Review

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Improvement of wound healing by the development of ECM-inspired biomaterial coatings and controlled protein release

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Abstract: Implant design has evolved from biochemically inert substrates, minimizing cell and protein interaction, towards sophisticated bioactive substrates, modulating the host response and supporting the regeneration of the injured tissue. Important aspects to consider are the control of cell adhesion, the discrimination of bacteria and non-local cells from the desired tissue cell type, and the stimulation of implant integration and wound healing. Here, the extracellular matrix acts as a role model providing us with inspiration for sophisticated designs. Within this scope, small bioactive peptides have proven to be miscellaneously deployable for the mediation of surface, cell and matrix interactions. Combinations of adhesion ligands, proteoglycans, and modulatory proteins should guide multiple aspects of the regeneration process and cooperativity between the different extracellular matrix components, which bears the chance to maximize the therapeutic efficiency and simultaneously lower the doses. Hence, efforts to include multiple of these factors in biomaterial design are well worth. In the following, multifunctional implant coatings based on bioactive peptides are reviewed and concepts to implement strong surface anchoring for stable cell adhesion and a dynamic delivery of modulator proteins are discussed.

Introduction

The long history of implantology dates back to ancient Mayans who replaced missing teeth with pieces of shell already around 600 AD (Abraham 2014). This arising field of biomaterials science has undergone substantial development, especially within the past 50 years. Presently, implants have to fulfill many more criteria besides mimicking the mechanical properties of the replaced tissue, lack of toxicity, and inertness towards the host response. Ageing of populations in developed countries and the demand of patients to maintain the same level of activity and livability increased the use of implants dramatically over the past years. Consequently, the requirement for high-performance biomaterials that can address unique challenges in cardiology, vascular therapy, orthopedics, trauma, dental, and wound care has also been increasing steadily. Biomaterials have the dual potential of treating disease and supporting healthy cells. Second generation materials were developed either to display bioactive components, eliciting a distinct action and physiological response, or to undergo breakdown and resorption, ultimately replacing the foreign body by regenerated tissue. In a combination of both approaches, third generation materials aim to help the body heal itself (Hench and Polak 2002). The circumvention of a foreign body reaction along with the integration into the tissue are crucial parameters constituting implant success. Herein, the bioactive cues displayed on the implant surface significantly influence the host response. Extracellular matrix (ECM)-mimetic coatings provide a sophisticated approach to direct cell adhesion, recruitment, and proliferation, ultimately leading to wound healing and the regain of tissue function. This review summarizes methods to produce multifunctional implant coatings, which combine bioactive peptide sequences

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with strong surface anchoring, leading to stable cell adhesion. Furthermore, affinity-based and non-affinity-based protein delivery systems are discussed, which will mediate desired regenerative stimuli from various implant material platforms. Current proceedings in this field raise hope for the translation of multifunctional peptide coatings and immobilized wound healing-related proteins to commercially available devices, providing a perspective for clinical applications.

Guiding the physiological response to foreign bodies

The implantation of a biomaterial into the body includes injury and blood-material interactions, leading to the adsorption of plasma proteins within seconds. Preferably, the first inevitable hemostasis and inflammation reaction is followed by progression to anti-inflammatory response and constructive tissue remodeling leading to the regain of tissue function. However, all involved cells interact with the provisional protein matrix rather than the foreign body surface, its composition is assumed to be of major relevance for all subsequent events during the foreign body reaction. For instance, monocytes adhere to the fibrin-dominated matrix and modulate the immunologic answer, which might result in a chronic immune response and fibrous encapsulation (Anderson et al. 2008). This is accompanied by macrophage fusion to multinucleated foreign body giant cells, resulting in destruction of the implanted material and the subsequent device failure (Klopfleisch and Jung 2017). The initial protein adsorption to a biomaterial surface is a very complex process that depends on numerous variables of the surface like wettability, topography, elasticity, chemical composition, and charge, but also on characteristics of the proteins like structure, isoelectric point, relative concentration in the plasma, and protein-surface affinity, determining the host response to the implant surface. Surface treatment of medical implants by various physical and chemical techniques are attempted in order to improve their surface properties to facilitate bio-integration and prevent bacterial adhesion. Therefore, functional biomaterial coatings need to control unspecific protein and bacterial adsorption and should promote the interaction with the desired tissue cells. Figure 1 depicts three strategies for host response guidance after biomaterial implantation. While strategies shown in Figure 1A and B mainly deal with repellence of host proteins and bacteria, Figure 1C illustrates how biomaterials can be used to send the appropriate signals to

Figure 1: Strategies to guide the host response following biomaterial implantation. (A) Bioinert surface coating minimizing interaction with the host tissue. The passive protein or cell repellent effect by physical or chemical surface modification limits unspecific adhesion of serum proteins, bacteria, and tissue cells. (B) Bioactive coatings actively influence the host response, e.g. by releasing therapeutic drugs or incorporation of antimicrobial silver particles. (C) Biomimetic coatings guide tissue regeneration inspired by the ECM. Components include natural or nature-derived adhesion ligands, anticoagulants, protease cleavage sites, and modulator proteins.

balance cell repellent and cell attracting properties. Herein, a more biologically-based method is considered, where the ECM provides a template to display the necessary cues on implant surfaces.

The natural cell environment

The ECM builds a highly complex three-dimensional network of extracellular macromolecules around the tissue cells with a unique composition and topography to the respective tissues and microenvironments, but shares common components like water, proteins, and polysaccharides (Figure 2). Herein, two main types of macromolecules are present: fibrous proteins, including collagens and elastin, as well as glycoproteins, including proteoglycans and the adhesives fibronectin, vitronectin, and laminin (Mouw et al. 2014). The multi-domain proteoglycans have a protein core where glycosaminoglycans (GAGs) are covalently attached. Their largest soluble representatives are versican, aggrecan, and perlecan, but there is also a class of cell surface-bound proteoglycans. Their main type, the syndecans, fulfill important biological functions through their covalently attached heparan sulfate chains, including cell adhesion, sequestration of heparin-binding ligands, and promoting the oligomerization of bound ligands (Mecham 2012). Collagen, the main

structural element of the ECM, provides tensile strength to the tissue, while the associated elastin is responsible for elasticity (Frantz et al. 2010). The multiple distinct domains of the large ECM proteins contain information of biological importance, ranging from providing a mechanical scaffold for tissue cells to the modulation of mediator protein presentation as well as regulation of hydration, osmotic pressure, and pH. Combining these, the ECM modulates the cell-activation status all-encompassing and actively participates in the establishment, separation, and maintenance of tissues and organs (Mouw et al. 2014).

It has been tested to decellularize ECMs for the generation of biomaterials. However, this approach bears a number of pitfalls, as complete removal of cells and unwanted residuals as well as comprehensive characterization remain difficult to achieve (Aamodt and Grainger 2016). Therefore, imitation of this complex network in a defined and controllable manner is of high interest. However, the use of full-length proteins for the coating of biomaterials is conflicting. Even though ECM proteins display more complex and dynamic binding motifs, isolation and purification remains challenging and bears the risk of contamination, adulteration, and subsequent immunogenicity (Hersel et al. 2003). Additionally, surface immobilization might change conformation and orientation of the adhesion motifs, impairing optimal cellular interaction. In contrast, short bioactive peptide sequences bear a number of advantages. Due to smaller size, higher packaging densities can be achieved, the production is more cost-

efficient, and the compounds are easier to characterize and store. Plus, peptides are more stable to heat, pH-changes, and sterilization, enabling translation into clinics (Hersel et al. 2003). Therefore, bioactive peptide sequences mediating implant integration are extensively exploited in biomaterials science. Identifying the bioactive domains and modularly assembling them in a distinct composition are the key principles of biomimetic implant coatings. In this regard, ECM-derived peptide sequences as well as presented mediator proteins provide a versatile tool to translate cell–matrix as well as matrix–matrix interactions on biomaterials. Next to simple adsorption of such motifs, immobilization using a strong anchoring and providing a distinct presentation should be considered.

Mussel-derived coating strategies for peptide and protein presentation

Strong anchoring of adhesion peptides is crucial to success, as the cells apply large forces to integrin ligands, which were even reported to rupture biotin-streptavidin binding (Jurchenko et al. 2014). As detached adhesion ligands have a strong apoptotic effect (Stupack and Cheresch 2002), appropriate elaboration of the surface immobilization technique is required. Therefore, simple adsorption is insufficient to promote robust implant integration. Especially approaches, which demonstrate synergistic signaling, anchor the adhesion peptides very strongly, either by covalent coupling or by high-affinity interactions. The latter mainly involve adsorption of thiolates to gold or titanium substrates (Hudalla and Murphy 2010; Mas-Moruno et al. 2014; Schenk et al. 2014) as well as L-3,4-dihydroxyphenylalanine (DOPA)-based interactions with titanium (Gunawan et al. 2007; Pagel et al. 2016a). The biological model for this vigorous surface anchorage can be found in the blue mussel (*Mytilus edulis*), which can virtually adhere to any kind of surface, organic or inorganic, regardless of the high pH and salt concentration in sea water or sea disturbance. Inspired by its adhesive proteins, rich in DOPA, hydroxyproline, and lysine (Waite and Tanzer 1981), surface coatings mediating the interaction with numerous biomaterials have been developed. Herein, the post-translationally modified amino acid DOPA was found to be essential for immobilization and to display a variety of different binding modes, which are summarized in Figure 3 (Kord Forooshani and Lee 2017). It is known for its exceptionally strong interaction with titanium dioxide, where the catechol unit forms a high-affinity

Figure 2: ECM interactions organizing mechanical cues and orchestrating biological signals to guide cell activation status. Structural proteins like collagen provide a three-dimensional scaffold, connected by adhesive proteins like fibronectin. Embedded proteoglycans (e.g. heparan sulfate) provide hydration and store mediator proteins. Integrin, syndecan, and different receptors act as cellular sensors transducing information stored in the ECM microenvironment.

Figure 3: Interfacial interaction modes of DOPA including coordinative binding, hydrogen bonding, hydrophobic interactions, and covalent cross-linking following oxidation.

coordination bond with the metal oxide (Li et al. 2014). Although the interaction is reversible as Messersmith and co-workers have shown in single-molecule experiments, it may not be the case for whole mussel adhesive proteins (Lee et al. 2006). The cooperativity of multiple DOPA-surface interactions might allow for tremendous force transmission across the interface, since the adhesion energies increase with DOPA concentration (Anderson et al. 2010). Interaction with an oxide surface of few DOPA units displays outstanding strength, leading to irreversible cohesion failure in atomic force measurements, which was also found for covalent bond breakage (Lee et al. 2006). This variable binding ability makes catechol chemistry a useful tool for the immobilization of functional peptides to various kinds of implant materials.

Today, the list of modifiable materials ranges from various metal oxides over synthetic polymers like polystyrene (PS), polyethylene (PE), polytetrafluoroethylene (PTFE), polyvinylidene fluoride (PVDF), polycaprolactone (PCL), poly lactic-co-glycolic acid (PLGA), and natural polymers including silk and cellulose to ceramics, viruses, yeast, and bacteria (Ryu et al. 2018). An extensively exploited approach herein is to modify the surface with polydopamine first and to cover it with the desired bioactives in a second step. The first example for such an ad-layer introduction was published in 2007, where the authors demonstrated that polydopamine coatings could be used for electroless metallization or grafting with antifouling and cell adhesive polymers (Lee et al. 2007). Since then, this method has found wide acceptance. It is suitable to stably immobilize entire ECM proteins like collagen, which was found superior to non-covalent protein adsorption (Yu et al. 2013), but also short adhesion peptides like GRGDS and YIGSR as well as growth factors. Polydopamine coatings are a simple and effective strategy to modify biomaterials, but the efficiency of the

functionalization and the exact composition of the resulting coating is difficult to characterize. Recent findings suggest a generally weak binding of polydopamine to titanium surfaces with some occasional adhesive points, implying that polydopamine coatings are not the first choice for stable binding properties (Delparastan et al. 2019). Coupling of DOPA to the bioactive moiety enables full analysis and sterilization before immobilization to the surface. DOPA units are either directly incorporated by solid phase peptide synthesis (SPPS) or generated from tyrosine by chemical or enzymatic modification (Taylor 2002). With this in mind, catechol units were directly coupled to antifouling poly(ethylene glycol) (PEG) units, peptidomimetics, or GAGs (Dalsin et al. 2003; Ham et al. 2013; Statz et al. 2005). In some approaches, these types of antifouling coatings act as a protein repellent linker that provides a functional group for further modification with adhesion peptides, antibodies, or fluorescent dyes after immobilization (Gao et al. 2010; Na et al. 2012; Zhu et al. 2014). However, incomplete conjugation leaves reactive groups, possibly causing side reactions. Therefore, comprehensive characterization of the compound prior to immobilization is desired. While Hwang and co-workers recombinantly fused DOPA-containing peptide sequences with integrin-binding ligands, Tang and Pan et al. elongated osteogenic peptide sequences with DOPA on solid support, before cleavage, purification, and immobilization (Hwang et al. 2007; Pan et al. 2016; Tang et al. 2014). Recently, a peptide platform was developed containing DOPA for surface anchorage, short ethylene glycol (EG) units for spacing and protein repelling as well as reactive amino acid side chains for orthogonal modification with bioactive peptides (Pagel et al. 2016a). This approach enables the combination of stable surface anchorage via DOPA with orthogonal modification strategies to produce fully characterizable, multifunctional peptides. This DOPA-containing multifunctional peptide was initially tested on titanium dioxide surfaces, where the peptide showed high binding affinities in the nanomolar range and furthermore outstanding stability over at least one week in cell culture supernatant of osteoblast-like cells (SaOs-2) (Pagel et al. 2016a). Based on this peptide, Clauder et al. conducted further studies on many more surfaces like PS (Clauder et al. 2020a), hydrophilized polycaprolacton-co-lactide (PCL-co-LC) scaffolds, and glass surfaces (Clauder et al. 2020b) as well as on 316L stainless steel, PTFE, and nitinol as examples for commercially available stent materials (Clauder et al. 2019).

Orthogonal modification strategies for multifunctional peptide coatings

In order to functionalize such biomaterial-binding peptides, selective side chain elongation by SPPS is the most straight-forward approach (Mas-Moruno et al. 2014). However, this strategy reaches its limits when long or complex peptides, for example cyclic integrin ligands, are attached or when whole proteins should be modified and subsequently immobilized. Separate synthesis and purification, combined with a sophisticated ligation strategy can yield complex molecules. When different compounds shall be conjugated to one peptide, orthogonal reaction strategies are exploited. The most frequently found reactions in this context are summarized in Figure 4. The inverse electron demand Diels–Alder reactions (DAR_{inv}) have the fastest reaction rate of all click reactions (Blackman et al. 2008). A possible downside is the decreased stability of the electron-poor tetrazine with increasing reactivity (Spicer et al. 2018) and the rather bulky linker. However, this could be of advantage for the introduction of integrin ligands in biomaterial coatings, as the natural presentation of the RGD sequence is in an exposed loop of the protein (Hersel et al. 2003) and the optimal receptor binding was found to require a spacer length of 11–32 Å (Beer et al. 1992). The tetrazine linker exploited by the group of Beck-Sickinger was found to optimally compromise ligand presentation and stability (Pagel et al. 2016b). Furthermore, the Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) is one of the most well-known click reactions. However, the required Cu(I) catalyst is the origin to several problems. Residual Cu(I) is cytotoxic and must therefore be removed before implementation in biological contexts. This can be overcome by performing the reaction on solid support. As on-resin reactions can be accompanied by sterical hindrance, resins with high swelling and low loading capacity as well as copper chelating ligands, like tris((1-benzyl-4-triazolyl)methyl)amine (TBTA) or tris(3-hydroxypropyltriazolylmethyl)amine (THPTA), can increase reaction kinetics (Tang and Becker 2014). Other click-type reactions like the well-established Michael-type addition reactions (Gennari et al. 2020) or the recently revisited thiazolidine chemistry (Yamada et al. 2020) have been proven as versatile tools for the catalyst-free preparation of bioconjugates under physiological conditions. A frequently used tool in protein modification is the native chemical ligation (NCL), combining C-terminal thioesters with N-terminal cysteine residues (Dawson et al. 1994). Especially the NCL has been

extensively used in semi-synthetic approaches like expressed protein ligation (EPL), where the N-terminal fragment of a protein is recombinantly expressed and the C-terminal fragment is synthesized by SPPS. The C-terminal part can be easily modified with artificial amino acids, functionalities, or labels (Beck-Sickinger and Panitz 2014). A detailed overview of possible orthogonal reaction combinations was summarized previously (Pagel and Beck-Sickinger 2017; Spicer et al. 2018). Combining orthogonal click reactions provides an opportunity to install several bioactive molecules in a defined ratio and spacing. Important considerations focus on possible cross-reactivity between the chosen functional groups as well as their stability under reaction conditions.

Mediating cell–matrix interactions

The imitation of structural ECM proteins in a defined and characterizable way provides support and presentation of bioactive domains and mediators, which can be achieved by short peptide sequences and small cytokines or growth factors. Approaching the natural situation, most of the ECM components display interaction sites for cellular receptors, triggering outside-in and inside-out signaling and thereby promoting the adaption of cells to matrix remodeling and *vice versa*. Herein, a dominating role is granted to the integrins, a family of heterodimeric glycoproteins, which express a subset of 18 α and 8 β subunits in 24 distinct combinations (Hynes 2002). While the major class recognizes the short tripeptide RGD present in fibronectin, others bind laminin and collagen-derived sequences. The extracellular domain interacts with a variety of ECM ligands and counter receptors on adjacent cells (Humphries et al. 2006), whereas the intracellular domain is linked to the actin cytoskeleton (Morgan et al. 2007). This connection enables matrix induced signal transduction events that modulate many aspects of cellular behavior including proliferation, survival/apoptosis, shape, polarity, motility, gene expression, and differentiation (Hynes 2002). By presentation of distinct integrin ligands, defined cell–matrix interactions can be imitated on biomaterial surfaces, recruiting desired cell types, switching from inflammation towards tissue regeneration and restoring tissue homeostasis.

The before mentioned RGD sequence was originally discovered in fibronectin, in an attempt to reduce the full protein to its minimal adhesion motif (Pierschbacher and Ruoslahti 1984), but was later found in many more adhesion

Figure 4: Orthogonal reactions for the selective modification of biomaterials. CuAAC: Cu(I)-catalyzed azide-alkyne cycloaddition; DAR_{inv}: Diels-Alder reaction with inverse electron demand; NHS: N-hydroxysuccinimide; SPAAC: strain-promoted azide-alkyne cycloaddition; NCL: native chemical ligation.

macromolecules. It was soon applied to direct the attachment of tissue cells on implant surfaces (Barrera et al. 1993) and was recently incorporated into self-assembling hydrogels as cell delivery vehicles (Yamada et al. 2019). The RGD-sequence has been one of the most effective and widely used adhesion peptides since its discovery (Hersel et al. 2003). While the simple tripeptide displayed greatly reduced integrin affinity when disengaged from the full protein, the importance of the flanking amino acids was recognized early, leading to high-affinity derivatives such as GRGDSP (Pierschbacher and Ruoslahti 1987) and AGTFALRGDNPQG (Truong et al. 2020). Owing to the promiscuity of RGD, extensive research has been carried out to tune selectivity. Implementing conformational restraint by peptide cyclization and the incorporation of D-amino acids has led to a variety of integrin specific derivatives, summarized elsewhere (Kapp et al. 2017). Hence, the opportunity to discriminate undesired cell types like platelets from tissue cells arose by sparing their main integrin receptor $\alpha_{IIb}\beta_3$ from the binding profile. One of the most successful compounds, c[RGDfK], was developed by Kessler and co-workers (Kantlehner et al. 1999) and selectively addresses integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$, expressed by many tissue cells including endothelial cells (Brooks et al. 1994) and osteoblasts (Hughes et al. 1993). Next to higher selectivity, cyclic adhesion peptides also display higher affinity and binding strength (Ishikawa et al. 2020; Xiao and Truskey 1996), and greater stability (Bogdanowich-Knipp et al. 1999). In addition to RGD, many other integrin-binding

peptides have been discovered. Hereunder, the fibronectin-derived sequences PHSRN (Aota et al. 1994), REDV (Humphries et al. 1987), and LDV (Komoriya et al. 1991), the laminin-derived YIGRS (Clément et al. 1990), (S)IKVAV (Freitas et al. 2007), and KVLTEQVL (Ishikawa et al. 2020), the collagen I mimetic DGEA (Staat et al. 1991) and GFOGER (Knight et al. 2000) as well as a number of rationally designed peptides, like RRETAWA (Koivunen et al. 1994), have found successful application. The spatial distribution of the motifs is of utmost importance, as precisely demonstrated for the classical RGD and its synergy site PHSRN in fibronectin, where synergism is easily lost when distances increase (Bachman et al. 2015). Co-immobilization of different adhesion peptides to imitate the natural ligand display has been attempted with peptide mixtures of defined ratios or bifunctional molecules.

Besides integrins, cells are equipped with a set of proteoglycan surface receptors, one of which are the syndecans. They likewise span the plasma membrane and are associated to the cytoskeleton, where they actively participate in signal transduction (Roper et al. 2012). On the extracellular side, the interaction is based on electrostatic attraction of the negatively charged sulfate and carboxyl groups of their GAG side chains with recognition sequences within ECM proteins, containing the basic amino acids histidine, arginine, and lysine. Therefore, synthetic peptides addressing syndecans can be derived from the heparin-binding regions of various structural proteins, such as fibronectin (PRRARV (Barkalow and Schwarzbauer

1991) and WQPPRARI (Woods et al. 1993)), laminin (RKRLQVLSIRT (Yamada et al. 2020)), and many more. Heparin-binding peptides were shown to promote adhesion and spreading attributed to their cell surface-binding properties, but demonstrated also differentiating effects (Rezania and Healy 1999; Sun et al. 2013). This was traced back to the binding of soluble GAGs and subsequent sequestration of growth factors (Hudalla et al. 2011). Interrelations of integrin and syndecan attracting peptides would hence cooperatively improve cell recruitment and tissue integration. Combinations yielding synergistic effects of integrin-binding sequences as well as combinations of integrin and syndecan-binding coatings are summarized in Table 1. Note, a similar table concentrating on bone regeneration has been published previously (Mas-Moruno 2018). The most often applied combination is the fibronectin mimetic RGD peptide together with PHSRN (Table 1a–d). Often, predominance of RGD peptide, a strong immobilization of the adhesion ligands, and a non-fouling base granted success. A further role model for the combination of integrin-binding peptides was found in laminin, a major component of the basal lamina. Its integrin-binding motifs YIGSR (integrin $\alpha_4\beta_1$) and (S)IKVAV (integrins $\alpha_3\beta_1$ and $\alpha_6\beta_1$) were combined with the well-known RGD sequence. Surprisingly, mixtures with fewer RGD content and higher amounts of alternative integrin-binding sites were more successful this time (Table 1f and h). In contrast, the spatially defined presentation of the two adhesion peptide sequences RGD and SIKVAV in a single molecule synergistically enhanced endothelialization more than a mixture of both (Table 1j). This stresses the complexity of the system and accounts not only for the importance of ligand type, but also for their distribution, density, and spacing. Besides cooperative signaling between different integrin heterodimers, synergy between integrins and syndecans are likewise encouraged. Many ECM proteins display a combination of cell and GAG-binding sites, synergizing in the mediation of cell adhesion. Through their large and flexible GAG chains, syndecans have the ability to bind ligands as far as 500 nm away (Weinbaum et al. 2007). Thereby, they promote the initial contact between cells and the adhesion site, leading to closer proximity, which then allows integrin clustering (Morgan et al. 2007). Integrin and syndecan cooperativity is fundamental for an all-encompassing adhesion response and many adhesion-directed processes including wound healing, angiogenesis, or axonal guidance depend on it (Morgan et al. 2007). Again, fibronectin and laminin lend themselves to ECM-mimetic peptide coatings (Table 1r and s). For the latter, the strongest influence on endothelialization was detected when ligands were presented as

heteroclusters, rather than homoclusters. This is similar to other approaches (Table 1l and o), where different ligand types are equally distributed rather than locally clustered. Pagel et al. modified a protein repellent surface-binding peptide with RGD and FHRRKA peptides using bioorthogonal-coupling methods as outlined above. This bifunctional display demonstrated significant increases on osteoblast adhesion, viability, and proliferation (Table 1o). Based on this, the doubling of the syndecan-binding motif (FHRRKA)₂ led to an improved cell viability of endothelial cells, but had no further impact on cell adhesion and spreading (Table 1p). By comparison of mixtures with the one compound approach, the importance of ligand spacing and distribution was stressed. As the optimal distance for integrin clustering was found to be within 58–73 nm, distinct ligand presentation is important to reliably match this specification (Arnold et al. 2004). An overall increased global ligand density might therefore be the rationale for the superiority of bifunctional peptides. Remarkably, if the short peptides are displayed optimally, cellular response can exceed that of the full-length ECM protein (Hoyos-Nogues et al. 2019; Jung et al. 2011; Mas-Moruno et al. 2014; Pagel et al. 2016a). This is attributed to the numerous advantages of small peptides described earlier and again emphasizing the great potential of multifunctional coatings in tissue engineering.

Integrating proteoglycans and mediator proteins into biomaterials

Complex ECM functions are determined by the composition and ultrastructure of its components, as single elements form supramolecular assemblies influence biological properties. In this context, the ECM modulates the abundance of cytokines, affects their activity, and establishes chemotactic gradients. Multifunctional peptide coatings, which mediate the interaction with implant surfaces, cells, and matrix molecules, therefore provide a sophisticated strategy to include proteoglycan and cytokine signaling in the biomaterial design. In this way, next to cell attachment also the recruitment of progenitor cells and their differentiation are promoted.

Next to fibrillar proteins, proteoglycans represent a major component of the ECM. Herein, the type of GAG and its extent of sulfation greatly varies within tissues. Proteoglycans are involved in tissue development, regeneration, and repair. Even though biomaterials science often focuses on adhesion and mediator proteins, the manifold regulatory functions of GAGs should not be neglected. On

Table 1: Combinations of integrin-binding peptides and syndecan-binding coatings inducing cooperative signaling.

A	Integrin ligand 1	Integrin ligand 2	Presentation	Effect	Reference
a	c[RGDfK]	PHSRN	Partly spatially constrained	Adhesion and spreading of fibroblasts	Schenk et al. (2014)
b	GRGDS		1:1 mixture	Adhesion of fibroblasts	J. Lee et al. (2013)
c	RGDS		Random mixture versus spatially constrained	Adhesion, spreading and proliferation of osteoblasts	Mas-Moruno et al. (2014)
d			10:1 mixture	Adhesion, proliferation and differentiation of osteoblasts	Chen et al. (2013)
e	cRGD (GCRGDGWCGY)	cLDV (GCWLDVCGY)	1:2 mixture	Adhesion of hematopoietic cells	Gunawan et al. (2007)
f	RGD	YIGSR	1:9 mixture	Spreading and viability of endothelial cell	Noel et al. (2015)
g	RGDS		1:1 mixture	Adhesion and proliferation of endothelial cells	Castellanos et al. (2017)
h		YIGSR/IKVAV	1:3.5 mixture	Differentiation of endothelial cells, matrix synthesis	Ali et al. (2013)
i		IKVAV	8:3 mixture	Viability of endothelial cells	Jung et al. (2011)
j	c[RGDfK]	SIKVAV	1:1 mixture versus spatially constrained	Adhesion, viability, proliferation and differentiation of endothelial cells	Clauder et al. (2019)
B	Integrin ligand	Syndecan ligand	Presentation	Effect	Reference
k	GRGDSP	KRSR	1:1 mixture	Viability of osteoblasts	Włodarczyk-Biegun et al. (2016)
l	RGDS		Spatially constrained	Differentiation of osteoblasts	Hoyos-Nogues et al. (2019)
m	GRGDSP		Mixtures	Adhesion and differentiation of osteoblasts	Bell et al. (2011)
n	RGDSP	KRSR or FHRIKA	1:3 mixture	Adhesion and proliferation of osteoblasts	Schuler et al. (2009)
o	c[RGDfK]	FHRIKA	Random mixture versus spatially constrained	Adhesion, spreading, viability and proliferation of osteoblasts	Pagel et al. (2016a)
p		FHRIKA or (FHRIKA) ₂	Spatially constrained	Adhesion and survival of endothelial cells	Clauder et al. (2020b)
q	RGD	FHRIKA	Mixtures	Adhesion and differentiation of osteoblasts	Rezania and Healy (1999)
r	GRGDS	RKRLQVQLSIRT or NSFALYLSKGR	1:1 mixture partly spatially constrained (homo- and heteroclusters)	Adhesion, spreading and proliferation of endothelial cells	Karimi et al. (2018)
s	GRGDSPA	SPRRARVT	1:1 mixture	Adhesion and proliferation of endothelial cell	Sagnella et al. (2005)
t	RGDSP	TYRSRKY	Mixtures	Spreading of MSCs	Hudalla and Murphy (2010)

the cell surface of the vascular endothelium, GAGs are well known anticoagulants (Bourin and Lindahl 1993). Next to that, they promote wound healing by exerting anti-inflammatory and radical scavenging effects (Salbach et al. 2012). They are involved in the differentiation of stem cells (Smith et al. 2011) and carry important functions in bone remodeling and resorption (Salbach-Hirsch et al. 2014). They modulate the immune response by directing the migration of neutrophils and

macrophages (Proudfoot et al. 2017). Immobilization of chemoattractants by GAGs is essential to the formation of chemotactic gradients and protects the mediator proteins from inactivating post-translational modifications or proteolytic degradation (Liang et al. 2016; Ziarek et al. 2013). A considerable proportion of proteoglycan function is carried out through GAG-binding proteins. Modulatory cytokines are bound and sequestered by proteoglycans to stimulate adhesion, survival/apoptosis, proliferation,

and differentiation during tissue homeostasis, wound healing, and inflammation (Martino et al. 2015). Therefore, incorporating proteoglycans in functional biomaterial coatings can create regenerative impulses synergizing with integrin and growth factor or cytokine signaling. However, GAGs were not only used to deliver modulator proteins, but can also act as a scavenger for pro-inflammatory chemokines in chronic wounds, shown in a collaboration of Franz and Freudenberg et al., who used a heparin-containing PEG hydrogel for binding MCP1, IL8, and MIP1 (Lohmann et al. 2017).

Strategies for controlled mediator protein delivery

Finding and implementing a suitable delivery strategy for ECM components is crucial to implant success and strongly depends on the purpose of the coating. While integrin signaling needs strong ligand anchoring by covalent or high-affinity binding, a more dynamic immobilization of GAGs and cytokines is desired that allows both binding and release of the mediator protein. Controlling their release is of utmost importance, as bolus injections of cytokines have raised serious safety concerns. For instance, BMP delivery induces ectopic bone formation and elevated cancer risks (Carragee et al. 2011), while extensive stimulation with VEGF leads to systemic hypotension and edema (Simons and Ware 2003). The major cause of these complications lies within the very high doses administered to achieve therapeutic concentrations, arising from the short biological half-life of the proteins and rapid clearance from the injured site. This in turn questions cost-effectiveness, demanding for more sophisticated delivery strategies (Martino et al. 2015). In the ECM, growth factors, including the VEGF, FGF, TGF- β and PDGF families, are stabilized and released depending on their binding affinity or through the action of proteases (Martino et al. 2015). Matrix interaction is likewise essential to cell migration, while this in turn is dependent on the formation of a chemotactic gradient. An important factor in progenitor cell recruitment for bone regeneration and angiogenesis is the chemokine CXCL12, hence its incorporation and release in biomaterials was studied and serves here as an example for protein immobilization strategies in Figure 5.

Simple protein adsorption to electrospun PCL/gelatin fibers was sufficient to increase bone formation *in vivo* by six-fold in comparison to blank fibers (Figure 5A) (Ji et al. 2013). However, the chemokine is released quickly and cleared from the site of injury. Encapsulation within

hydrogels or polymers represents a likewise straightforward approach, but significantly slows down release kinetics. Herein, the proteolysis of the scaffold determines the release of the protein (Figure 5B). Other groups used the physical entrapment of mediator proteins like FGF2 as well, but tuned the long-term delivery by modulating mesh size and swelling (Tong et al. 2015). Furthermore, fast and slow degrading hydrogels have been developed for the time-controlled delivery of chemokines, which suppressed neutrophil infiltration in early phases and promoted angiogenesis and survival in the infarcted myocardium in later stages (Projahn et al. 2014). Development of a chemically cross-linked DNA hydrogel led to electrostatic immobilization of CXCL12 (Figure 5D) (Basu et al. 2019). Additional bridging of the DNA strands by silicate nanoparticles enabled interaction with the chemokine and was found to significantly slow release kinetics in comparison to encapsulation. But protein release depends on their isoelectric point (pI) and can be fine-tuned by incorporating positively or negatively charged peptides into hydrogels, thus electrostatic interactions retard or promote protein release (Nagy-Smith et al. 2016). In any case, encapsulation and non-covalent interactions of proteins with biomedical substrates maintain the protein's native conformation and thereby retain their biological activity. The selective covalent immobilization of a protein requires its chemical modification, which should not interfere with the biological function or structure. Nevertheless, such approaches bear the chance for a triggered release upon a given stimulus and consequently covalently joined proteins are often combined with a protease-cleavable linker. As a proof of concept, Kloxin and colleagues modified proteins with azide functionalities by genetic code expansion for bioorthogonal immobilization chemistry utilizing strain-promoted azide-alkyne cycloaddition (SPAAC) in dibenzocyclooctyne (DBCO)-modified PEG hydrogels. Furthermore, the protein was decorated with a thrombin-cleavable linker in order to release the whole protein from the hydrogel (Guo et al. 2017). Applying the EPL technique, the group of Beck-Sickinger coupled CXCL12 to polymer surfaces using a matrix metalloproteinase-9 (MMP9)-sensitive linker, a protease, which is highly abundant in wound healing (Figure 5C) (Steinhagen et al. 2014). In addition to the C-terminal cleavable linker sequence, they stabilized the chemokine N-terminus against MMP2 and MMP9-mediated degradation and subsequent inactivation based on concepts of Lee and colleagues (Segers et al. 2007), as the truncated CXCL12(5–68) showed impaired chemotactic activity (Jin et al. 2008; Peng et al. 2012) and was found to be neurotoxic (Vergote et al. 2006). Since MMP2 and MMP9 levels are

Figure 5: Strategies for delivery of modulatory proteins by biomaterials, exemplified by CXCL12. (A) Adsorption to PCL/gelatin nanofibers (Ji et al. 2013). (B) Encapsulation in degradable hydrogels and proteolytic release (Projahn et al. 2014). (C) Covalent surface coupling and triggered release through cleavage of an MMP9-sensitive protease cleavage site (PCS) (Steinhagen et al. 2014). (D) Electrostatic immobilization with the help of silicate nanoparticles into cross-linked DNA hydrogels (Basu et al. 2019). (E) Affinity-based delivery by incorporation of heparin, a natural binding partner, into starPEG hydrogels (Prokoph et al. 2012). (F) Dual affinity system comprised of a covalently immobilized heparin-binding peptide, heparin, and the chemokine (Clauder et al. 2020b).

elevated after myocardial infarction as well as in injured or wounded tissue areas (Madlener 1998; Peterson et al. 2000), these additional stabilizing features of the chemokine are highly valuable. As the N-terminal S4V stabilization against protease degradation only leads to a slight loss in potency at the CXCL12 endogenous receptor CXCR4 (Steinhagen et al. 2014), these improvements make a therapeutic use of the chemokine accessible. Furthermore, sterically demanding amino acids at the N-terminus, like lysine in case of CXCL12, prevent the N-terminal methionine excision by methionyl-aminopeptidase in *Escherichia coli* (Hirel et al. 1989). Subsequently, this additional methionine leads to a further stabilization against dipeptidyl peptidase-4 (DPP4), which is present in very early and later wound healing stages (Schürmann et al. 2012). This serine exopeptidase releases Xaa-Pro and Xaa-Ala dipeptides from the N-terminus, thereby limiting the efficacy of CXCL12 upon truncation (Segers et al. 2007). A very smart approach was established in 2012, where the CXCL12 activation was coupled to DPP4 by addition of a AAV tripeptide to the N-terminus of the chemokine (Baumann et al. 2012). By excision of the Ala-Ala dipeptide by DPP4, the fully active CXCL12 was achieved and led to efficient migration of early endothelial progenitor cells (eEPCs) *in vitro*. By combining stabilization of the N-terminus with the MMP9-cleavable linker at the C-terminus of CXCL12,

which was further elongated by a mussel-derived surface-binding peptide, Spiller and co-workers achieved outstanding results in *ex vivo* studies in epithelial wounds shown in a porcine skin organ culture model (Data unpublished). Coated PCL-co-LC scaffolds with the releasable protein significantly improved re-epithelialization and wound closure compared to uncoated scaffolds as well to coated scaffolds with a non-releasable protein variant. This effect was CXCL12-specific, since it has been blocked by the addition of AMD3100, a CXCR4 inhibitor (Rosenkilde et al. 2004). These findings were underpinned by elevated keratinocyte migration *in vitro* and encourage proceeding with further *in vivo* studies and consideration of translational aspects of this strategy.

Another aspect of ECM function is the regulation of growth factor signaling by affinity binding to recognition sites in proteoglycans and structural proteins. Covalently immobilizing a binding ligand to the material which then reversibly binds a mediator protein represents another bioinspired approach in controlling cytokine activity (Mas-Moruno 2018). Specific recognition sequences known to bind CXCL12 have been described (Peled et al. 2013). However, distinct binding partners for all mediators are not yet known and recombinant or chemical modification of the proteins to display binding sequences is laborious and potentially interferes with protein function. Therefore,

selection of a universal binding partner such as heparin is preferred. A possible delivery matrix is presented by starPEG-heparin hydrogels (Figure 5E) or hyaluronan-based hydrogels, which are versatile platforms to deliver a variety of heparin-binding proteins, including growth factors like FGF2, VEGF, and EGF (Thönes et al. 2019; Xu et al. 2018; Zieris et al. 2010). The amount of delivered protein is thereby adjustable by adapting the loading concentrations and by protease-mediated hydrogel cleavage (Bauermann et al. 2012; Prokoph et al. 2012). In order to modulate the release kinetics of the bound protein, other types of GAGs, like chondroitin or dermatan sulfate, can be incorporated (Wang et al. 2013). To further tune the affinity, natural GAGs may even be substituted with analogs like sulfated hyaluronic acid (Thönes et al. 2019) or fucoidan (Huang and Liu 2012). Influences like sugar length and the number and position of sulfates on the binding kinetics of various chemokines and growth factors should not be underestimated (Atallah et al. 2018; Clauder et al. 2020a; Köhling et al. 2019). Therefore, assembling of different GAGs can be used to generate long-term gradients out of protein-loaded hydrogels. The other way around, Spiller and Panitz et al. designed CXCL12 variants with higher and lower binding affinity towards heparin by mutating the heparin-binding sites of the chemokine, leading to a prolonged chemotactic gradient out of starPEG-heparin hydrogels and a subsequent eEPC migration *in vitro* (Spiller et al. 2019). Furthermore, they compared heparin as a naturally occurring GAG as well as nonasulfated hyaluronic acid in order to address artificial approaches replacing heparin. These mutagenesis studies were based on the previous characterization of a high-affinity heparin-binding site and a low-affinity heparin-binding site at the CXCL12 interface by a combination of NMR spectroscopy, molecular modeling, and molecular dynamics simulations (Panitz et al. 2016). A different GAG-free approach equipped model proteins with a positively charged interaction domain and subsequently immobilized them in a compatible negatively charged peptide hydrogel via defined linker lengths, attenuating gel-based protein denaturation (Miller et al. 2019). Controlled release of therapeutic proteins from biomaterials is critical to promote tissue regeneration and the period as well as the duration of release of the applied protein should be considered with regard to the effective concentration range. Since an excessive amount of chemokine might lead to fast receptor internalization and subsequent signal termination, it is highly relevant to modulate and balance the local chemokine concentration as in the before mentioned studies. The utilization of biodegradable biomaterials like starPEG-heparin hydrogels with high storage

capacity in the combination with modified chemokine variants with an altered binding pattern to these materials may offer the opportunity for deeper understanding of beneficial wound healing parameters.

Next to covalent coupling, strategies for the incorporation of GAGs into functional biomaterials encompass electrostatic interactions, for instance using polylysine (Liu et al. 2014). Introducing specific binding sequences with an affinity for heparin or similar polymers enables further fine-tuning of the release kinetics (Figure 5F) (Spicer et al. 2018). Syndecan-binding peptides described and summarized in Table 1B are numerous and suitable for heparin binding with subsequent mediator protein delivery (Sakiyama-Elbert 2013). With the help of a bi-domain peptide for cross-linking to fibrin matrices and heparin binding based on antithrombin III, Sakiyama-Elbert and colleagues opened up a field of novel growth factor delivery strategies in the year 2000 (Sakiyama-Elbert and Hubbell 2000b). While the first study immobilized and released FGF2, following investigations proved the versatility of this approach by transfer to NGF (Sakiyama-Elbert and Hubbell 2000a), PDGF (Thomopoulos et al. 2007), and GDNF delivery (Wood et al. 2009). Self-assembling peptide nanofibers displaying the rationally designed LRKKLGKA heparin-binding motif were likewise exploited for immobilization of heparin and subsequent delivery of VEGF, FGF2, and BMP2 (S.S. Lee et al. 2013; Webber et al. 2010). The previously established heparin-binding peptide FHRRKA immobilized by a mussel-derived surface-binding peptide (Pagel et al. 2016a), was expanded and tested in a layered set-up with non-covalently bound heparin and loaded CXCL12 (Clauder et al. 2020a). This dual affinity approach proved more successful than covalent coupling of heparin analogs, as optimal degree and pattern of sulfation are synthetically difficult to achieve. Elongation of the heparin-binding peptide sequence resulted in a significantly higher amount of immobilized heparin and CXCL12. Interestingly, this higher chemokine loading did not lead to an increased Jurkat migration, as simultaneously released heparin supposedly scavenged CXCL12. In such dual affinity systems, the release kinetics are dependent on the affinity between the heparin-binding peptide and heparin, as well as the affinity between heparin and the cytokine. Accordingly, the cytokine can diffuse by itself or in complex with the GAG. Hence, the affinity of the heparin-binding peptide towards heparin significantly influences protein release (Maxwell et al. 2005). Likewise, the ratio between heparin and the cytokine can be optimized, to minimize free diffusion (Vulic and Shoichet 2014).

Table 2: Selected multifunctional coatings combining adhesion ligands, proteoglycans, and cytokines or growth factors.

Cell–matrix interaction	Matrix–matrix interaction		Other bioactive	Material	Effect	Reference
Adhesion ligand	Proteoglycan	Mediator protein				
a RGD	Chondroitin sulfate	NGF	PEG	Hydrogel	Primary cortical neurite outgrowth	Butterfield et al. (2011)
b RGD	Heparin	FGF2 FGF/VEGF	PEG	Hydrogel	Survival of neural stem cells	Freudenberg et al. (2009)
c RGD	Heparin mimetic polymer	FGF2	PEG	Silicon	Angiogenesis	Zieris et al. (2010)
d FNIII9-10	Hyaluronic acid	BMP2		Hydrogel	Endothelial adhesion	Kolodziej et al. (2012)
e Laminin	Heparin	CXCL12		Nanoparticle on stainless steel	Bone formation	Kisiel et al. (2013)
f ICAM-1	Heparan sulfate	CXCL12	EG	Gold	Antithrombosis and endothelialization	Liu et al. (2017)
g RGD	Heparin	CXCL12/ FGF2/VEGF	EG	PCL-co-LC	T-lymphocyte adhesion	Migliorini et al. (2014)
					Endothelial adhesion	Clauder et al. (2020b)

Cellular response to multifunctionality

For tissue engineering approaches that chaperon multiple stages of regeneration, from cell adhesion via proliferation to differentiation, diverse stimuli should be applied. Therefore, multifunctional material coatings combining adhesion sequences, degradation domains, biophysical and mechanical cues, growth factors and alike, are required (Hamley 2017). Following this concept, varieties of multifunctional biomaterials have been developed. Selected examples of such constructs are described and summarized in Table 2. A sophisticated approach on multifunctionality was presented by Clauder and colleagues (Table 2g). The peptide coating, which simultaneously mediated surface binding, presentation of an RGD motif for cell adhesion, and a syndecan-binding peptide for heparin immobilization, enabled the release of CXCL12, FGF2, or VEGF from biodegradable scaffolds (Clauder et al. 2020b). In consequence, endothelial cell viability, differentiation, and migration was greatly improved in comparison to uncoated scaffolds and further increased in comparison to peptide alone. Similar effects have been observed for the viability and differentiation of neural stem cells (Table 2b), the survival of cells recruited by chemotactic signaling (Table 2c and f), or the extent and quality of bone formation (Table 2d).

Closing remarks

By the regulation of the location and kinetics of signal molecule presentation and release, the ECM allows a tight spatio-temporal control of the activation status of tissue cells (Mecham 2012). Several examples discussed in the previous sections stress the advantages of simultaneously addressing integrin and cytokine signaling by multifunctional biomaterial coatings. Simultaneous or sequential stimulation of multiple key players in regeneration can achieve high efficiency with low doses of cytokines, which in turn would reduce side effects and enhance cost-effectiveness. Utilization of peptide-based anchoring with incorporated DOPA units enables a strong surface immobilization on a variety of substrates. This binding peptide can be modularly decorated in a customizable way with releasable and non-releasable mediator molecules, which can be adapted to the patient's needs of acute or chronic wounds.

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