimetic process to co-precipitate CPP and BMP-2 and compared this type of coating with CPP coated and adsorbed BMP-2. CPP 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 or the other control groups. Unfortunately, they did not present data concerning the release kinetics of the growth factor but claimed that adsorbed BMP-2 was released more rapidly than incorporated BMP-2 (Liu et al. 2007a).

Similar results were obtained by Schliephake et al. (2009), who investigated a multi-component system on etched titanium implant surfaces, comprising adsorbed collagen I, chondroitin sulphate (ChS) and BMP-2, in a dog model. ChS was incorporated into the collagen fibrils during fibrillogenesis and is supposed to act as a binding molecule for the growth factor. BMP-2 was adsorbed on the collagen/ChS surfaces. In this study no enhancement of the peri-implant bone formation or bone to implant contact could be attributed to the growth factor coating. The authors 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 immobilization

Covalent attachment of BAMs to surfaces has the advantage of stable immobilization and is therefore widely used (Xiao et al. 1997; Morra et al. 2003; Zreiqat et al. 2003; Porté-Durrieu et al. 2004; Bagno et al. 2006). 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. This can be illustrated using the examples of silanization with 3-aminopropyltriethoxysilane (APTES), as was described by Xiao et al. (1997) for coupling a RGD sequence on Ti-coated glass, and by Martin et al. (2004) for attachment of chitosan as an antibacterial agent. A prerequisite for that technique is the existence of free surface hydroxy groups, which have to be generated by treatment with HNO₃ or piranha solution. APTES, dissolved in water or toluene, may than react with amino groups of the protein or peptide. Besides the laborious procedure, the strong bond to the surface noted as an advantage above may turn to a disadvantage because of its irreversible nature, which makes this technique unsuitable for molecules requiring controlled release.

2.3. Self-assembled monolayers

Self-assembly of monolayers is a principle often used for immobilization of molecular chains at surfaces. It is based on the interaction between anchor groups of the molecules and specific interaction sites on the surface. A well-known example is the immobilization of thiocarbonylthio-modified molecules on gold surfaces (Huang et al. 2001; Chaki & Vijayamohanan 2002), mostly used for sensor applications. The technique has been adapted for titanium-based biomaterials by pre-coating their surface with gold (Huang et al. 2003). 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 negative effects. Fortunately, there exist other possible anchor groups for binding organic molecules to titanium surfaces.

Among the molecules with high affinity towards metal oxides are phosphates and especially phosphonates. Therefore, they are predestined as anchor groups. A number of studies deal with the adsorption of phosphates or phosphonates alone. For alkyl phosphonates formation of self-assembled monolayers (SAMs)
was observed (Hähner et al. 2001; Hofer et al. 2001; Gnauck et al. 2007; Liu et al. 2008). Spori et al. (2007) found that alkyl phosphates with a chain length between 10 and 18 C atoms adsorbed at TiO₂ layers on silicon display a higher degree of ordering with longer chain length (above 15 C atoms). Furthermore, they suggested a strong bidentate binding mode, bridging between two Ti atoms, though they performed no stability tests with the adsorbed molecules.

Philippin et al. (2003) compared the formation of monolayers of alkyl phosphonic acid with that of alkyl trichlorosilanes, finding that the latter form better ordered monolayers according to electrochemical impedance spectroscopy results. Gao et al. (1996) investigated alkyl phosphonic acids bound to anatase, ZrO₂ or Al₂O₃ with solid-state nuclear magnetic resonance and found only weak interaction between Ti and P–O owing to not completely deprotonated OH-groups. Contrary to that, Viorney et al. (2002) interpreted their 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 (Zorn et al. 2005, 2007; Adden et al. 2006a,b; Clair et al. 2008). There are currently not many studies dealing with the stability of SAMs on titanium. Silverman et al. (2005) compared phosphate-anchored SAMs after heat treatment with siloxane-anchored molecules and found the former to be more stable against hydrolysis in water at pH 7.5 and exhibiting a 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 phosphonate groups and concluded that binding of

2.4. Electrochemical methods

Because of the nature of the titanium surface, both cathodic and anodic procedures can be used to immobilize BAMs and to modify their properties. Because of the principal differences in the underlying mechanisms, both processing routes are applicable for different tasks and will be treated separately. The current status in this field has been reviewed by Scharnweber et al. (2009).

2.4.1. Cathodic polarization.

During cathodic polarization, the pH value near the electrode increases owing to hydrogen evolution. This can be used to deposit CPP on titanium surfaces from supersaturated solutions because their solubility decreases with rising pH value (Shirkanzaadeh 1998; Rössler et al. 2003; Cheng et al. 2004). The structure of the deposited CPP encompasses amorphous CPP, brushit, octacalcium phosphate and HAP, depending on electrolyte composition, temperature and electrochemical parameters. Rössler et al. (2001) improved their near-physiological process to achieve mineralized collagen coatings on titanium surfaces. This process has further been adapted by Scharnweber et al. (2007) to co-precipitate chlorhexidine (CHD) as an antibacterial agent on TiAl6V4 surfaces using the pH-dependent solubility of CHD.

Furthermore, the process can be generally applied to all substances showing a pH-dependent solubility, as has been shown by Scharnweber and co-workers for chitosan coatings (Scharnweber et al. 2009).

2.4.2. Anodic polarization.

With anodic polarization at low potentials (+0.7 VAg/AgCl), conducting polymers may be deposited on metal substrates. De Giglio and co-workers used this method to coat titanium surfaces with polypryrole (PPY) films as an anchor for the coupling of RGD peptides, collagen and HAP (De Giglio et al. 1999, 2000, 2001).

At higher anodic potentials the thickness of the passive layer can be increased in a controlled manner to thicknesses ranging from a few up to more than 100 nm. During the 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 in our groups to develop a method to immobilize collagen I (Scharnweber et al. 2004) and a cyclic phosphonate-anchored RGD peptide (Beutner & Sewing 2007) and is the basis of the modular immobilization system presented below. For this reason the basic principles of the formation of anodic oxide layers on titanium will be shortly summarized, though they are well known and have already been reviewed on various occasions (Aladjem 1973; Schutz 1997; Kunze et al. 2005; Yao & Webster 2006).

The thin passive film on titanium surfaces consists mainly of a sub-stoichiometric oxide of the general formula \( \text{T}_i \text{O}_z \), exhibiting n-type semiconducting properties. Zhang et al. (2007) determined a band gap
of 3.3 eV for amorphous, sputtered TiO$_2$ films on glass substrates; that of the native passive film may be expected to be in the same range. Local oxygen point defects act as electron donors (Göpel et al. 1984; Diebold 2003; Kunze et al. 2005). Scharnweber et al. (2002) investigated the semiconductor properties of the three alloys commercially pure Ti, Ti6Al4V and Ti6Al7Nb in phosphate buffer at pH values between 4.4 and 9.2 and determined comparable donor densities for commercially pure Ti and Ti6Al4V with $(1.3 - 1.6) \times 10^{20} \text{ cm}^{-3}$ and $(1.2 - 1.9) \times 10^{20} \text{ cm}^{-3}$, respectively, but 50 per cent lower values for Ti6Al7Nb (7.8–9.8) $\times 10^{19} \text{ cm}^{-3}$.

Because of these properties, charge transfer during anodic polarization occurs in the first instance by migration of Ti$^{4+}$ 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 two-layered system. The total oxide layer thickness depends linearly on the applied potential, with a growth parameter of 1.4–2.3 nm V$^{-1}$ (Aladjem 1973; Ohtsuka et al. 1985; Khalil & Leach 1986; Lausmaa et al. 1988, 1990a,b; Shibata & Zhu 1995; Ohtsuka & Nomura 1997). 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 (Khalil & Leach 1986). For the low potentials $< 10 \text{ V}_{\text{SCE}}$ used here, both transfer numbers for Ti$^{4+}$ and O$^{2-}$ may be estimated as approximately 0.5. Thus approximately 50 per cent of the total oxide layer thickness may be available for incorporation of molecules.

Besides oxide formation, oxygen evolution has to be considered as a parallel reaction at the oxide/electrolyte interface owing to the already mentioned possibility for electron transfer processes. Thus, the possible reaction pathways at that interface can be summarized according to reactions (2.1)–(2.3). The dissociation of water generates oxygen ions, which are able to migrate through the oxide (reaction 2.1) or to react with molecular oxygen via intermediate oxygen radicals (reaction 2.3). Titanium ions approaching the interface are oxidized via the intermediates titanyl ions and hydroxylated titanium oxide according to reaction 2.2(a–c)

$$
\begin{align*}
3\text{H}_2\text{O} & \rightarrow \text{O}^{2-} + 2\text{H}_3\text{O}^+, \quad (2.1) \\
\text{Ti}^{4+} + 3\text{H}_2\text{O} & \rightarrow \text{TiO}_2^2+ + 2\text{H}_3\text{O}^+, \quad (2.2a) \\
\text{TiO}^{2+} + 4\text{H}_2\text{O} & \rightarrow \text{TiO(OH)}_2 + 2\text{H}_3\text{O}^+, \quad (2.2b) \\
\text{TiO(OH)}_2 & \rightarrow \text{TiO}_{2-} + \text{H}_2\text{O}, \quad (2.2c) \\
6\text{H}_2\text{O} & \rightarrow 2\text{O}^\bullet + 4\text{H}_3\text{O}^+ + 4e^- \rightarrow \text{O}_2. \quad (2.3)
\end{align*}
$$

A number of side effects may be caused by these reaction pathways in general, and particularly with regard to immobilization of BAMs.

(i) Generation of hydronium ions during reactions (2.1), (2.3) and (2.2a), (2.2b) results in a decrease in the pH value near the electrode. This may have a direct impact on the surface-bound molecules and their immobilization behaviour and should be compensated by an appropriate buffer capacity of the electrolyte.

(ii) 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 of the BAMs 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.

(iii) Generated oxygen radicals can cause direct damage to bound BAMs. 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. Generally, the extent of oxygen evolution is lowest for galvanostatic polarization at high current densities (Delplancke & Winand 1988; Blackwood & Peter 1989; Scharnweber et al. 2002). However, this recommendation may conflict with the pH drop owing to hydronium ion generation, which is higher for higher current densities.

3. SELF-ORGANIZATION OF NUCLEIC ACIDS AS A STRUCTURAL TOOL FOR SURFACE MODIFICATION

Self-organization is ubiquitous in nature, and life in itself is a huge self-organized system. Biological examples have always been the reference for investigation and creation of self-organized systems that have found rising interest in recent decades.

The appeal of self-organization is based on the potential to switch from a ‘top down’ process to a ‘bottom up’ 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, NAs are probably those with the highest potential for forming a large variety of structures. This assumption 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 (polyhedra, etc.) (Seeman 1982, 1999, 2003; Chworos et al. 2004; Park et al. 2006; Rothemund 2006; Andersen et al. 2008; He et al. 2008). Such defined structures can be used to bind other materials or molecules like gold nanoparticles (Le et al. 2004) or proteins in a defined regular pattern and to grow silver nanowires (Yan et al. 2003).

So-called DNA ‘nanomachines’ and ‘nanomotors’ often rely on switching from a quadruplex to a duplex structure and back (Alberti et al. 2006; Beissenhirtz & Willner 2006). Most of them are fuelled by added NAs. ‘Fuel 1’ hybridizes to the initial state of the nanomotor, thus inducing motion. The recovery of the initial state is then propelled by adding ‘fuel 2’ (Alberti & Mergny 2003; Choi et al. 2007). Such nanomotors can also be driven by other processes like metal ion complexation, as used by Fahlman et al. (2003) for quadruplexes stabilized by Sr$^{2+}$ and destabilized by EDTA. Thus, processes on surfaces modified with NAs could be controlled by external stimuli.

Nucleic acid self-organization is therefore seen as a powerful tool for surface structuring, controlling processes and drug delivery that should be applied to biosurface engineering of titanium implant materials.

4. INTRODUCING MODULARITY TO THE BIOMATERIAL/TISSUE INTERFACE

As discussed in the previous section, currently various methods exist for immobilization of BAMs on titanium-based biomaterials. Among them, covalent coupling results in stable immobilization at the expense of a complex procedure and higher hurdles for approval by the authorities owing to the involvement of several potentially toxic substances in the preparation process, e.g. irritant and reactive amino- or mercaptoalkyl alkoxy-silanes or linkers such as the reactive and carcinogenic glutardialdehyde. Furthermore, this method is irreversible, which renders it inapplicable for growth factors and other molecules which must be released to be effective. On the other hand, adsorption as the simplest coating method does not offer appropriate binding stability. Though release is favoured for some BAMs, 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 (e.g. fibrillar collagen) with sufficient stability is combined with other components (e.g. growth factors), which may be released. However, this approach may also not result in defined release behaviour as discussed in the aforementioned example of Schliephake et al. (2009). The method developed in our laboratories which uses anodic polarization to immobilize BAMs by their partial entrapment into the thickened oxide layer is promising, because the process is simple, can be carried out under near physiological conditions, and results in stably bound molecules comparable to covalent coupling. But again, no defined release behaviour can be achieved this way.

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

Our suggestion to overcome these drawbacks is to combine the electrochemical immobilization as a fundamental method with the huge possibilities offered by the self-organization potential of NAs. Therefore, we recently developed a modular immobilization system which is presented below and has been described and investigated in more detail elsewhere (Michael et al. 2007, 2009; Beutner et al. 2009).

This modular system can be considered as a flexible toolbox for surface bio-functionalization based on one universal immobilization technique that allows the immobilization of a higher number of different BAMs.

The principle of the immobilization system is depicted in figure 1. In a first step NA 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 (figure 1a). Interaction via the sugar–phosphate backbone or the bases is undesirable at this point, because this would lead to reduced hybridization efficiency in the last step. Adsorption is followed by anodic polarization, during which the adsorbed P-ASs are fixed by partial incorporation into the anodic oxide layer (figure 1b). Adsorption and fixation are considered as one step because they are carried out successively in the same electrolyte and vessel. In a second step the immobilized ASs are hybridized with complementary strands (CSs) conjugated to biologically active molecules, enabling a prearranged functionality (figure 1c,d). In some cases (e.g. RGD peptides, figure 1d) a stable fixation is intended, while other BAMs (e.g. antiphlogistics, antibiotics; figure 1f) have to be released. This can be controlled via the stability of the chosen NA sequences (see (iii) below). All NAs applied have to be checked for undesired biological activity prior to use by sequence comparison with known functional NAs, e.g. 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.

(i) It is a convenient and toxicologically harmless method for surface modification with ASs.

(ii) It allows for immobilization of different BAMs in one step using hybridization. The functionalization can be tailored to the specific needs of different indications if different mixtures of BAM conjugates with adjusted molar ratios are applied.

(iii) The release behaviour of the BAMs can be controlled in a wide range 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 (figure 1d).

(iv) The specific functionalization, i.e. the hybridization of mixtures of CS conjugates adapted to certain medical indications with the fixed ASs, may be carried out immediately prior to implantation, which enables a higher flexibility of the medical treatment.

It has been shown in Beutner et al. (2009) that ASs can be immobilized electrochemically by their partial incorporation into an anodic titanium oxide layer and are available for hybridization, if certain effects which can compromise the integrity of the strands are considered. Most importantly, the generation of oxygen radicals during anodic polarization according to reaction (2.3) has to be inhibited by choosing appropriate polarization conditions. Generated radicals must be rendered innocuous by the addition of antioxidative substances such as ethanol to the electrolyte in a sufficiently high concentration.

This is demonstrated exemplarily in figure 2 for electrolytes with or without ethanol. Surface densities are compared for immobilized ASs as well as hybridized CSs and NSs, where NS is a non-complementary control for surface hybridization with ASs. Surface densities of the strands were determined by labelling with $^{32}$P the 3' -terminus of a precursor strand using an $\alpha^{32}$P radioactive labelled mononucleotide and terminal transferase. Light grey rectangle, CS(3'-32P); unfilled rectangle, NS(3'-32P).

Figure 2. Surface density of $^{32}$P-labelled P-ASs, CSs and NSs for immobilization electrolytes (acetate buffer, pH 4.0) with and without ethanol after hybridization in PBS (pH 7.4). Black-filled rectangle, P-AS(5',32P): 5'-phosphorylation of the non-phosphorylated strand using T4-kinase and radioactive labelled $\gamma^{32}$P-ATP; dark grey rectangle, P-AS(3',32P); 3'-elongation of a precursor strand using an $\alpha^{32}$P radioactive labelled mononucleotide and terminal transferase. Light grey rectangle, CS(3'-32P); unfilled rectangle, NS(3'-32P).

Figure 1. Principle of the modular immobilization method. (a) Adsorption of single-stranded anchors or functional nucleic acids (e.g. aptamers) via their terminal phosphate groups on the native oxide layer of titanium materials. (b) Partial entrapment of the adsorbed nucleic acids in an anodically formed oxide layer. (c) Hybridization of nucleic acid conjugates of bioactive molecules to the fixed ‘anchor strands’ (ASs). (d) Unfolding a therapeutic effect by attracting cells or binding molecules from the environment via reversibly bound BAMs (peptides or aptamers) and triggering cellular reactions by the release of BAMs in a defined way. Reproduced with permission from Michael et al. (2009). Copyright 2009, ACS Publications.
although the location of rupture within the strand cannot be identified. From figure 2 it can be deduced that surface coverage with P-ASs (P-AS(5'0'-32P) and P-AS(3'0'-32P)) is generally higher if the immobilization electrolyte contains ethanol, although a certain amount of P-AS damage is indicated by the fact that the surface density of P-AS(3'0'-32P) is only 46 per cent of that of P-AS(5'0'-32P). This does not affect the hybridizability of the ASs, as the surface density of the hybridized strand CS(3'0'-32P) is comparable to that of the P-AS(5'0'-32P) label immobilized into the oxide layer, indicating that the fragmentation leading to the loss of the AS(3'0'-32P) label occurred close to the 3' terminus.

A hexapeptide with the sequence GRGDSP was chosen as the first BAM to be conjugated to NAs (Michael et al. 2009), and the functionality of the conjugates was verified. The essential preservation of conjugate hybridizability was tested by competitive hybridization (figure 3), where the signal reduction for CS* by approximately 50 per cent proves that hybridization of the conjugate and its precursor takes place (more detailed results are available in Michael et al. (2009)).

The ability of the conjugates to bind to osteoblast-like cells (SAOS-2) was demonstrated in a blocking assay. Incubation with the conjugates inhibited cell adhesion to polystyrene (PS) (figure 4), which is attributed to blocking the integrin adhesion receptors with the GRGDSP sequence. However, NA also contributed to decreased cell adhesion on PSs, possibly owing to non-specific binding.

On titanium surfaces with immobilized ASs and subsequently hybridized with CS_C6-GRGDSP, osteoblasts show increased binding compared with the anodic oxide layer without ASs and CS_C6 hybridized with immobilized ASs (figure 5). This enhanced binding highlights the specific interaction between integrins and GRGDSP of the surface hybridized conjugate.

5. SUMMARY AND OUTLOOK

Immobilization of ECM proteins and their peptide derivatives to generate bioactive behaviour of titanium-based materials in order to enhance osseointegration is the main field in the current biomaterials research. In vivo osseointegration and bone remodelling can be accelerated especially in the early healing phase by a number of molecules and substances. However, at later stages the initial advantage compared with uncoated surfaces is often lost, suggesting that there is still a lack of decisive stimuli. This may be attributed partly to the fact that there is at the moment no method available for the concurrent immobilization of multiple components that allows for defined release behaviour at the same time. Our approach to overcome current limits is a new, modular immobilization system for BAMs. It uses the nanomechanical fixation of single-stranded NAs into anodic titanium oxide layers and their hybridization ability for loading the surface with BAMs conjugated to CSs. The feasibility of self-organization based on hybridization of NA conjugates to anodically immobilized NAs has recently been established successfully using an RGD-peptide as a first BAM molecule.

Further development of the immobilization system requires on the one hand its adaptation to surface conditions of real implants. This means the use of rough surfaces, which are currently the gold standard for cement-free orthopaedic implants. Also, all major titanium-based implant materials should be tested. Until now commercially pure Ti (Beutner et al. 2009)
and the alloy Ti6Al7Nb (Michael et al. 2007) have been investigated, but Ti6Al4V is still widely used and the β- and near-β-Ti alloys, such as TiNb13Zr13 or TiNb30, are promising materials because of their low Young’s modulus. Such changes in surface properties may require adaptation of the immobilization procedure. Otherwise, other BAMs (e.g. growth factors) will be used for conjugation with CSs and conditions allowing defined release behaviour of the conjugates will be investigated.

Beyond hybridization of NA conjugates on biomaterial surfaces, self-organization of NAs still offers great opportunities with respect to our system. Two-dimensional structures in various patterns and shapes (Park et al. 2006; Rothemund 2006) could possibly be used for controlled surface patterning of implants. DNA dendrimers (Shchepinov et al. 2008) may offer new opportunities with respect to our system. Two-dimensional structures in various patterns and shapes (Andersen et al. 2008; He et al. 2008) could also be created using self-organized NA polyhedra (Andersen et al. 2008; He et al. 2008).

Besides the use of NAs as a tool they may offer further advantages, since NAs can be functional by themselves as is known from aptamers, ribozymes, deoxyribozymes, siRNA or miRNA. First results using an aptamer for the anodically supported immobilization on titanium, i.e. with respect to the modular immobilization system using a functional AS and to relinquish the BAM–CS conjugate, were promising (Guo et al. 2007b).

REFERENCES


Choi, S. W., Makita, N., Inoue, S., Lesoil, C., Yamayoshi, A., S102 Review. Nano-bio-functionalization of Ti


