Review

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Multifunctional biomaterial coatings: synthetic challenges and biological activity

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Abstract: A controlled interaction of materials with their surrounding biological environment is of great interest in many fields. Multifunctional coatings aim to provide simultaneous modulation of several biological signals. They can consist of various combinations of bioactive, and bioinert components as well as of reporter molecules to improve cell-material contacts, prevent infections or to analyze biochemical events on the surface. However, specific immobilization and particular assembly of various active molecules are challenging. Herein, an overview of multifunctional coatings for biomaterials is given, focusing on synthetic strategies and the biological benefits by displaying several motifs.

Keywords: biomaterials; click chemistry; coating; implant; multifunctional; surface chemistry.

Introduction

Materials that are designed to positively interact with a living biological system for a therapeutic or diagnostic purpose are defined as biomaterials (Williams, 2009). Their applications range from vascular grafts, orthopedic and dental implants to analytical devices. A common problem is the poor interaction of cells and the non-specific adsorption of proteins on the surface (Schwartz-Arad et al., 2008). These events can lead to the so-called foreign body reaction, which can eventually cause loosening of the implant, inflammation, coagulation or, in a secondary event, infections (Figure 1A) (Anderson et al., 2008; Trindade et al., 2016). To circumvent these issues, biomaterials are coated with components of the extracellular matrix (ECM) such as proteins, specific peptide sequences or glycosaminoglycans (GAGs) (Geissler et al., 2000; LeBaron and Athanasiou, 2000; Lee et al., 2011b; Salbach et al., 2012; Chlupac et al., 2014; Agarwal et al., 2015). However, to address additional biological cues or to increase specificity and activity, multifunctional coatings are required (Figure 1B). By immobilizing different cell adhesive motifs, or molecules that induce beneficial cellular effects, a diverse biofunctional surface is created that aims for the controlled and multisided interaction between the surface and the surrounding biological environment. Beside the attempts to mimic the highly complex ECM, bioinert modifications are also required. To prevent inflammation at the implanted site or coagulation at vascular grafts, non-specific protein adsorption has to be minimized. Furthermore, bacterial adhesion has to be prevented to ensure a successful healing of the implant. Cell- and protein-repellent backgrounds also play a major role in the analysis of several biological events and interaction of different biomolecules. In order to investigate these biological systems, label or reporter molecules are introduced into the coating, resulting likewise in a multifunctional surface modification. Importantly, distinct immobilization methods have to be used to ensure the balance of strong but reversible/degradable surface modification. Considering all these requirements, the benefits but also the challenges of designing multifunctional coatings become obvious. Herein, crucial aspects such as immobilization techniques and synthetic strategies are discussed to enable a specific and active assembly of multifunctional surface modifications. Moreover, an overview of applied coatings and their impact on the particular biological system is given (Figure 1D).

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Immobilization of functionalities

The immobilization of functional molecules on surfaces can be realized by various methods. These strategies differ in binding strength, modularity and complexity.

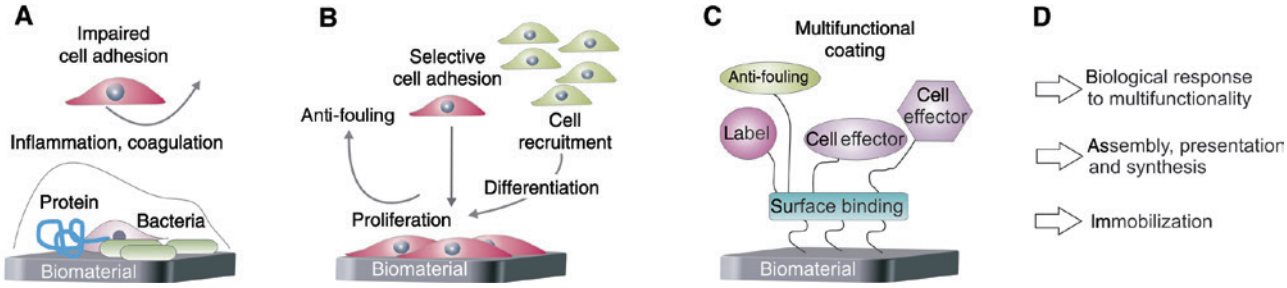


Figure 1: Basic principle of the design of a multifunctional biomaterial coating.
(A) Schematic representation of problems arising with implanted materials. (B) The aim of a biomaterial coating is the homing of selective cell types and subsequent integration of the implant in the surrounding tissue by triggering several cellular cues. (C) Multifunctional coating that consists of bioinert (antifouling or anti-microbial) and/or bioactive molecules and labels. (D) Crucial points that should be considered when building up a multifunctional coating.

Poor binding affinity can lead to inefficient immobilization or to loosening of bioactive molecules. Uncontrolled release of the cell adhesive motif RGD, for example, would be highly undesirable as the detached peptide can block integrin mediated adhesion and thereby provoke apoptosis (Mas-Moruno et al., 2010). Conversely, strong anchoring of biomolecules can hamper biological signals such as the recruitment of cells by chemokines. Hence, a feasible balance between controlled release and firm attachment is requested. The naturally occurring and simplest immobilization technique is adsorption. Proteins can be absorbed on surfaces by electrostatic, hydrophobic or hydrogen bond interactions. However, the adsorptive immobilization is rather weak, can be easily displaced by other molecules or can alter the activity of the proteins by conformational changes (Steinhagen et al., 2011). Moreover, proteins possess not only properties that beneficially affect cell function. Their non-specific adsorption can potentially lead to the previously described complications associated with the foreign body reaction (Anderson et al., 2008).

Electrostatic interactions are a commonly used method to anchor positively charged molecules to negatively charged metal surfaces for example (Figure 2). The affinity of electrostatic interactions can be tuned by raising the number of charged residues as in poly-lysine (Harris et al., 2004; Hassert and Beck-Sickinger, 2013). It has been shown that the affinity of a silicon-binding peptide can be strongly increased by repeating a specific amino acid sequence, which is rich in basic amino acids (Hassert et al., 2012). Other such cooperative effects could be coordinative and hydrogen bond interactions (Micksch et al., 2014). Surface binding peptides can be replaced by salts or proteins or degraded under physiological conditions, thus it is important to study the binding stability of the peptide (Micksch et al., 2014). The advantage of peptide-based approaches is the easy introduction of bioactive motifs to the anchor molecule (Khoo et al., 2010).

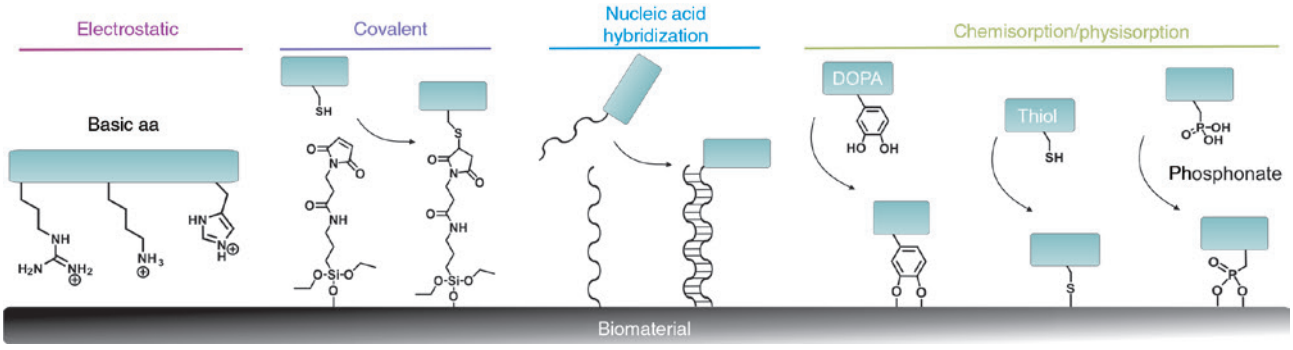


Figure 2: Strategies to immobilize bioactive functionalities.
Electrostatic interactions are mostly applied between a negatively charged surface such as TiO_2 and positively charged amino acids (aa). **Covalent** linkages can be achieved through chemical modification of the surface as silanization. The resulting functional groups (e.g. amino groups) can be further modified with biomolecules directly or via a crosslinker (maleimide). The **method of nucleic acid hybridization** consists of single-stranded nucleic acids strands, that are bound to the surface via phosphate groups grafted into an anodic TiO_2 layer. Complementary strands carrying the biomolecule are subsequently immobilized. **Chemisorptive** and **physisorptive** reactions of L-3,4-dihydroxyphenyalanine (DOPA), thiols or phosphonic acids to metals, metalloids or metaloxides can be used to directly modify surfaces.

The strongest and presumably most stable immobilization method is the covalent modification of biomaterial surfaces. The usual strategy is the introduction of functional groups by silanization (Figure 2). Hydroxyl groups on the surface are modified with an alkoxysilane such as (3-aminopropyl) triethoxysilane (APTES), which introduces amino groups. Subsequently, the surface can be modified directly or a crosslinker can be used to enable the immobilization of thiol-containing biomolecules, as shown in Figure 2 (Bauer et al., 2013; Godoy-Gallardo et al., 2014). This covalent strategy is, however, associated with downsides as complex and sensitive surface treatment and potential hydrolysis of the siloxane bond, which would result in a release of the bioactive moiety (Aissaoui et al., 2012).

A particularly modular method relies on self-organization of nucleic acids. This immobilization strategy consists of the adsorption of single nucleic acids strands by their terminal phosphate, followed by fixation in an anodic oxide layer (Beutner et al., 2010). The incubation with complementary strands that carry a biomolecule consequentially functionalizes the titanium or gold surface (Michael et al., 2009; Schliephake et al., 2015). Differences in hybrid stability can be exploited to release bioactive motifs such as growth factors (Schliephake et al., 2012).

Specific chemisorption can be used to immobilize bioactive molecules without surface treatment, which is an advantage compared to silanization or nucleic acid hybridization. A well-known example is the anchorage of thiols to gold surfaces upon H_2 release (Figure 2). This method is often applied for self-assembled monolayers modified with cell-attracting molecules (Vericat et al., 2010). A major advantage is the abundance of thiols in proteins or peptides and the easy introduction of cysteines in tailor-made biomolecules. Applications of thiol mediated modification of gold are predominately focused on analytical investigations (Huang et al., 2009; Lee et al., 2013a; Migliorini et al., 2014). Interestingly, thiols were used for the immobilization of multifunctional motifs to titanium-surfaces as well (Mas-Moruno et al., 2013; Mas-Moruno et al., 2014). However, the interaction is probably mediated by physisorption and not by chemisorption, resulting in less affinity. Another chemisorptive interaction is the immobilization of phosphonates on metal oxides. Biomolecules are easily modified with phosphonates and their direct immobilization is stable and strong (Pujari et al., 2014). This led to the design of several phosphonic acid containing biomaterial coatings that favor cell adhesion or create bioinert surfaces (Auernheimer et al., 2005; Adden et al., 2006; Zoulalian et al., 2010; Mas-Moruno et al., 2013).

The strong interaction of L-3,4-dihydroxyphenyalanine (DOPA) to metal surfaces has been applied for the coating of different biomaterials (Lee et al., 2011a). This post-translationally modified amino acid was found to be the pivotal moiety in mussel adhesive proteins that can stick to a variety of surfaces under wet conditions (Waite, 1983). The binding mode of DOPA has not yet been completely elucidated. Depending on the surface, DOPA can bind by coordinative or hydrophobic interactions or via hydrogen bonds (Li et al., 2014b). The binding to titanium is supposed to be coordinative, exceptionally strong, yet reversible (Lee et al., 2006). The anchorage of DOPA-modified molecules is highly stable. Incubation of coated titanium-plates in cell supernatant at 37°C displayed a remained surface coverage of around 80% over 7 days (Pagel et al., 2016). Other investigations demonstrated a half-life of 14 days on titanium in buffer and at room temperature (Tang et al., 2014). Furthermore, cell-repellent properties of DOPA- modified PEG-coatings were stable over 14 days, highlighting the strong anchorage under cell culture conditions (Dalsin et al., 2003). Two different approaches are mainly used to apply mussel adhesive properties in the field of biomaterials. On the one hand, modular peptides containing DOPA can be synthesized and easily modified by solid phase peptides synthesis (SPPS) (Ceylan et al., 2012; Ham et al., 2013; Tang et al., 2014). On the other hand, DOPA is incorporated into polymers or recombinantly expressed proteins (mussel foot proteins from the blue mussel (*Mytilus edulis)* by enzymatic hydroxylation of tyrosine residues (Hwang et al., 2007). Whereas in small artificial peptides DOPA is primarily responsible for adhesion to the surface, it is also relevant in proteins and polymers for crosslinking with itself or with amino groups to obtain a three-dimensional network (Yu et al., 1999; Burzio and Waite, 2000; Dalsin et al., 2003; Byun et al., 2014). Beyond that, different strategies to introduce functional moieties in DOPA-containing molecules are applied. Bioactive motifs can be fused to mussel adhesive proteins or integrated in polydopamine polymers by reactions of amino or thiol groups to the oxidized catechol unit (Hwang et al., 2007; Lee et al., 2007a). The resulting drawback is the difficult characterization of the product and the possible presence of unreacted toxic functionalities (Schweigert et al., 2001). Moreover, quinones result in a substantial loss of affinity to surfaces like titanium (Lee et al., 2006). Mussel derived peptides are easily modified with additional functionalities by SPPS or orthogonal click reactions, providing the possibility of exactly defining the chemical composition (Ceylan et al., 2012; Ham et al., 2013). The mussel adhesive approach was used to anchor cell-attracting as well as protein-repellent

effects (Gong et al., 2012; Wei et al., 2014; Kakinoki and Yamaoka, 2015).

Components of multifunctional biomaterial coatings

Beneficial influence of cellular fate is a crucial requirement for the successful implantation of biomaterials. Titanium implants, usually used for orthopedic and dental implants, are generally well accepted by the human body. Nevertheless, implant mobility, infection and eventually failure are severe issues that can result from non-specific protein adsorption, bacteria adhesion and poor osseointegration (Esposito et al., 1998). Problems arising in context of vascular grafts are protein and platelet adhesion that can lead to infections and occlusion as well as insufficient endothelialzation (Qi et al., 2013). Beyond that, directed and selective cell adhesion is required to elucidate various biological events (Figure 5) (Esch et al., 2015; Khalili and Ahmad, 2015). Hence, new strategies to gain selective cell adhesion, recruitment of progenitor cells and subsequent differentiation, are developed. Simultaneously, bioinert surfaces are designed to decrease non-specific protein and bacteria adhesion. The bioactive molecules that have been applied to solve the above-mentioned critical points are peptides, proteins and GAGs (Figure 3).

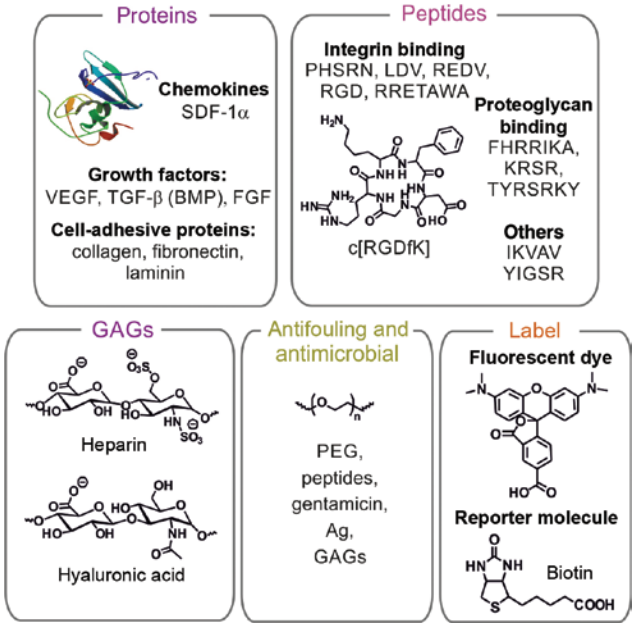


Figure 3: Components of multifunctional coatings.
Cellular function can be mediated by different proteins: VEGF (vascular endothelial growth factor), BMP (bone morphogenetic protein), TGF-β (transforming growth factor), FGF (fibroblast growth factor), SDF-1 (stromal cell-derived factor 1; PDB: 1A15), peptides (sequences are shown by the one letter code) or glyosaminoglycans (GAGs). To prevent infections or unspecific cell and protein adhesion, anti-fouling and anti-microbial agents are immobilized on surfaces (PEG, polyethylene glycol). Reporter molecules and labels introduced in biomaterial coatings are applied to detect several biological cues.

Collagen, vitronectin, fibronectin and laminin possess several beneficial domains such as integrin or proteoglycan binding sites and are therefore feasible mediators to attach cells to biomaterials and regulate signaling events (Siebers et al., 2005). The family of growth factors is widely applied to stimulate cell growth and differentiation of primary and progenitor cells to osteoblasts or endothelial cells (Park et al., 2010; Zieris et al., 2010). Other cellular events that favor implant healing and integration, such as increased collagen synthesis or enhanced cell adhesion and proliferation, were also induced by growth factors (Hempel et al., 2012; Shim et al., 2014). In particular, bone morphogenetic protein-2 (BMP-2), which belongs to the transforming growth factor β (TGF-β) superfamily, is applied in the field of dental and orthopedic implants because of its strong osteoinductive effect (Migliorini et al., 2016). In addition, TGF-β1 is known to regulate bone remodeling (Jansen et al., 2005; Tang et al., 2009). Vascular endothelial growth factors (VEGFs) stimulate the formation of new blood vessels and are used for vascular materials as well as for bone regeneration (Liu et al., 2014a; Yang et al., 2012). Proteins belonging to the group of fibroblast growth factors (FGFs) such as the basic FGF (bFGF or FGF-2) are known to be pro-angiogenic and increase the proliferation of cells (Sonmez and Castelnuovo, 2014). Chemokines trigger cell migration in the direction of their gradient to home specific cell types to the injured site or the biomaterial surface. This has been employed to recruit endothelial progenitor cells by a SDF-1-gradient, to support neovascularization at injured cardiovascular sites (Baumann et al., 2012). Another beneficial function of SDF-1 is the chemotaxis of mesenchymal stem cells and the stimulation of osteogenic differentiation (Kitaori et al., 2009; Eman et al., 2014). Beside the induction of cell migration, chemokines play a major role in the immune response of biomaterials, which is well summarized elsewhere (Franz et al., 2011). The critical point of the application of chemokines and growth factors is their gradual release from the coating. Even though proteins display various helpful functions to modulate cell fate on biomaterials, it is rather difficult to include them in a multifunctional coating. Modification of proteins with anti-fouling agents or tags implies challenging synthesis strategies and can alter the activity of the biomolecule. Moreover, ECM-proteins can, under certain circumstances, induce

unfavorable immune responses and undesired clotting in vascular grafts (Franz et al., 2011).

Peptides are helpful alternatives. These rather small molecules can be synthesized in a tailor-made way to realize directed immobilization and multifunctionality (Delaittre et al., 2012). Thereby, immobilization anchors such as thiols (Huang et al., 2009), DOPA (Tang et al., 2014; Pagel et al., 2016), phosphonic acids (Auernheimer et al., 2005), nucleic acids (Michael et al., 2009) and basic amino acids (Harris et al., 2004; Hassert et al., 2012) are easily introduced or ligated to bioactive peptides. Moreover, additional cell-attracting or repelling functionalities can be easily incorporated by specific reactions. Peptides can favor cell adhesion, proliferation, migration and differentiation by binding to receptors in the cell membrane (Figure 3). Their sequences are often derived from proteins occurring in the ECM such as fibronectin (RGD, PHSRN, LDV, REDV, KRSR) (Yamada, 2000), collagen (DGEA, GFOGER) (Lee et al., 2011b), bone sialoprotein (FHRRIKA, RGD) (Rezania and Healy, 1999), FGF-2 (TYRSRKY) (Lee et al., 2007b) and laminin (YIGSR, IKVAV) (Sreejalekshmi and Nair, 2011). The selective endothelial cell binding motif RRETAWA was interestingly not derived from an ECM-protein. This peptide was discovered by an $\alpha_5\beta_1$ integrin targeted phage display (Koivunen et al., 1994). The RGD motif is the peptide most often used to improve tissue integration of biomaterials and to investigate integrin-mediated cell adhesion on surfaces (Hersel et al., 2003). Since linear RGD peptides bind with moderate affinity to various integrins, it is important to design receptor-selective ligands. The cyclic c[RGDfK] binds with high affinity to the integrin $\alpha_v\beta_3$ and can be used in soluble form as chemotherapeutic and immobilized on a surface to mediate cell adhesion (Pfaff et al., 1994; Mas-Moruno et al., 2010). Thereby, pronounced selective and affine cell adhesion can be achieved in contrast to the application of linear sequences (Patel et al., 2012). Cyclic RGD peptides have been applied to mediate adhesion of endothelial cells and osteoblasts as well as for analytical studies (Mas-Moruno et al., 2013; McNichols et al., 2014; Pallarola et al., 2014). The peptides REDV and YIGSR were immobilized on vascular graft materials to improve endothelialization and simultaneously prevent smooth muscle cell hyperplasia (Ren et al., 2015). Increased spreading and proliferation of osteoblasts was gained by coating biomaterials with the peptides FHRRIKA and KRSR, which bind to proteoglycans in the cell membrane and thus mediate cell attachment (Rezania and Healy, 1999; Balasundaram and Webster, 2007; Sun et al., 2013). Whereas the sequence IKVAV is mainly applied to promote neural cell adhesion, the integrin binding peptide PHSRN is mostly used in combination with RGD to either study synergy towards $\alpha_5\beta_1$ or to improve cell attachment (Sreejalekshmi and Nair, 2011; Chen et al., 2013; Pulsipher et al., 2014).

Proteoglycans are a major component of the ECM and are characterized by their modification with GAGs. Negatively charged GAGs mediate and regulate various functions, mainly by electrostatic interactions with proteins in the ECM. Hence, they are a promising tool to increase the biocompatibility of biomaterials (Salbach et al., 2012). The structures of GAGs differ in their saccharide composition, glycosidic linkage and their chemical modification such as sulfation (Figure 3). It could be shown that the degree of sulfation exhibits a tremendous influence on osteogenic differentiation (Hempel et al., 2014; Salbach-Hirsch et al., 2014). Moreover, GAGs are able to store and release growth factors and chemokines and thus regulate their functions (Hintze et al., 2014; Zieris et al., 2014). The anti-coagulative property of heparin is especially useful for vascular materials (Begovac et al., 2003; Liu et al., 2014b). Beside bioactive functions, GAGs exhibit non-fouling properties and are therefore a natural alternative to PEG (Xu et al., 2011; Bauer et al., 2013). The introduction of GAGs to multifunctional coatings is mainly realized in a non-specific manner. In most of the approaches electrostatic interactions are used to graft GAGs on positively charged peptides or proteins as collagen (Wieduwild et al., 2013; Miron et al., 2014). Other strategies covalently link GAGs by their functionalities like carboxyl and amino groups (Kim et al., 2011; Lee et al., 2012; Ao et al., 2013). Anchored in the cell membrane, proteoglycans (e.g. syndecan) modulate cell attachment, formation of focal contacts and several other cellular events (Tumova et al., 2000; Woods and Couchman, 2001). Furthermore, it is described that cell membrane proteoglycans synergistically interact with integrins, which displays a promising target for multifunctional coatings (Morgan et al., 2007; Pagel et al., 2016).

Beside the above mentioned variety of cell-attracting motifs, cell-repelling molecules are incorporated into multifunctional biomaterial coatings. PEG is the most commonly used moiety to prevent undesired cell and protein adhesion. Although increasing PEG size correlates with enhanced bioinert properties, short ethylene glycol units can induce cell-repellent effects as well (Banerjee et al., 2011; Pagel et al., 2016). Surfaces have been coated with PEG by various immobilization methods, such as chemisorption, physisorption and covalent strategies which is well-reviewed elsewhere (Meyers and Grinstaff, 2012). In addition to the already described non-fouling properties of polysaccharides, peptides (mostly zwitterionic) are known to mitigate unspecific protein and bacteria adhesion (Cui et al., 2014; Maity et al., 2014; Yu et al., 2015). Beyond

Table 1: Biomaterial coating modified with reporter molecules for analytical investigations.

	Label	Bioactive motif	Surface	Immobilization	Investigation	Reference
a	Fluorescent dye	cRGD + FHRRIKA	Ti	DOPA	Immobilization, distribution of coating	Pagel et al. (2016)
b	Fluorescent dye	RGD	Glass	Silanization	Colocalization with FA	Hoesli et al. (2014)
c	2 fluorescent dyes	cRGD	Silica	Silanization/affinity	Integrin-RGD interaction	Jurchenko et al. (2014)
d	Fluorescent dye	klyrvraa, klhrlra	Si	Silanization	Immobilization	Vutti et al. (2015)
e	Biotin	RRETAWA	PS	Adsorption	Immobilization	Meyers et al. (2011)

Small one letter codes indicate the use of D-amino acids. PS, polystyrene; cRGD, cyclic RGD peptide; FA, focal adhesion.

that, specific peptide sequences can feature anti-microbial activity (Peyre et al., 2012; Krizsan et al., 2014). Classic and novel antibiotic reagents are mostly used through a release mechanism to circumvent infections of biomaterials (Fullenkamp et al., 2012; Lee et al., 2015).

To elucidate biochemical and physical aspects on biomaterial surfaces, label and reporter molecules are introduced into biomaterial coatings. A cRGD containing silicon-binding peptide has been fluorescently labeled to test the immobilization of the biofunctional construct (Hassert et al., 2012). The homogenous distributions of a titanium-binding peptide, which carries two cell-binding motifs, could be confirmed by a fluorescent label (Table 1, line a). Fluorescence microscopy pictures could additionally show a remained homogenous peptide layer in presence of adhered cells (Pagel et al., 2016). A similar assembly consists of a linear RGD peptide, which was modified with a fluorescent dye and immobilized by silanization on glass (Table 1, line b). With this tag it was possible to calculate bound peptide, to visualize generated micropatterns and to investigate their potential co-localization with focal contacts by fluorescence microscopy (Hoesli et al., 2014). Fluorescence resonance energy transfer (FRET) between two fluorescent dyes is used to evaluate distances between a donor and an acceptor chromophore and thereby investigate interactions of biomolecules. Jurchenko et al. used this technique to assess the interaction of an integrin with its RGD ligand (Jurchenko et al., 2014). Therefore, a highly complex molecule was synthesized that contains a biotin for affinity-based immobilization, the integrin ligand c[RGDfK], a PEG linker and one (respectively two) fluorescent dye (Table 1, line c). The results show, how the strong biotin-streptavidin affinity was ruptured due to the tight integrin-RGD interaction and thus emphasize the need of strong immobilization of bioactive molecules (Jurchenko et al., 2014). Recently, Vutti et al. applied a fluorescent dye that reacts with amino groups to control functionalization of the oxidized layer of silicon. Thereby, the surface modification with azide groups and the subsequent cell-adhesive-peptide immobilization was verified by the fluorescent dye (Table 1, line d) (Vutti et al., 2015). Biotinylation can be used to assess affinity and stability of immobilized molecules. This could be applied to study the anchor strength of the conjugate, listed in Table 1, line e., consisting of a polystyrene-binding peptide and the integrin-ligand RRETAWA (Khoo et al., 2010; Meyers et al., 2011).

Combining multifunctionality on a biomaterial surface

Designing a multifunctional coating is challenging and requires a sophisticated assembly or synthesis strategy. It is important that the methods, which are used to combine functional molecules, are critically evaluated to conclude

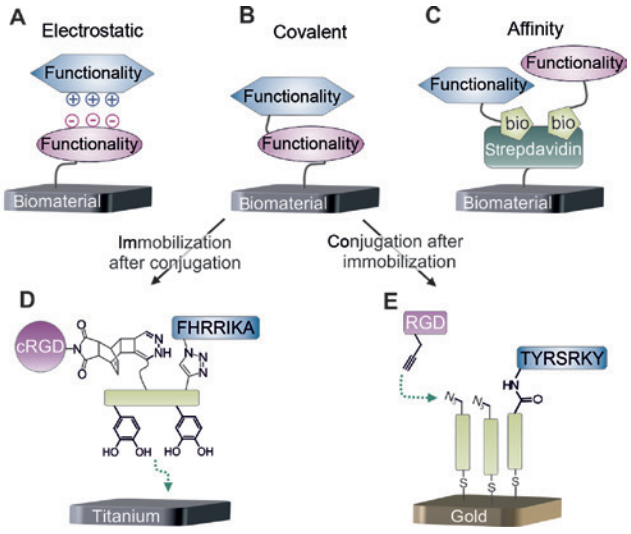


Figure 4: Different strategies for the assembly of multifunctional coatings.
(A) Electrostatic interactions which are often used in GAG/protein coatings (B) Covalent linkages are used to strongly connect multiple functionalities. (C) Affinity-based systems offer the possibility of a facile combination of different (e.g. biotinylated) molecules. (D) A covalently conjugated molecule that was analyzed, characterized and subsequently immobilized (Pagel et al., 2016). (E) Covalent modification directly on the surface to introduce different motifs (Hudalla and Murphy, 2010).

from biological results. A simple and commonly used method is the assembly of different modulators by electrostatic interactions (Figure 4A). This method can consist of single and multiple layers (layer by layer approach). An advantage is that the sequestered molecules, especially chemokines and growth factors, can be released over time. However, these mechanisms are hardly controllable, even though growth factor release can be customized, for instance by changing the sulfation degree of the sequestering GAGs (Zieris et al., 2014). A selective release strategy is, for instance, the enzymatic sensitive approach, which biochemically regulates the secretion of a chemokine (Steinhagen et al., 2014). Interestingly, Ao et al. could demonstrate that a layer-by-layer system can be stabilized by crosslinking the charged layers of collagen and hyaluronic acid through the generation of an amide bond (Ao et al., 2013). In addition, differently charged peptide helices, which can build a coiled coil, can be used for a directed presentation of molecules for several biochemical applications (Reinhardt et al., 2014; Lequoy et al., 2016). An advantage of peptide self-assembly strategies is the generation of highly modular fibrils that can additionally create a structural ECM-mimetic to support cell adhesion (Collier, 2008; Jung et al., 2011; Ceylan et al., 2012).

Functional molecules can be also combined by the high affinity between biotin and streptavidin (avidin) (Figure 4C) (Freitas et al., 2012; Gorbahn et al., 2012). The interaction is strong and specific, and biotin can be easily introduced into biomolecules. Furthermore, streptavidin can bind several biotinylated moieties, creating a highly versatile system. Thus, this method is especially suitable for analytical investigations as shown in Figure 5F (Gunawan et al., 2007; Migliorini et al., 2014). Another affinity-based approach is the already described strategy that employs the self-organization of nucleic acids. If differently modified complementary strands are used, a multifunctional coating can be easily obtained (Beutner et al., 2010; Zhang et al., 2013).

Covalent linkages between multiple functionalities are advantageous due to their high stability, which circumvents uncontrolled release of the modulators. There are two main principles of how a covalent linked coating can be generated. On the one hand, the multifunctional coating can be synthesized before the immobilization to the biomaterial surface (Figure 4D). This strategy facilitates the characterization and analysis of the multifunctional molecule prior to the coating and provides the possibility to store and sterilize the bioactive compound. This approach has been demonstrated by synthesizing a multifunctional peptide, carrying DOPA as surface anchor and two cell binding peptides before the coating step, which resulted in improved cell adhesion on titanium (Figure 4D) (Pagel et al., 2016). On the other hand, molecules, already attached to a surface, can be modified out to obtain a covalently linked multifunctional coating. The characterization of the coating is often rather difficult and relies mostly on indirect techniques. However, this approach often benefits from higher modularity. This strategy has been implemented by immobilizing two cell binding peptides on gold via covalent conjugation to self-assembled monolayers (Figure 4E) (Hudalla and Murphy, 2010). The characterization of the reaction outcome was carried out by infrared spectra. Peptide density on the biomaterial could be easily adjusted by varying the mole fraction of the modified thiols. The distance and distribution of bioactive motifs can be crucial for successful cell adhesion and in particular for additive and synergic effects (Huang et al., 2009). Furthermore, ECM-proteins, as fibronection or vitronectin, naturally display their different binding sequences in a defined spacing (Dalton et al., 1995). When the multifunctional coating is synthesized prior to immobilization the distance of the bioactive molecules can be controlled by a distinct spacer. If bioactive molecules are covalently coupled or anchored directly on the biomaterial, patterned surfaces can be exerted to investigate different distances of bioactive molecules. The cooperative effect of RGD and PHSRN was investigated using different approaches. Results from Mas-Moruno et al. (2014) underline the advantage of a multifunctional molecule presenting the bioactive motifs adjacent to each other. They immobilized the integrin binding motifs PHSRN and RGD in one molecule on titanium and could show beneficial influence on osteoblast-like cell behavior over a mixture of both peptides. Defined spatial distributions could be also realized by a patterned surface displaying PHSRN and RGD, which also resulted in a positively synergic effect on cell fate (Schenk et al., 2014).

Click chemistry for multifunctional coatings

Covalent linkages can be used to connect several moieties but also to immobilize bioactive molecules directly on the surface. Beside classical reactions such as amide and disulfide bond formation, bioorthogonal reactions gained more and more importance in the field of biomaterials (Nimmo and Shoichet, 2011). Click chemistry summarizes reactions that can be selectively performed in the presence of natural occurring functional groups (bioorthogonal), that result in high yields and proceed in

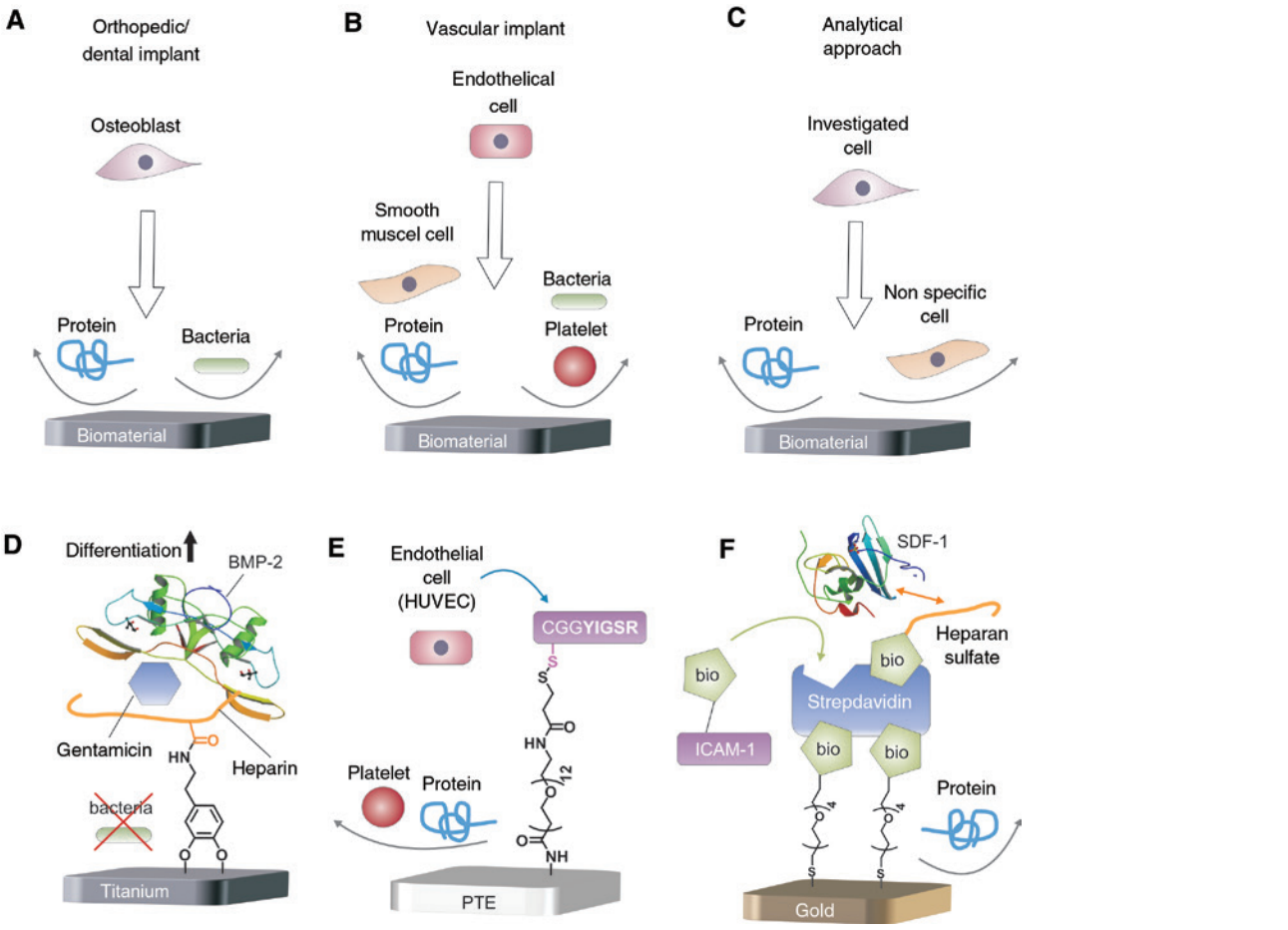
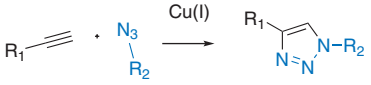
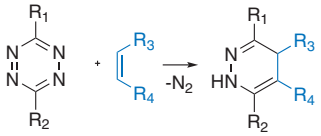
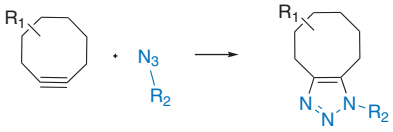
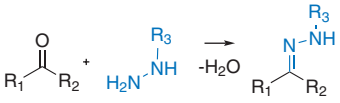
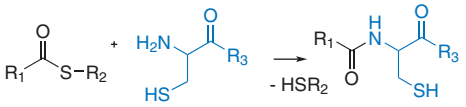
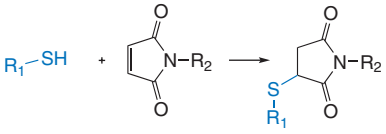
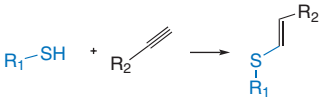


Figure 5: Comparison of three applications combining bioinert with cell-attracting properties (A–C) and corresponding examples of recent literature (D–F).
(A) To lower the inflammatory potential of orthopedic or dental implants unspecific cell, bacteria and protein adhesion has to be decreased while increasing the osseointegration of the biomaterial. (D) Titanium was coated with dopamine covalently coupled to heparin. The antibiotic gentamicin and the growth factor BMP-2 (PDB: 3BMP) were immobilized by electrostatic interaction. Premature osteoblasts showed higher differentiation while bacterial growth was decreased (Lee et al., 2012). (B) Vascular materials require protein, bacteria and platelet repulsion and the decrease of smooth muscle hyperplasia. The adhesion of endothelial cells is desired. (E) An approach using PEG covalently attached to PTE (polyethylene terephthalate) to decrease protein and platelet adhesion is shown. The selective homing of endothelial cells is mediated by the conjugation of the peptide sequence YIGSR (Noel et al., 2015). (C) To analyze cellular events triggered by immobilized effectors, a bioinert background has to be established. (F) This could be realized by the introduction of short PEG-units to a multifunctional coating. Modularity was achieved by the affinity-based immobilization of biotinylated biological active molecules as the chemokine SDF-1α (PDB: 1A15), the adhesion protein ICAM-1 (intercellular adhesion molecule-1) and heparan sulfate to streptavidin (Migliorini et al., 2014).

benign solvents such as water (Kolb et al., 2001). Thus, click chemistry is highly suitable to covalently ligate molecules for biomaterial coatings. Since these reactions are generally orthogonal to each other, they can be combined to obtain multifunctional constructs. However, side and cross reactions define whether the reactions are carried out simultaneously in one pot or stepwise (Table 2).

The best known click reaction, the CuAAC, can be combined stepwise with a classic amide coupling (Table 2, lines a and b). Hudalla and Murphy could thereby selectively immobilize an integrin and a heparin binding peptide on gold, coated with self-assembled monolayers (SAM) as shown in Figure 4E (Hudalla and Murphy, 2010). Whereas TYRSRKY was coupled via its N-terminus to carboxyl-functionalized SAMs, the alkyne modified RGD peptide was ligated to azido bearing SAMs. The same combination of reactions was used in the work by Vutti et al. (2015) (Table 1, line d and Table 2, line b). Cell adhesive peptides containing propargylglycine (alkyne functionality) were ligated to azido modified silicon wafers. The reaction outcome was monitored by the coupling of peptidic amino groups with an active ester modified fluorescent dye (Vutti et al., 2015). Due to orthogonality, the CuAAC can be easily combined with other click reactions

Table 2: Combinations of click reactions to introduce multifunctionality into biomaterials.

	Reaction 1	Reaction 2	Reference
a	CuAAC	Amide bond	Hudalla and Murphy (2010)
b	CuAAC	Amide bond	Vutti et al. (2015)
c	 CuAAC	 DAR_{inv}	Hassert et al. (2012); Pagel et al. (2016)
d	CuAAC	Native chemical ligation	Steinhagen et al. (2014)
e	 SPAAC	 Oxime ligation	Zheng et al. (2015)
f	 Native chemical ligation	 Maleimide-thiol Michael addition	Jurchenko et al. (2014)
g	CuAAC	 Thiol-yne	Sun et al. (2016)

CuAAC, copper(I) catalyzed azide alkyne cycoladdition; SPAAC, strain promoted azide alkyne cycloaddition; DAR_{inv}, Diels-Alder reaction with inverse electron demand.

as the Diels-Alder reaction with inverse electron demand (DAR_{inv}). The DAR_{inv} gained a lot of attention in recent years since its potential for ligating biomolecules was discovered (Knall and Slugovc, 2013). In contrast to the normal Diels-Alder reaction, the DAR_{inv} is not reversible, as N_2, which is the only by-product, is released. The modularity of the reaction has been shown by a combination with the CuAAC and the thiol-maleimide Michael addition to obtain multifunctional silicon- and titanium- coatings (Hassert et al., 2012; Pagel et al., 2016). Beside immobilizing or conjugating peptides, the CuAAC can be used to anchor proteins on biomaterial surfaces. The chemokine SDF-1 was modified by native chemical ligation with a peptide consisting of an alkyne group and a specific enzymatic cleavage site (Table 2, line d). By performing the CuAAC, SDF-1 could be immobilized and upon specific enzymatic cleavage, subsequently released (Steinhagen et al., 2014). A related study could demonstrate that the CuAAC and the native chemical ligation are orthogonal to each other and can thus be applied in a simultaneous one pot reaction (Steinhagen et al., 2011). Azides can undergo cycloadditions with strained alkynes without the need of a catalyst. This strain promoted azide alkyne cycloaddition (SPAAC) was discovered by the lab of C. Bertozzi and could be performed in combination with the oxime ligation to yield di-functionalized nanofibers (Agard et al., 2004). Two fluorescent dyes were thereby exploited to test both reactions in a one pot approach, followed by the nanofiber decoration with the bioactive peptide motifs RGD and YIGSR (Table 2, line e) (Zheng et al., 2015). In line c in Table 1, a complex biomaterial coating was described to analyze the RGD-integrin interaction. The applied multifunctional coating was build up by two click reactions: the native chemical ligation and maleimide-thiol Michael addition. Both reactions rely on the reaction of thiols and are therefore carried out in a stepwise manner (Table 2, line f). A thioester carrying RGD peptide was conjugated with a thiol bearing peptide that was modified by SPPS with a PEG linker, biotin and a fluorescent dye. Through the native chemical ligation, a free cysteine was created which then reacted in a Michael-addition with a maleimide functionalized fluorophore (Jurchenko et al., 2014).

Sun, Tai and colleagues combined the CuAAC with the maleimide Michael addition and thiol-yne click reaction, which was driven by UV-irradiation (Table 2, line g) (Sun et al., 2016). With the use of these orthogonal reactions cell binding (RGD) as well as cell-repellent (PEG), moieties could be immobilized on a polymer surface in a stepwise manner.

Biological response to multifunctionality

On the one hand, multifunctional biomaterial coatings aim to mimic the complex system of the ECM. On the other hand, anti-fouling as well as anti-microbial surfaces are applied to decrease the potential of infections and to ensure selective and improved cell adhesion. The balance between cell-repellent and cell-attracting properties is often difficult to adjust. In addition, the combination of multiple cell attracting molecules can result in cooperative effects but also diminishing interactions. In the next two paragraphs, *in vitro* (and a few *in vivo*) studies are shown, which apply several cell mediating motifs in a bioinert background and their biological outcome is shortly described.

Trigger cell function in a bioinert background

Protein and cell repelling properties are required in the entire field of biomaterials (Figure 5). Whereas orthopedic and dental implants primarily request protein and bacteria repulsion, vascular implants demand in addition the reduction of pathologic smooth muscle cell proliferation and growth, which can lead to vascular stenosis, hypertension or atherosclerosis (Amann et al., 1995; Qiu et al., 2014). Furthermore, analytical studies of cell-ECM or cell-biomaterial interactions can only be well interpreted if unspecific cell or protein intervention can be excluded.

The strategy applied most often is the combination of cell-attracting properties of the RGD peptide with the well-studied non-fouling properties of PEG. Groll et al. have demonstrated that cell adhesion of osteoblast-like cells (SaOS-2) could be controlled by regulating the concentration of the RGD (gRGDsc) peptide. In absence of RGD, PEGylated surfaces showed decreased protein and cell adhesion. Moreover, it was demonstrated that human mesenchymal stem cells could undergo osteogenic differentiation on RGD-PEG modified surfaces (Groll et al., 2005). Another approach uses the affinity of poly-lysine to several surfaces to graft PEG, which is modified with a linear RGD (GCRGYGRGDSPG) peptide via thiol addition to vinyl sulfones (Table 3, line a). Thereby, protein adhesion could be decreased and adhesion of fibroblasts increased, verified by a RDG-control peptide (VandeVondele et al., 2003). The introduction of additional cell effectors is applied to enhance cell adhesive properties or to introduce further cellular trigger. Schuler et al. tested the potential synergistic effect of the GAG binding peptides FHRRIKA and KRSR with a linear RGD- peptide in a bioinert background. The authors found an unexpected weak improvement of cell adhesion by combining these motifs. Cell adhesion was improved the most by a RGD/KRSR mixture (Table 3, line b) (Schuler et al., 2009).

The cooperative effect of RGD and FHRRIKA was also tested in previous work by our group (Figure 4D). The multifunctional coating was built up of three peptides. A DOPA-containing peptide was applied as surface anchor and could induce a cell-repellent effect by the incorporation of two short oligoethylene glycol (OEG) units. The covalent conjugation with the cell binding motifs to this anchor peptide improved cell adhesion and viability of osteoblast-like cells (SaOS-2) as well as of a precursor cell line (MG-63). The cooperative effect of both cell binding peptides was less pronounced on a mixture coating and could be verified by disturbing the interaction of FHRRIKA to membrane-bound proteoglycans. The promising interaction of RGD and KRSR was tested in a PEG-induced non-fouling background on titanium by poly-lysine anchoring (Table 3, line d). A mixture of both cell adhesive peptides increased the number of adherent cells (MG-63), but simultaneously decreased the alkaline phosphatase activity and osteocalcin level; both marker for osteogenic differentiation (Bell et al., 2011). To study the described synergic behavior of RGD and PHSRN, both integrin ligands were immobilized in a distinct spatial distance (line e in Table 3). While employing a bioinert background by PEG chains, cell-attracting cRGD was immobilized on gold nanoparticles and PHSRN was coupled by CuAAC to the silanized glass surface. The defined distribution of these peptides had a pronounced impact on cell number, area and the generation of focal contacts (Schenk et al., 2014). In Figure 5E it is schematically shown how the requirements of cardiovascular biomaterials can be implemented in an experimental setup. Noel and coworkers functionalized polyethylene terephthalate (PET) surfaces with PEG and modified them in different concentrations with either RGD, REDV or YIGSR via disulfide-formation (Table 3, line f). Bioinert PEG-surfaces showed an inhibition of

Table 3: Combinations of bioinert and bioactive motifs in biomaterial coatings.

	Bioinert	Bioactive	Surface	Immo-bilization	Assembly	Biological outcome	Reference
a	PEG	RGD	Nb_2O_5 PS	Electrostat.	Covalent	Protein ad.↓ Cell adhesion ↑	VandeVondele et al. (2003)
b	PEG	RGD + FHRRIKA or + KRSR	Ti	Electrostat.	Covalent	Cell adhesion↑	Schuler et al. (2009)
c	OEG	cRGD + FHRRIKA	Ti	DOPA	Covalent	Cell adhesion viability and proliferation↑	Pagel et al. (2016)
d	PEG	RGD + KRSR	Ti	Electrostat.	Covalent	Cell attachment↑ Cell different.↓	Bell et al. (2011)
e	PEG	cRGD + PHSRN	Au/glass	Thiol/silanization	Covalent	Protein ad.↓ Cell adhesion↑	Schenk et al. (2014)
f	PEG	RGD or YIGSR or REDV	PET	Covalent	Covalent	Protein ad.↓ Cell adhesion↑	Noel et al. (2015)
g	PEG	RGD + TYRSRKY	Au	Thiol	Covalent	Cell adhesion↑	Hudalla and Murphy (2010)
h	PEG	cRGD + cLDV	TiO_2	DOPA	Affinity	Cell adhesion↑	Gunawan et al. (2007)
i	PEG	Anti-CD34 antibody	Ti	Silanization	Covalent	Platelet adhesion↓ Cell spreading↓ Cell proliferation↓	Chen et al. (2012)
j	PEG	SDF-1 + heparan sulfate + ICAM-1	Au	Thiol	Affinity	Cell attachment↑	Migliorini et al. (2014)
k	$(EK)_3E$	RGD	Au	Thiol	Covalent	Protein ad.↓ Cell adhesion↑	Nowinski et al. (2012)
l	Ag	Hydroxyapatite + chitosan + heparin + BMP-2	Ti	Electro- deposition	Electrostat.	Bacterial growth↓ Cell different.↑ Bone formation↑	Xie et al. (2014)
m	Gentamicin	Heparin + BMP-2	Ti	DOPA	Electrostat.	Bacterial growth↓ Cell different.↑	Lee et al. (2012)
n	CS	VEGF + EGF	PS	Covalent	Covalent/ electrostat.	Cell viability↑	Lequoy et al. (2016)

PEG, polyethylene glycol; OEG, oligoethylene glycol; PS, polystyrene; SDF-1, stromal cell-derived factor; ICAM-1, intercellular adhesion molecule 1; BMP-2, bone morphogenetic protein; VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; PET, polyethylene terephthalate; ad., adsorption; electrostat., electrostatic; CS, chondroitin sulfate; different., differentiation; + indicates the combination of certain molecules, ↓= decrease, ↑= increase.

human umbilical vein endothelial cells (HUVEC), platelet and protein adsorption. Introduction of RGD and YIGSR resulted in an increase of endothelial cell attachment but to a certain degree also in platelet adhesion (to greater extend on RGD). The integrin ligand REDV had no effect on HUVEC or platelet adhesion (Noel et al., 2015). As previously described, Hudalla and Murphy applied the CuAAC to present a mixture of RGD and the heparin-binding sequence TYRSRKY on gold (Figure 4E). When both peptides were attached in a distinct ratio, adhesion of human mesenchymal stem cells in a cell-repelling environment was favored (Table 3, line g) (Hudalla and Murphy, 2010). A non-covalent, affinity-based system was used to immobilize cRGD and cLDV on DOPA-modified PEG (Table 3, line h). Since avidin is able to bind several biotinylated peptides, both integrin- ligands could be displayed in a specific ratio, yielding a beneficial synergistic cell response (Gunawan et al., 2007).

In addition to peptides, PEG functionalized surfaces were coated with proteins such as the anti-CD34 antibody to recruit endothelial progenitor cells from the blood stream (Table 3, line i). The antibody was coupled to PEG via amide bond formation and thus favored adhesion of endothelial progenitor cells on the surface. However, the PEG-induced anti-fouling effect also disturbed spreading and proliferation of these cells, which indicates the complicated adjustment of the balance between cell-attracting and -repellent effects. Smooth muscle cells and platelets were successfully not attracted by the coated surface (Chen et al., 2012). In Figure 5F it is schematically shown how the interaction between ECM-molecules bound to streptavidin can be studied in a bioinert background. Biotinylated heparan sulfate could be used to sequester SDF-1 to study cell adhesion of T-lymphocytes on this complex. Additional presentation of the adhesion molecule ICAM-1 could synergistically improve the heparin sulfate/SDF-1-meditated cell attachment (Migliorini et al., 2014). Apart from PEG, specific peptide sequences can also feature anti-fouling properties. Nowinski et al. coupled the non-fouling peptide EKEKEKE to a cysteine containing linker peptide (poly-proline) for chemisorptive immobilization on gold (Table 3, line k). This construct led to a reduced protein adsorption, remarkably depending on the applied spacer. Modification of the anti-fouling peptide with RGD resulted in improved fibroblast adhesion (Nowinski et al., 2012). The antibacterial properties of silver could be utilized in combination with a complex assembly of hydroxyapatite, chitosan, heparin and BMP-2 (Table 3, line l). Thereby silver was engrafted to chitosan via electrostatic interactions and immobilized with hydroxyapatite by electrodeposition. A heparin/BMP-2 complex was then adsorbed electrostatically on this coating. Sustained release of silver and BMP-2 mediated antibacterial properties as well as osteogenic differentiation of bone marrow stem cells. These *in vitro* results could be emphasized by *in vivo* studies showing favored bone formation by coating titanium with the described multifunctional construct (Xie et al., 2014). A BMP-2/heparin complex was also used in the study summarized in Table 3, line m. Notably, Lee and co-workers immobilized heparin via the strong DOPA-titanium interaction (Figure 5A). Additionally, the antibiotic gentamicin was adsorbed to the BMP-2/heparin complex. Thereby the authors could achieve the induction of osteogenic differentiation (of MG-63 cells) by excretion of BMP-2 and a lowered bacterial growth by the release of gentamicin (Lee et al., 2012). Lowered protein adsorption and platelet adhesion can be mediated by GAGs such as chondroitin sulfate (Thalla

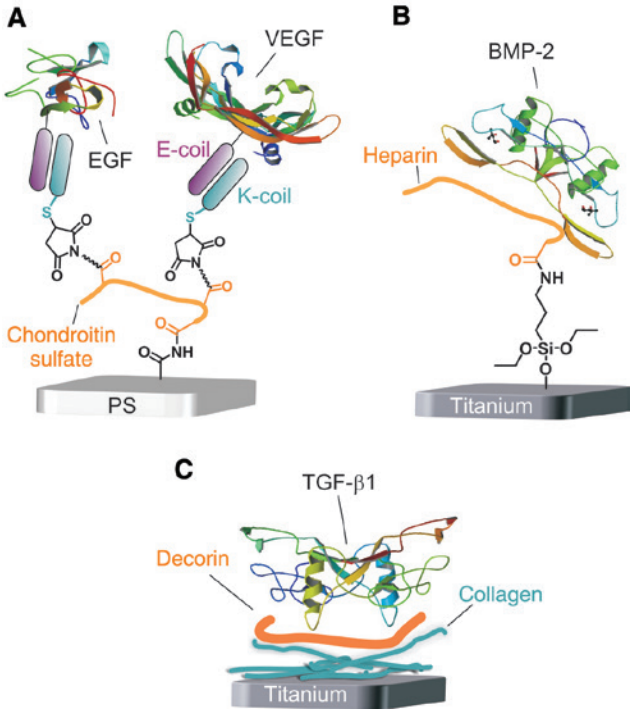


Figure 6: The assembly of proteins and GAGs/proteoglycans. (A) The strong interaction between a K-coil and an E-coil is used to immobilize VEGF (PDB: 1VPF) and epidermal growth factor (EGF, pdb 1JL9) on covalently immobilized chondroitin sulfate (Lequoy et al., 2016). (B) Heparin was immobilized covalently by silanization. This enabled the accumulation and simultaneously the gradual release of the growth factor BMP-2 (PDB: 3BMP) to promote osteogenic differentiation (Kim et al., 2011). (C) Collagen fibers were adsorbed on titanium and grafted with the proteoglycan decorin. Through negatively charged GAG chains of decorin, TGF-β1 (PDB: 1KLC) could be immobilized to increase the bone-implant contact (Stadlinger et al., 2008).

Table 4: Display of various cell effecting molecules to improve cell-surface interactions.

	Bioactive	Surface	Immobilization	Assembly	Favored cellular functions	Reference
a	RGD + FHRRIKA	Si	Silanization	Covalent	Spreading, mineralization	Rezania and Healy (1999)
b	RGD + PHSRN	Ti	Thiol	Covalent	Spreading, proliferation	Mas-Moruno et al. (2014)
c	RGD + BMP-2 + hydroxyapatite	Ti	DOPA	Covalent/electrostat.	Adhesion, mineralization	Chien and Tsai (2013)
d	RGD + bFGF	Si	Spin coating/thermal annealing	Covalent/electrostat.	Spreading, focal adhesion	Kolodziej et al. (2012)
e	OGP + fibronectin	Ti	Adsorption/co-precipitation	Electrostat.	Adhesion, proliferation, differentiation	Chen et al. (2015)
f	Heparin + laminin + bFGF	PLLA	Covalent	Covalent/electrostat.	Neurite outgrowth	Patel et al. (2007)
g	Heparin + BMP-2	Ti	Silanization	Covalent/electrostat.	Anti-inflammatory, proliferation, mineralization	Kim et al. (2011)
h	Heparin + VEGF + fibronectin	Ti	Electrostat.	Layer by layer electrostat.	Anti-coagulative, adhesion, proliferation	Wang et al. (2013)
i	Heparin + SDF-1	PGS	Electrostat.	Electrostat.	Progenitor cell recruitment	Lee et al. (2013b)
j	Chitosan + BMP-2	Ti	Covalent	Electrostat.	Differentiation	Han et al. (2014)
k	Hyaluronic acid + collagen	Ti	Silanization	Layer by layer covalent	Adhesion, proliferation, differentiation	Ao et al. (2013)
l	Hyaluronic acid + collagen	Ti	Electrostat.	Electrostat.	Non-pathological smooth muscle cell phenotype	Li et al. (2014a)
m	Collagen + lactoferrin	PLGA	Electrostat.	Electrostat.	Adhesion, proliferation, differentiation	Vandrovcova et al. (2015)
n	Collagen + CS + BMP-4	Ti	Electrostat.	Electrostat.	Increase of bone-implant contact	Stadlinger et al. (2008)

BMP-2, bone morphogenetic protein; bFGF, basic fibroblast growth factor; OGP, osteogenic growth peptide; VEGF, vascular endothelial growth factor; SDF-1, stromal cell derived factor; TGF, transforming growth factor; PLLA, poly(L-lactide); PGS, poly(glycerol sebacate); PLGA, poly(lactic-co-glycolic acid); CS, chondroitin sulfate; + indicates the combination of certain molecules.

et al., 2014). Lequoy et al. covalently linked a positively charged K-coil to immobilized chondroitin sulfate. The strong electrostatic interaction to a negatively charged E-coil permits the modular tethering of two growth factors (Figure 6A; Table 3, line n). Thereby, survival of HUVEC and human aortic smooth muscle cells (AoSMC) could be synergistically increased (Lequoy et al., 2016).

Multifunctional coatings to simultaneously modulate diverse cell functions

In the following section the cellular response to a combination of various bioactive molecules is described, without the use of non-fouling or anti-microbial agents. The interaction of potential synergistic motifs to RGD was tested with several systems. A strong cooperative effect on spreading and mineralization of rat calvaria osteoblast-like cells was found by combining RGD with FHRRIKA (Table 4, line a) (Rezania and Healy, 1999). Similar observations could be obtained by displaying RGD and PHSRN in a defined distance on titanium (Table 4, line b) (Mas-Moruno et al., 2014). The absence of PEG in multifunctional coatings enables the efficient adsorption of proteins by, for instance, electrostatic interactions, and thereby the facile combination with further bioactive molecules. The growth factor BMP-2 was attached to titanium in an one-pot approach with the RGD-peptide and hydroxyapatite, which resulted in pronounced adhesion and mineralization of human bone marrow stem cells (Table 4, line c) (Chien and Tsai, 2013). A mixture of RGD and basic fibroblast growth factor (bFGF) synergistically improved cell spreading and promoted the generation of focal contacts of endothelial cells (HUVEC) (Table 4, line d) (Kolodziej et al., 2012). Another peptide/protein combination is presented in Table 4, line e. The osteogenic growth peptide (OGP) and fibronectin were either adsorbed or co-precipitated on functionalized titanium, leading to a diverse release pattern. A dual presentation of these biomolecules increased adhesion, proliferation and osteogenic differentiation of rat mesenchymal stem cells (Chen et al., 2015). The storage of proteins using GAGs is widely used and a promising approach to display these molecules in a biomimetic manner. Laminin and bFGF were grafted on heparin functionalized nanofibers and synergistically increased neurite outgrowth (Table 4, line f) (Patel et al., 2007). Figure 6B shows how Kim et al. covalently immobilized heparin on titanium using silanization (Table 4, line g). Anti-inflammatory effects were conveyed by heparin since mRNA levels of TNF-α and Il-6 were down- regulated on coated titanium, compared to controls. The electrostatic

adsorption and subsequent slow release of BMP-2 promoted proliferation and mineralization of premature osteoblasts (MG-63) (Kim et al., 2011). To improve blood compatibility and endothelialization of vascular materials as titanium, a layer-by-layer system was built up consisting of heparin, VEGF and fibronectin (Table 4, line h). Diminished platelet activation and aggregation as well as improved cell attachment and proliferation were obtained by the resulting multifunctional coating (Wang et al., 2013). The strong interaction of heparin and SDF-1 was also applied for the coating of cardiovascular devices. Yu et al. (2012) and Lee et al. (2013b) described the increased stability of the chemokine SDF-1 when present in a coacervate with heparin. Recruitment of endothelial progenitor cells was enhanced through the cumulative release of SDF-1. In addition, Yu et al. (2012) showed increased migration and differentiation of smooth muscle progenitor cells.

The growth factor BMP-2 can bind to several polysaccharides (Tables 3 and 4). Through silanization, chitosan was covalently bound to titanium and subsequently BMP-2 was sequestered (Table 4, line j). The protein was steadily released and thus stimulated differentiation of bone marrow stem cells. *In vivo* experiments with a rabbit femur implantation could demonstrate osteoinductive properties of the chitosan/BMP-2 coating (Han et al., 2014). In Table 4, lines k and l, two different approaches are shown to coat titanium with hyaluronic acid and collagen. Ao et al. stabilized their layer-by-layer assembly of hyaluronic acid and collagen by covalent crosslinking to create a novel titanium-coating for orthopedic and dental implants. This strategy resulted in an improved response (attachment, spreading, proliferation and differentiation) of human mesenchymal stem cells compared to an adsorbed complex of GAG and collagen (Ao et al., 2013). Conversely, Li et al. (2014a) describe how a hyaluronic acid/collagen complex can be applied for coating of vascular materials. The therein demonstrated investigation revealed a favoring effect towards the non-pathological phenotype of smooth muscle cells. The glycoprotein lactoferrin presents a novel motif in the field of bone regeneration and has been described to enhance osteoblast growth and differentiation of mesenchymal stem cells (Naot et al., 2005; Montesi et al., 2015; Vandrovcova et al., 2015). Table 4, line m summarizes how lactoferrin can be grafted to collagen fibrils and thus favors adhesion, formation of focal contacts, proliferation and differentitation of osteoblast-like cells (SaOS-2) (Vandrovcova et al., 2015). In addition to GAGs, proteoglycans can be used to immobilize signaling molecules as growth factors (Table 4, line n). Whereas BMP-4 was immobilized on collagen/chondoritin sulfate coatings, TGF-β was assembled on collagen fibers containing the proteoglycan decorin (Figure 6C). Moreover, both complexes were combined in a multifunctional coating. *In vivo* experiments with functionalized bone implants exhibited a combination of collagen/chondroitin sulfate and BMP-4 as the most promising titanium modification since the bone-implant contact was enhanced the most on this coating (Stadlinger et al., 2008).

Conclusion

Multifunctional biomaterial coatings are a versatile tool to improve and study cell-material interactions. In recent years various approaches have been developed and applied to mimic the ECM and to prevent protein adsorption and bacterial growth by anti-fouling and anti-microbial agents. The success of multifunctional coating is not only dependent on the rational selection of active components. It is rather important to carefully analyze the entire set-up of a multifunctional coating to draw final conclusions for further investigations and the development of novel strategies. Different immobilization methods as well as the material itself can have a major impact on the biological outcome. Furthermore, the combination of bioactive motifs, the density and distribution of the functionalities as well as their assembly represent crucial factors that can influence cellular fate. Therefore, it is not surprising that approaches, which consist of the same combination of bioactive molecules, but apply different set-ups, have led to diverse results. Moreover, complex systems may also impede the individual functionalities of the building blocks by steric hindrance or an inadequately balanced equilibrium of bioinert and bioactive properties. The herein-described studies emphasize the complex and often difficult synthesis, analysis and last but not least discussion of multifunctional coatings. Nevertheless, they also demonstrate that the concept of multifunctionality is a promising approach, which may lead to individually adapted biomaterials and help to overcome disadvantages of monofunctionalized materials.

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