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Multi-functional biomaterials: combining materials modification strategies for engineering of cell contacting surfaces

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Abstract

In the human body, cells in a tissue are exposed to signals derived from their specific extracellular matrix (ECM) cues from architectural structure, mechanical properties and chemical composition (proteins, growth factors). Research on biomaterials, in tissue engineering and regenerative medicine aim to recreate such stimuli with engineered materials in order to induce a specific response of cells at the interface. While traditional biomaterials design has been mostly limited to varying individual cues, increasing interest has arisen on combining several features in recent years in order improve the mimicry of extracellular matrix properties. Tremendous progress in

combinatorial surface modification exploiting for example topographical features or variations in mechanics combined with biochemical cues enabled the identification of their key regulatory characteristics on various cell fate decisions. Especially gradients facilitated such research by enabling the investigation of combined continuous changes of different signals. Despite unravelling important synergies for cellular responses, challenges arise in terms of fabrication and characterization of multifunctional engineered materials. This review summarizes recent work on combinatorial surface modifications that aim to control biological responses. Modification and characterization methods for enhanced control over multi-functional material properties are highlighted and discussed. Thereby, this review deepens the understanding and knowledge of biomimetic combinatorial material modification, their challenges but especially their potential.

1. Introduction

The interaction of implant materials or engineered tissues with their environment and surrounding cells is decisive for their success. Depending on the application, different levels of integration in and interaction of the materials with the surrounding are needed. While bio-inert materials should interact minimally with the surrounding tissue, bioactive or biomimetic materials are designed to actively engage with their biological environment by directing, supporting and stimulating cellular responses.¹⁻⁵ Bio-inert materials are important in many applications, e.g. blood contacting devices that have to minimize protein adsorption and prevent blood coagulation on their surfaces.^{3, 6-7} Surface modification of materials with active or inductive properties create biointerfaces that have to be specifically designed and functionalized according to the desired target tissue or application in the body.⁸⁻¹³

Biomaterials design for tissue engineering and regenerative strategies have been traditionally inspired to mimic single aspects like mechanics, topography or a chemical cue from the complex natural extra cellular matrix (ECM). By combining at least two of those ECM characteristics on multifunctional engineered materials, mimicry of the ECM is enhanced and elucidation of the interplay between various cues and their effect on cellular behavior can provide a better understanding of material-tissue interactions.

Tissues are sophisticated and highly dynamic systems. Their multifunctional microenvironments instruct cellular behavior *via* a complex interplay of multiple biochemical and physical cues from the ECM, soluble factors, such as growth factors, cell-cell contacts as well as hydrostatic pressure.¹⁴⁻¹⁵ Depending on the tissue type, distinct compositions of these components induce different mechanics or topography, due to altered protein fibril structures and composition of the ECM, influencing cell behavior.¹⁶⁻²⁴ Cells interact with the ECM in a complex interplay of biochemical and mechanobiological processes. For instance, the adhesion to ECM proteins is mediated *via* transmembrane proteins (integrins) that recognize specific amino acid sequences (adhesion peptides).²⁵ However, the generally inactive integrins need to be activated by focal adhesion proteins, which in turn are activated by transmembrane kinases or growth factor receptors.^{17,26} The resulting focal adhesion complexes respond to physical and mechanical stimuli by interaction with and alteration of cytoskeletal organization.²⁷

Most tissues, however, are not defined by a definite mechanical value, one topographical feature or fixed ECM and growth factor composition, but consist of gradients or heterogenic distributions. Biophysical and biochemical gradients are known to steer cellular migration and differentiation during embryogenesis, tissue remodeling and regeneration, inflammation or disease.²⁸⁻³¹ Gradual distributions of such properties are also a prominent structural hallmark in tissues that bridge between two distinct types of tissues, such as in the tendon-to-bone transition zone or in the osteochondral tissue.³²⁻³³

Since most unmodified synthetic materials do not present suitable surface properties for the interaction with living cells and tissues, they require modification or functionalization. To this end, many strategies have been developed to design specific biointerfaces mimicking individual characteristics of natural ECM of the target tissue.^{5, 11, 34-40} (Fig. 1)

Approaches to control cell behavior through the design of biomaterial surfaces have been reviewed recently^{11, 41-42} and include, for example, surface functionalization with ligands for direct interaction with cellular transmembrane receptors, such as ECM proteins, growth factors or their functional fragments (peptides). Cellular adhesion, cytoskeleton formation and differentiation can also be influenced by engineering mechanical properties of biomaterials to match the respective cellular environment.⁴³⁻⁴⁵

Figure 1 Schematic representing the complexity of the extracellular environment in general and the natural extracellular matrix in particular. Recreating this, combinatorial materials functionalization represents a strategy towards biomimetic materials design.

Cell behavior is furthermore strongly influenced by the surface roughness (density and dimension of presented topographical surface patterns) of biomaterials,⁴⁶ with a highly cell-type specific response to the range and scale of topographical features.⁴⁷⁻⁵¹ Especially, differentiation, gene expression and cell fate decisions of different cell types are affected by various surface topographies.⁵¹⁻⁵⁵ Although the size of individual cells is in the micrometer range, they interact with submicron-topographical features by actin-fibril-driven filopodia⁵⁵, which contain integrin receptors that identify surface features of just 10 nm in height.⁵⁶⁻⁵⁷ In this context, studies with ordered and disordered RGD-functionalized gold nanopatterns showed that a distance of around 70 nm between RGD ligands was the crucial ligand density for adhesion of various cell types.⁵⁸⁻⁶²

Furthermore, biochemical, mechanical or structural gradients can be created to mimic naturally graded tissue or to study cell migration as a function of changing substrate properties.^{39, 63-69} In the following sections, synergistic effects of combinatorial material modifications on different biological systems and characterization of such systems are presented and discussed, while the ones that currently provide the best control over material properties are highlighted.

2. Synergistic effects of multifunctional materials on cellular interaction

2.1 Combining chemical surface modifications

Combinations of various ECM proteins, growth factors and their fragments

Modification of a surface by a combination of several ECM proteins usually promotes cell attachment, spreading and survival. This was for instance shown by increased neurite formation and length of neural cells on combinations of collagen I (Col I) with fibronectin (FN) or laminin (LM) compared to cells seeded on pure collagen I modified surfaces.⁷⁰ In addition, combinations of adhesion peptides enhanced the attachment, spreading and survival of endothelial cells⁷¹⁻⁷⁵, neuronal cells⁷⁶, fibroblasts⁷⁶⁻⁷⁷ or osteoblasts⁷⁷.

Co-immobilizing RGD (from fibronectin) and YIGSR (from laminin), enhanced for example endothelial cell adhesion, as measured by cell number and spreading, compared to immobilized single peptides.⁷²⁻⁷⁵ This combination has also been shown to promote neurite outgrowth of dorsal root ganglions.⁷⁸ (Fig.2a) The presence of other peptide combinations, such as RGD and KRSR or combinations of laminin fragments also cooperatively enhanced the attachment, spreading and proliferation of fibroblasts,⁷⁶⁻⁷⁷ demonstrating a strong dependence of the cell response on the exact ratio of incorporated peptides.⁷⁶ Interestingly, however, Castellanos *et al.* demonstrated that the combination of RGD and REDV, where the latter is known as an endothelial specific peptide derived from fibronectin, did not enhance adhesion of endothelial cells.⁷¹ Hence, the choice of molecule(s), as well as their concentration and ratio need to be adjusted for each cell type, desired functionality and target tissue.

Figure 2 Combination of adhesion motifs, growth factors or both for synergistic effects on cells: a) Co-immobilization of RGD and YIGSR peptides enhanced neurite outgrowth from dorsal root ganglions (scale bar = 500 μm ; adapted with permission from Zhu *et al.*⁷⁸. Copyright © 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim). b) Combination of epidermal growth factor and VEGF enhanced survival of endothelial cells (scale bar = 200 μm ; adapted and reprinted from Lequoy *et al.*⁷⁹. Copyright (2016), with permission from Elsevier). c) Surface functionalization with BMP-2 mimetic peptide and RGD increased osteogenic marker expression (i.e. RunX2) in human bone marrow stem cells (scale bar = 50 μm ; adapted and reprinted from Bilem *et al.*⁸⁰. Copyright (2016), with permission from Elsevier).

Additional to steering attachment and adhesion, the stimulation of cell proliferation, quiescence or differentiation is essential for the performance of a biomaterial. To this end, different strategies to stimulate angiogenesis⁸¹, endothelial cell functionality^{79,82}, hepatocyte⁸³ but also bone or cartilage differentiation⁸⁴ have been successfully demonstrated by modifying materials with specific combinations for growth factors. (Fig. 2b) For instance, the functionalization of collagen scaffolds with a combination of VEGF and Angiopoietin-1 induced enhanced angiogenesis compared to their

single factor functionalized controls, which was particularly evident, when the molecules were covalently immobilized rather than being physisorbed.⁸¹ The co-immobilization of epidermal growth factor and VEGF at a molecular ratio of 4:1 on chondroitin sulfate scaffolds promoted significantly enhanced endothelial attachment and survival. However, other ratios had the opposite effect, resulting in decreased cell attachment compared to VEGF-only controls.⁷⁹ This concentration- or ratio-dependent outcome highlights the importance of gaining a better understanding on possible synergistic/antagonistic effects of combined biochemical surface modifications to improve biomaterial designs.

The combined stimulation of adhesion and differentiation by co-immobilization of growth factors and adhesion molecules has been demonstrated in concepts including but not limited to osseointegration⁸⁵⁻⁸⁷, endothelialization and angiogenesis^{82, 88-89} or liver regeneration⁸³ (see one example in Fig. 2c). While co-immobilizing RGD with epidermal growth factor enhanced hepatocyte survival and functionality,⁸³ combining it with VEGF or basic fibroblast growth factor improved endothelial cell functionality.^{82, 88} The same effect could be observed for combinations of RGD with different growth factor derived peptides: The combination of RGD with VEGF mimetic QK peptide synergistically enhanced the angiogenic potential of endothelial cells.⁹⁰ Targeting a different tissue, RGD in combination with a BMP-7 mimicking peptide (CGQGFSYPYKAVFSTQ) had a beneficial effect on osteogenic differentiation of mesenchymal stem cells.⁹¹ Brigaud *et al.* studied the synergistic effect of fibronectin with BMP-2, BMP-6 or BMP-7 adsorbed on titanium-hydroxyapatite samples on the osteogenic commitment and differentiation of C2C12 cells. They observed that BMP-6 was most potent at lower concentrations alone, but BMP-2 was most potent when combined with fibronectin.⁸⁵ This highlights the importance of addressing both the concentration as well as the very nature of the molecule.

Combinatorial gradients of biochemical components

As it was observed in various of the studies above, not only the choice of combined chemical signals, but also their specific concentration and ratio influences the response of cells. To this date, the exact concentrations and ratios of such signals in natural tissues are unknown. In order to strategically analyze mixing ratios and different concentrations of immobilized molecules, preferably arrays and gradients can be used instead of preparing large numbers of samples with different, independent mixing ratios and concentrations. Arrays offer a multitude of different

discrete spots to which a cocktail of molecules can be tethered on one single substrate.⁹² Gradients on the other hand exhibit gradually increasing concentrations or densities of immobilized molecules on a sample and thereby allow for screening of different ratios, concentrations or groups within a single experiment, thus providing higher throughput of experiments at higher levels of complexity.^{84, 86, 92-93} Notably, surface gradients can be prepared according to the experimental design along the x- or y-axis of a sample in parallel, orthogonal or reverse parallel manner.^{84, 86, 93} Using an array approach Koepsel *et al.*⁹² and Zhang *et al.*⁹⁴ compared different mixing ratios of different BMP mimicking peptides with RGD or YIGSR to demonstrate the dependence of osteogenic induction by BMP peptides on the amount of the adhesion peptides. With their arrays, they achieved up to 120 spots on one sample, with up to 16 different groups of peptide mixtures and 9 replicates per condition.^{92, 94} This work could facilitate the further understanding of peptide combination and concentration specific effects. However, since array spots contain discrete concentrations over a certain range, optimized concentrations or ratios might be missed.

Dual gradients with continuous concentration changes on the other hand were successfully used for identification of molecule ratios showing synergistic effects on osteogenic differentiation (orthogonal or parallel gradients)^{86, 93, 95}, for migration studies (parallel gradients)⁹⁶ or recreation of tissue transition zones, such as the tendon-to-bone region (reverse gradients)^{66, 84, 97-98}. Using a parallel concentration gradient of RGD and BMP-2 mimetic peptide, Moore *et al.*⁸⁶ demonstrated that the combination of both components at the highest concentration (130 pmol/cm² combined) synergistically promoted mineralization of human bone marrow stromal cells *in vitro*.⁸⁶ Additionally, Ma *et al.*⁹⁵ created orthogonal gradients of RGD on the x-axis and BMP-2 on the y-axis of the samples and could reveal that osteogenic differentiation is highest at higher RGD (83 pmol/cm²) concentrations combined with lower BMP-2 concentrations (0.5 pmol/cm²) (Fig. 3a). The results of these studies are partially contradictory. They highlight the strong dependence of the outcome on the study design (parallel vs. orthogonal gradient). Furthermore, the exact concentration of each molecules is not known on the parallel gradient.⁹⁵

By creating parallel gradients of fibronectin and VEGF and single component gradients of each as control, Liu *et al.*⁹⁶ could show that cell migration from low to high concentrations was significantly increased on the dual gradient, compared to the single gradients. While this demonstrates that a combination of chemical cues can enhance cell migration on parallel gradients,

it is interesting that the slope of the concentrations along the gradient did not have a significant influence.⁹⁶ Using reverse dual gradients with BMP-2 and platelet derived growth factor type BB (PDGF-BB), Ki *et al.*⁸⁴ demonstrated osteogenic differentiation of adipose derived stem cells (ADSCs) on the BMP-2 rich end and differentiation towards tenocytes on the PDGF-BB rich end (Fig. 3b).⁸⁴ Such reverse gradients are a powerful tool to study or recreate tissue interfaces, such as the here presented bone-tendon interface.

Figure 3 Biochemical ligand gradients: a) Orthogonal gradient of RGD and BMP-2 peptides allowed to identify optimized combinations and ratios for osteogenic differentiation (adapted and reprinted with permission from Ma *et al.*⁹⁵. Copyright (2016) American Chemical Society). b) Interfacial tissues, such as tendon to bone can be replicated with reverse parallel gradients of for instance BMP-2 and FGF (Adapted and reprinted with permission from Guan *et al.*⁹⁷. Copyright (2016) American Chemical Society).

Various studies clearly demonstrated a strong dependency of the cell response on concentration and ratio of the presented peptides or proteins.^{58, 76, 79, 85, 99} Although current studies focused on the combination of two molecules, the search for enhanced understanding forces researchers to increase the number of “signals” to the point where cues from natural tissues are better reflected. In the next step, it will be interesting to study how the interplay of three molecules influences cell behavior. While methods for the fabrication of three-component gradients have already been developed¹⁰⁰⁻¹⁰¹, immobilization of the biological molecules and subsequent gradient

characterization as well as analyzing cell interaction have yet to be achieved. Furthermore, multi-component chemical surface modification mimics only one aspect of natural tissues. Table 1 summarizes studies combining different chemical surface modifications. For recapitulation of natural tissues, researchers also need to consider surface topography and substrate mechanical properties.

Table 1 Overview of different approaches for chemical surface modification and the respective characterization method. Studies are divided into four groups, depending on the immobilization or characterization approach: molecule specific immobilization and characterization, molecule specific immobilization and unspecific characterization, unspecific immobilization and molecule-specific characterization, and unspecific immobilization and characterization.

Specific immobilization, molecule-specific characterization

Substrate	Chemistry	Molecules	Characterization method	Cell type	Biological effect	Ref.
poly[(4-methylpropiolate-p-xylylene)-co-(p-xylylene)]	alkyne-azide click reaction and thiol-yne reaction; gradients	FGF and BMP-2	IRRAS, WCA, fluorescence	fibroblasts (3T3)	FGF supported cell proliferation on one side of the reverse gradient, BMP-2 induced bone formation on the other side of the gradient.	97
PEA and PMA	adsorption	VEGF, affinity bound to FN	AFM, ELISA, VEGF specific immunogold reaction	endothelial cells (HUVEC)	FN and VEGF induce tube formation of HUVEC. <i>In vivo</i> evaluation in a murine fat pad model induced vascularization.	89

Specific immobilization, unspecific characterization

Substrate	Chemistry	Molecules	Characterization method	Cell type	Biological effect	Ref.
UV-ozone activated glass slide	thiol-ene and SPAAC reactions	RGD and BMP-2	XPS	mesenchymal stem cells (hMSC)	Synergistic and concentration dependent upregulation of osteogenic specific genes after 14 days.	95
PEG-ylated and gold nanoparticle decorated glass slide	CuAAC click reaction and gold-sulfur affinity	RGD and PHSRN	chymotrypsin assay, QCM	Fibroblasts (REF WT)	RGD and PHRSN synergistically enhanced cell attachment, spreading and focal adhesion assembly.	102
PLLA	EDC/NHS coupling and direct solution spinning	RGD, BCP	XPS, SEM, thermogravimetric analysis	mesenchymal stem cells (hMSC)	RGD and BCP synergistically enhanced adhesion, spreading and ALP activity.	87

Unspecific immobilization, molecule-specific characterization

Substrate	Chemistry	Molecules	Characterization method	Cell type	Biological effect	Ref.
Dextran	click chemistry and coiled coil (K5-E5)	RGD (click) and VEGF (coiled coil)	¹ HNMR, Ellmanns assay of peptide supernatant, orange II assay,	endothelial cells (HUVEC)	Combining VEGF with RGD enhanced proliferation and attachment of HUVEC.	88

Chondroitin-sulfate	Coiled-coil (Kcoil/Ecoil)	EGF and FGF	ELISA	endothelial cells and smooth muscle cells (HUVEC and AoSMC)	EGF and VEGF synergistically enhanced HUVEC and SMC attachment and viability.	79
Poly(L-lysine)/Hyaluronic acid	LbL adsorption and microfluidics; gradients	BMP-2 and BMP-7	fluorescence	skeletal myoblasts (C2C12)	Synergistic effect of BMP-2 and BMP-7 on osteogenic differentiation.	103
Collagen	EDC/NHS coupling	VEGF and Angiopoietin-1	indirect ELISA (supernatant)	murine heart endothelial cells (H5V)	VEGF and Ang-1 synergistically enhanced cell proliferation and tube formation.	81
PCL/F127	heparin immobilization; gradient	PDGF-BB and BMP-2	toluidine blue and ELISA	adipose derived stromal cells (ASC)	A reverse gradient revealed increased tendon specific protein expression on the PDGF-BB side, and increased bone specific protein expression on the BMP-2 side.	84
PS plates	Sulfo-SANPAH chemistry	EGF and IGF	fluorescence and ELISA)	keratinocytes (HaCaT)	EGF and IGF collectively influenced keratinocyte migration and morphology.	104

Unspecific immobilization, unspecific characterization

Substrate	Chemistry	Molecules	Characterization method	Cell type	Biological effect	Ref.
Gold coated glass cover slips	SAMs and EDC/NHS coupling	RGD and BMP-2 or heparin mimicking peptides	fluorescence	mesenchymal stem cells (hMSC)	Strongest osteogenic differentiation on RGD combined with BMP-2 peptide.	92
UV-ozone activated glass slide	SAMs and bifunctional PEO linker	RGD and BMP-2 derived peptide (KPSS)	XPS, WCA	mesenchymal stem cells (hMSC)	Synergistic upregulation of osteogenic markers by RGD and BMP-2 peptides.	86
Quartz substrates	SAMs and Michaels addition	cyclic RGD and bone forming peptide-1	WCA, XPS	mesenchymal stem cells (MSC)	Synergistic upregulation of osteogenic markers when combining RGD with BMP-7 derived peptide.	91
Gold coated glass cover slips	SAMs and CuAAC click chemistry	RGD, YIGSR, KRSR, KPSS	IR, XPS, Ellipsometry, AFM	adipose derived stromal cells (hADSC) and mouse embryonic fibroblasts (MEF)	Combination of adhesion peptides (RGDS and YIGSR) with BMP-7 derived peptide upregulated bone specific protein expression.	94

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3	Gold coated glass	SAMs and	FN and VEGF	FTIR, XPS, SPR	bovine aortic	BAEC migrated significantly faster on	96
4	cover slips	EDC/NHS			endothelial cells	combined gradients of FN and VEGF compared	
5		coupling			(BAEC)	to single component gradients and compared to	
6						non-gradient controls.	
7	Titanium-	adsorption	FN and BMP-2 or	BCA assay (amount of molecule),	Skeletal myoblasts	Synergistic effect of FN with either BMP-2,	85
8	Hydroxyapatite		BMP-6 or BMP-7	SPR (adsorption kinetics)	(C2C12)	BMP-7 or BMP-6 on the osteogenic	
9						differentiation.	
10							
11	PU-PEG	EDC/NHS	RGD, YIGSR	WCA, AFM, amino acid analysis,	endothelial cells	RGD and YIGSR synergistically enhanced EC	73
12		coupling		XPS	(HUVEC)	viability.	
13							
14	Silk-fibroin	EDC/NHS	RGD and KRSR	absorbance of peptide solutions	osteoblasts (NHO),	RGD and KRSR peptide synergistically	77
15		coupling		(UV/VIS),	and fibroblasts	improved cell attachment, spreading and	
16					(NHDF)	viability.	
17	Aminated PU-PEG	EDC/NHS	RGD, YIGSR,	toluidin blue, amino acid analysis	endothelial cells	Combination of RGD, YIGSR and heparin led	74
18		coupling	Heparin		(HUVEC)	to enhanced cell viability and vascular graft	
19						patency.	
20							
21	PLCL	Polydopamine	RGD and bFGF	Fluorescamine assay and ELISA,	endothelial cells	Immobilized RGD and bFGF enhanced	82
22		(Michael type		XPS	(HUVEC)	HUVEC viability and interconnected	
23		addition)				monolayer formation.	
24							
25	Cobalt chrome	CPTES silanization	RGD, REDV,	XPS, surface charge, QCM-d	endothelial cells	Only the combination of RGD and YIGSR	71
26		and thiol reaction	YIGSR		(HUVEC)	induced strong synergistic increase in cell	
27						attachment.	
28	PCL-PLGA	urea bond between	RGD, YIGSR	fluorescamine assay	endothelial cells	Synergistic improvement on cell attachment of	75
29		peptide and NCO-			(HUVEC and	RGD and YIGSR.	
30		PEG			HPMEC)		
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2.2 Combining biochemical and topographical surface modification

Effects of different spatial surface topographies combined with biochemical ligands on cell behavior

The density and also the size (macro, micro, nano dimensions) of topographical patterns, which are introduced on the surface of materials, strongly influence cell behavior. Diverging observations have been described regarding the effects of macro-, micro- or nano-roughness on cell function, with responses being highly cell type specific.^{5, 105-106} For instance, an increased surface roughness (nano- to micrometer-range) promoted proliferation of osteoblasts⁵⁰, but decreased fibroblast proliferation.⁴⁷⁻⁴⁸ Additionally, surfaces with micro-scale features led to decreased proliferation, but enhanced gene expression of osteoblasts.^{48, 107-108} These studies came to similar conclusions, although topographies differed in their height and width as well as in the fabrication approach. Besides the roughness dependent differentiation of MSC^{49, 109}, also early inflammatory responses of macrophages were enhanced on 2D-films or micro-fibrous PLLA scaffolds compared to nano-fibrous substrates¹¹⁰. In these approaches the cell responses to the materials were generally influenced by the present topography as well as by unspecific protein adsorption from serum-supplemented culture medium to the sample surface.

In this context, Macgregor *et al.*¹¹¹ demonstrated that the dimension of topographical surface nanopattern (16 nm, 38 nm or 68 nm) could influence the conformation of the protein immobilized on the surface. This fact influenced the cell-surface interaction, in this case binding of mouse embryonic stem cells (mESC) to FN. They showed that FN could not bind to the surface when 16 nm surface topographies were present, as FN displayed similar size (15 nm length, 9 nm width), which in turn didn't occur for larger patterns.¹¹¹ Besides surface chemistry, existing surface topographies could lead to protein denaturation and conformational changes after adsorption on the surface, which in turn could lead to a loss of function or bioactivity impairing cell interaction.^{5, 9, 106, 112-113} Furthermore, such conformational changes could lead to toxic effects or inflammation¹¹³⁻¹¹⁴, which are unwished side effects. However, controlled protein adsorption allowed immobilization of attachment sites on a material surface for guiding cellular interaction.^{9, 106, 112} Thus, to better control the surface characteristics, surface topography can be further complemented

by the functionalization with biochemical ligands. In general, combining different surface topographies and ECM-derived stimuli enables the investigation of synergistic or antagonistic effects on cell behavior and ultimately to generate ECM mimicking cellular microenvironments.¹¹⁵⁻¹¹⁸

It was shown that the size of topographical structures from nano- to micro-patterns influenced cell adhesion, spreading and proliferation, while chemical ligands had an impact on self-renewal and differentiation of osteoblasts and stem cells.^{111, 119} For example, the outward surface modification with spatial hut, dome or pyramidal nanostructures, functionalized with RGD or FN-coating, led to enhanced proliferation of MSC and drove endothelial cell adhesion. While the cytoskeleton of endothelial cells was arranged around the pyramids, the focal adhesion formation of MSC was highest on FN-modified, hut-nanostructured (3 nm height, 35 nm width) surfaces, verifying the important influence of nanostructures for these cells.¹¹⁵⁻¹¹⁶ However, surface functionalization with RGD-modified pyramids in the micro-scale (height $\sim 2 \mu\text{m}$) hindered endothelial cell attachment. Le Saux *et al.*¹¹⁶ showed that the density of surface exposed RGD drove spreading, focal adhesion formation and internal organization of cells, while size and scale of surface topography mainly influenced the number of surface attached cells. This led the authors to propose a two-step model of cell adhesion: initial cell contact to a substrate surface is dominated and guided by topographical surface features, but the attachment is driven by the interaction of cellular surface receptors with surface exposed chemical cues.¹¹⁶ Another study showed that osteogenic differentiation of hMSC could be enhanced on surfaces with BMP-2 peptide modified nanopatterned surfaces in presence and absence of osteogenic differentiation medium.¹²⁰

Combined gradients of nanopatterns and proteins, for instance FN and vitronectin, have been investigated to understand synergistic effects on cell behavior, which is especially relevant for tissue transition zones.¹²¹⁻¹²³ For example, it was shown that spreading and cytoskeleton organization of C2C12 myoblasts increased along topographical gradients of different nanopatterns (bars, rings and dot pairs) functionalized with vitronectin, and that the pattern length, but not its area, was the critical parameter for cell spreading.¹²² On a parallel gradient of BSA/lysozyme and surface roughness in the nano-scale, fibroblasts showed increased cell adhesion in direction of the gradient.¹²³ Osteoblasts, however, showed decreased spreading and proliferation along a uniformly RGD-decorated nanoparticle-density gradient.¹²⁴

Biochemical ligand functionalized indentations and grooves support cell alignment and differentiation

One aim of combining surface topography with chemical functionalities is to understand and mimic (at least partially) the influence of the ECM on stem cell differentiation^{111, 120, 125}, skeletal muscle tissue engineering¹²⁶⁻¹²⁷, peripheral nerve regeneration¹²⁸ and development of functional cells, such as neurons¹²⁹⁻¹³⁰, or on cellular transformation into cancer¹³¹. Thereby, shape-restriction of biochemical ligands could be used to guide and control cell attachment and differentiation on biomaterial surfaces. For example, MSC displayed more and larger focal adhesions as well as enhanced differentiation towards the osteogenic fate on 100 nm deep indentations, modified with RGD, compared to those with 10 nm depth.¹²⁵ While outgrowth of neuronal cells was rapid on continuous, shallow poly-L-lysine surface features in the micrometer-range, it was delayed on interrupted ones. This demonstrated that controlled neuron adhesion and outgrowth were regulated by a chemical compound, whereas topography provided guidance for neurons.¹³⁰

Furthermore, grooves (from the nanometer- to the micrometer-scale) combined with biochemical ligands were shown to promote alignment and differentiation of cells, including MC3T3-E1 osteoblasts¹³², MG-63 osteoblast-like cells¹³³, C2C12 myoblasts^{126-127, 134}, PC12 cells¹²⁹, hMSC¹²⁰, anterior cruciate ligament fibroblasts (ACLF)¹³⁵ and human corneal epithelial cells (HCEC)¹³⁶⁻¹³⁷. Studies combining such grooves with RGD^{126-127, 133, 135-137}, YIGSR¹²⁷, a LM-derived peptide PPFLMLLKGSTR¹²⁹, BMP-2 peptides¹²⁰, FN¹³⁴, Col I^{129, 133} or neuronal GF¹²⁸ all demonstrated beneficial and conducive effects on adhesion, growth, differentiation or alignment of myoblasts (Fig. 4)^{126-127, 134}, HCEC¹³⁶⁻¹³⁷, hMSC¹²⁰, ACLF¹³⁵, osteoblast-like cells¹³³ or PC12 cells¹²⁸⁻¹²⁹. The adhesive ligand significantly enhanced attachment, growth and differentiation of cells, while grooves directed the cellular morphology, alignment and elongation along the topographical pattern (Fig. 4).^{126-129, 133, 135}

On surfaces combining microgrooves (4 μm height, 8 μm width) with orthogonally arranged FN microlanes (10 μm width), osteoblasts were predominantly influenced by the topographical feature and aligned preferentially along the grooves.¹³² Similarly, fibroblasts showed clearly increased cell elongation and alignment along a parallel, linear, micro-structured FN gradient.¹²¹ Another study demonstrated that a microgroove (2 μm height, 20 μm width) induced contact guidance of MG-63 osteoblast-like cells could only be interrupted by an amino functionalization, but not by coupling Col I or RGD on the patterned surface.¹³³

In addition, one study, combining grooves in the sub- to micrometer-range (200 nm to 2 μ m width) with RGD and different cell culture media, confirmed that cell responses to functionalized surfaces were not only guided by topographical and chemical cues, but were also dependent of present soluble factors in the cell culture media, as HCEC alignment differed in serum-containing or GF-defined, serum-free media.¹³⁶ This observation could be confirmed on BMP-2 peptide functionalized nano-grooves, where osteogenic differentiation and cell behavior of hMSC was affected by present soluble factors of an osteo-inductive medium: While the cells aligned to modified grooves in standard growth medium, their spreading and calcium deposition was enhanced in presence of an osteogenic induction medium.¹²⁰

Figure 4 Synergistic influence of spatial surface topography (grooves) and biochemical ligands on cell behavior. Sub-micron grooves on a polystyrene (PS) surface guided alignment and elongation of C2C12 myoblasts along the grooves, while RGD functionalization directed their spreading, growth and differentiation by interaction with cellular surface receptors. The grooves displayed widths from 670 nm to 920 nm and depths of around 670 nm (Cartoon for illustration only, not for scale). Immunofluorescent staining of nuclei (DAPI) and F-actin in C2C12 myoblast was done after 24 h in culture (A, a, C, c). The scale bars in the fluorescence (A, a, C, c) and SEM (B, b, D, d) images are 25 μ m and 10 μ m, respectively. The arrow displays the groove direction. (Images adapted and reprinted with permission from Wang *et al.*¹²⁶. Copyright © 2012 Wiley Periodicals, Inc.).

Fibers combined with biochemical ligands support cellular responses and function

Fibrous scaffolds as an overall system represent hierarchical surface topography and structures mimicking the physical properties of the ECM. However, single fibers of the substrate show their own inherent surface topography adding an additional dimension of topographical cues, such as different fiber surface roughness or pore depth, to influence cell response. For instance, this nano-roughness and nano-pores led to different osteogenic gene expression of hMSC¹³⁸, enhanced growth of A-172 neuronal cells¹³⁹, different astrocyte mediated neurite outgrowth¹⁴⁰ or increased adhesion, proliferation and gene expression of vascular SMCs¹⁴¹. Moreover, fibrous topographies from the nano- to the micro-scale combined with ECM-derived ligands, such as laminin, RGD and VEGF, have been shown to support engineering of endothelial¹⁴²⁻¹⁴⁹ or neuronal tissue.¹⁵⁰⁻¹⁵² Compared to unmodified fibers, endothelial cell adhesion, proliferation, migration and function were clearly enhanced on electrospun nano- or micro-fibrous poly(ϵ -caprolactone) (PCL), poly(L-lactic acid-co- ϵ -caprolactone) (PLLCL) or blended PCL/gelatin and PCL/poly(3-hydroxybutyrate-co-3-hydroxyvalerate) non-woven meshes modified with RGD^{142, 144} or with immobilized VEGF^{143, 145-146, 148-149}. The spatial functionalization of fibrous PCL scaffolds in the sub- and micrometer-range with osteopontin-derived and heparin-binding peptides by thiol-maleimide coupling enabled cell-specific adhesion of endothelial cells (HUVEC) and pericytes to promote endothelization and angiogenesis.¹⁴⁷ In another example, adhesion, proliferation, migration and axonal growth of Schwann cells was shown to be enhanced on laminin-modified core-shell poly(L-lactide-co-caprolactone) (PLCL) nanofibers¹⁵² and guided on PCL/PEG blend microfibers functionalized with RGD compared to scaffolds without RGD.¹⁵¹ PC12 cells also demonstrated increased viability on RGD-modified poly-(L-lactic acid) (PLLA) nanofibers and higher neurite outgrowth on blended LM-PLLA nanofibers (diameter ~ 100 nm to 500 nm).¹⁵⁰ The fibrous structure of such substrates was shown to enable the adhesion, migration and growth along the fibers, while the biochemical ligands represent chemo-attractive, cell-stimulating factors.¹⁴²⁻¹⁵³ A recent study showed that initial adhesion of bone murine stromal ST-2 cells was topography-driven and even possible on unmodified electrospun PCL microfibers (diameter ~ 1.3 μ m to 1.6 μ m), but proliferation rate was clearly enhanced on FN functionalized PCL fibers.¹⁵⁴ Table 2 summarizes studies combining chemical and topographical surface modifications and the respective characterization methods as well as the biological response.

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Table 2 Overview of studies combining chemical and topographical surface modifications and the respective characterization methods. The biological response *in vitro* is briefly stated.

Different spatial surface topographies combined with biochemical ligands

Substrate	Preparation method	Topography & Range	Topographical characterization	Immobilized molecules	Immobilization method	Chemical characterization	Cell type	Biological effect	Ref.
PLA with hydroxyapatite (HAP)	HAP precipitation on Gly, Arg or Asp	rough and irregular surface patterns in the nanometer-range	SEM, AFM, WCA	serum proteins	protein adsorption	BCA assay	human osteoblasts (hFOB 1.16)	Increased hFOB proliferation with increased topological roughness.	50
Titanium disks with aluminum particles	sandblasting of Al ₂ O ₃ particles	rough and irregular surface patterns in the sub- and micro-meter-range	profilometry, SEM, EDX	FN, RGD and/or PHSRN	coupling by tresyl chloride	ELISA	osteoblasts (MC3T3)	FN and peptides enhanced MC3T3 adhesion, proliferation and differentiation.	119
Tantalum coated Ge-Si surfaces	molecular beam epitaxy (MBE) deposition and tantalum coating	variety of nanometer-scaled hut and dome structures	AFM, XPS	FN	protein adsorption	ellipsometry, antibody binding	human dental pulp derived stem cells (DP-MSC)	DP-MSC proliferation was enhanced on topographical surfaces.	115
PEI	electrostatic adsorption silica particles to PEI	nanoparticle-density gradient	SEM, AFM	RGD	PLL-g-PEG-RGD	cell staining after adhesion	rat calvarial osteoblasts (RCO)	RCO adhesion and spreading increased on unstructured surfaces, decreased with increasing particle gradient.	124
Silicon oxide	hole mask colloidal lithography and angled physical vapor deposition	nanopattern-gradients with varied bar length, ring diameter, dot pair separation	SEM, QCM	vitronectin	protein adsorption	cell staining after adhesion	skeletal myoblasts (C2C12)	Best at beginning of the gradient, ring: increased spreading with increasing ring diameter, bar: FA elongated with elongation of the pattern.	122
Silicon	time-dependent wet chemical etching	nano- and micro-scaled pyramids	AFM	RGD	covalent coupling by urethane bonding	cell staining after adhesion	bovine aortic endothelial cells (BAEC)	RGD density drove spreading, focal adhesion formation of EC, while size and scale of pyramids guided adhesion.	116

PAAM	microcontact printing of PDMS stamp	various shapes (round, star-shaped, oval) in the micrometer range	immunofluorescence imaging	FN, Col I, LM	covalent coupling of aldehyde-hydrazine groups	cell staining after adhesion	mesenchymal stem cells (hMSC)	Neurogenesis occurred protein-dependent and protein-independent on anisotropic patterns, adipogenesis observed on FN and if spreading is restricted.	155
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Grooved and longitudinal surface topographies combined with biochemical ligands

Substrate	Preparation method	Topography & Range	Topographical characterization	Immobilized molecules	Immobilization method	Chemical characterization	Cell type	Biological effect	Ref.
HMDS	patterning by reactive ion etching	continuous and interrupted patterns in the micrometer-range	SEM, AFM	PLL patterns	acetone liftoff after adsorption of PLL into etched trenches	FITC-PLL	rat hippocampal cells (E18)	Rapid neurite outgrowth on continuous lines, delayed outgrowth on interrupted lines.	130
PET	photolithography	linear patterns in the micrometer-range	FITC-labeled peptides, XPS	SVVYGLR peptide	EDC/NHS coupling	FITC-labeled peptides, XPS	endothelial cells (HUVEC)	Narrow linear SVVYGLR-micropatterns enhanced spreading, orientation, migration and morphogenesis of HUVEC.	156
ODTS	multiphoton excited photochemistry	parallel series of individual linear micro-structured gradients	immunofluorescence imaging	FN	multiphoton excited photochemistry coupling	fluorescence	fibroblasts (NIH 3T3)	Increased fibroblasts elongation/alignment along a linear, structured gradient with increasing FN concentration.	121
PLLA	dry phase inversion, casting of polymer particles in longitudinal patterns	longitudinal, topographical surface micropatterns	SEM	β -NGF	coupling by ester-amine reaction, self-assembly, protein-protein interactions	XPS	neuroblasts (PC12)	PC12 aligned along the topographical pattern, β -NGF enhanced growth, highest neurite extension at end with highest β -NGF amount.	128
PC	hot embossing with a silicon master	holes, grooves and ridges in the micrometer-range	SEM, AFM	FN	coupling to CH ₃ -by HDT	immunostaining	primary and skeletal (C2C12) myoblasts	Alignment of primary and C2C12 myoblasts to ridges and grooves, no influence on myogenesis.	134
PUA/pGMA	dispensing on a silicon master fabricated by photolithography	grooves and dots in the nanometer-range	SEM, AFM	BMP-2 peptide	epoxy-amine coupling via ring-opening reaction	fluorescamine assay of supernatants, XPS	mesenchymal stem cells (hMSC)	Enhanced osteogenic differentiation in presence of BMP-2 peptides and nanopatterns.	120

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3	PS	casting on a master silicon wafer	grooves in the nanometer-range	AFM	RGD	interaction with dopamine coating	ESCA	anterior cruciate ligament fibroblasts (ACLF)	Elongation and alignment of cells increased with increasing groove depth, RGD enhanced proliferation and spreading, best cell behavior on 700 nm grooves and RGD.	135
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9	PET	time-dependent UV-exposure (254 nm)	micropatterns with depth variation in the nanometer-scale	optical 3D profiler system	RGD	EDC/NHS coupling	XPS, high resolution beta-imager ([³ H] lysine)	mesenchymal stem cells (hMSC)	100 nm pattern enhanced adhesion and osteogenic differentiation of hMSC, 10 nm pattern enhanced adhesion.	125
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13	PLGA	solvent-assisted micromolding method of PLGA:PLL-peptide	ridges and grooves in the sub-micrometer-range	AFM, SEM	RGD, YIGSR	EDC/NHS coupling to PLL	ESCA, fluorescence	skeletal myoblasts (C2C12)	Grooves induced C2C12 alignment; RGD had a higher impact than YIGSR on growth and differentiation.	127
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18	PS	electron beam lithography and dry etching a PDMS mold	grooves in the sub-micrometer-range	AFM, SEM,	RGD	EDC/NHS coupling	ESCA, water contact angle	skeletal myoblasts (C2C12)	C2C12 cell attachment increased after RGD modification, alignment along the grooves, highest differentiation when combined.	126
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23	PEGDA	casting on a PDMS mold	grooves in the sub- and micrometer-range	AFM	RGD	Michael-type addition	cell staining after adhesion	human corneal epithelial cells (HCEC)	Parallel alignment of HCEC along the grooves.	137
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28	PEI/PVDMA	layer-by-layer coating on a PDMS stamp	grooves in the sub- and micrometer-range	AFM	RGD	coating with peptide and D-glucamine	cell binding assay with soluble peptides	human corneal epithelial cells (HCEC)	Cell alignment dependent on the scale of the topographical cue, best on 400 and 4000 nm pitches	136
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32	PLGA	solution casting and laser ablation	grooves in the micrometer-range	surface profile control technique	Col I, LM-peptide (PPFLMLLKGS TR)	coating	FITC-conjugated LM-peptide	Neuroblasts (PC12)	Enhanced neurite alignment and parallel outgrowth in grooves, LM-peptide and 5 μm grooves showed parallel outgrowth, decreased neurite number and length on micropatterns	129
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38	Polyimide	Hot embossing with a silicon master	grooves in the micrometer-range	SEM	FN	μCP by PDMS stamps coated HDT for	SEM, immunostaining	osteoblasts (MC3T3-E1)	Single modification: osteoblasts aligned along the pattern.	132
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					covalent coupling			Combined: alignment along the topographical pattern.	
Silicon wafers with Titanium	deep reactive ion etching (DRIE)	grooves in the micrometer-range	SEM, AFM	Col I, RGD, Amino-groups	Acetic coating and drying, coupling to APTES/SPDP, plasma process	WCA, zeta potential	human osteoblast-like cells (MG-63)	Cell elongation and alignment along the groove/plateau direction on Col I and RGD, amino modification interrupted topography induced contact guidance.	133

Fibrous surface topographies combined with biochemical ligands

Substrate	Preparation method	Topography & Range	Topographical characterization	Immobilized molecules	Immobilization method	Chemical characterization	Cell type	Biological effect	Ref.
PLLA	(blended) electrospinning	nano-fibrous non-wovens	SEM	LM	EDC/NHS coupling or physical adsorption	FITC-LM, SCM, XPS, MicroBCA protein assay	neuroblasts (PC12)	Enhanced viability on post-modified nanofibers, enhanced neurite outgrowth on blended fibers.	150
PCL	electrospinning	nano- and micro-fibrous non-wovens	SEM	VEGF	EDC/NHS coupling	XPS, ELISA, immunohistochemistry	endothelial cells (HUVEC)	Immobilized VEGF enhanced endothelial cell adhesion and proliferation.	143
PCL, PLLCL	electrospinning	non-wovens in the sub- and micrometer-range	SEM	GRGDSP	covalent grafting of photoreactive peptide after UV irradiation	XPS, WCA	endothelial cells (HUVEC)	Dose-dependent increase of HUVEC adhesion and control over proliferation.	144
PCL	electrospinning	non-wovens in the sub- and micrometer-range	SEM	RGD	grafting with Nap-FFGRGD through self-assembly	fluorescence	rabbit endothelial cells and smooth muscle cells (EC and SMC)	RGD modified grafts enhanced hemocompatibility, EC cell migration, and SMC regeneration.	142
PCL	electrospinning	non-wovens in the sub- and micrometer-range	SEM	adhesion peptide: SVVYG LRGCC, heparin binding peptide:	covalent coupling to Mal-modified PCL	fluorescence	endothelial cells (HUVEC) and human saphenous vein-derived	Enhanced HUVEC-specific adhesion; increased pericyte adhesion and secretion of VEGF.	147

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PCL/gelatin	electrospinning	blended micro- fibrous non- wovens	SEM	VEGF	coupling to heparin after heparin immobilization on gelatin	toluidine blue	endothelial cells (HUVEC)	VEGF enhanced proliferation of HUVEC.	145
PCL	electrospinning	blended micro- fibrous non- wovens	SEM	VEGF-HGFI	hydrophobic interactions	fluorescence	endothelial cells and smooth muscle cells (HUVEC and SMC)	Lower fibrinogen adsorption, platelet activation and aggregation. Increase of HUVEC functionality, endothelization, enhanced expression of SMCs marker genes.	149
PCL	electrospinning	micro-fibrous non-wovens	SEM	FN	coating to hydrolyzed PCL or additional ECD + NHS coupling or coaxial electrospinning	ATR-FTIR, ToF- SIMS	bone murine stromal cells (ST-2)	Enhanced ST-2 proliferation, especially on coaxial spun fibers.	154

2.3 Combining biochemical modifications with different mechanical properties

Substrate composition and mechanical properties control initial cell material contacts and differentiation

Synergistic effects of physical (mechanical properties) and biochemical cues (growth factors, proteins) on cell behavior are well known, and several studies have focused on different substrates with varying elastic moduli combined with diverse biochemical ligands, such as full-length ECM proteins, GFs or peptide sequences derived therefrom.¹⁵⁷⁻¹⁶⁸ In general, with increasing substrate rigidity and presence of ECM proteins (e.g. Col I and FN), spreading, actin cytoskeleton organization and proliferation of hMSC^{161, 163}, smooth muscle cells (SMCs)¹⁶⁹ and keratinocytes¹⁶³ as well as adhesion and neurite formation of PC12 cells¹⁶⁰ were enhanced. Furthermore, the modification of substrates with a range of elastic moduli between 0.5 kPa and 423 kPa with peptide sequences, such as RGD¹⁵⁷⁻¹⁵⁸, KQAGDV¹⁵⁷ and BMP-2mp¹⁵⁸, revealed in some cases synergistic, but sometimes also discrete effects on the formation and organization of the F-actin cytoskeleton, proliferation and differentiation of cells.¹⁵⁷⁻¹⁵⁸ It was shown previously that SMC spreading and focal adhesion formation were promoted by the ligand density and identity as well as substrate stiffness. However, there, an increase in elastic modulus led to enhanced proliferation, but decreased differentiation independently of the present biochemical cue.¹⁵⁷ Furthermore, the combination of substrates showing mechanical properties matching the target tissue, with hepatocyte GF¹⁶⁷, PDGF¹⁶⁸, BMP-2^{165, 168}, and VEGF¹⁶⁶ revealed synergistic effects on cell adhesion, migration and proliferation as well as increased metabolic activity, of endothelial cells¹⁶⁶⁻¹⁶⁷, myoblasts¹⁶⁵ and ADSC¹⁶⁸. Taken together, the functionalization of substrates with growth factors can enhance cellular functions or support cellular interactions with an otherwise cell-unfavorable substrate.^{165-167, 170} Furthermore, the surface-bound presentation of GFs enhanced the efficacy and function of growth factors, such as VEGF¹⁷¹, BMP-2¹⁶⁵ and insulin-like GF¹⁷², and is closer to physiological conditions in the nature, as many GFs are bound to proteins or glycosaminoglycans in the ECM^{19, 165, 173-175}. In this context, researchers hypothesized that the spatial arrangement of the GF on the surface led to a restricted diffusion and increased local

availability of the GF as well as an interplay of different cell receptors, such as cell adhesion receptors^{165, 176-178}. Nevertheless, it was also shown that the concentration of a GF, such as VEGF, is a critical factor for increased, stable vascularization or aberrant angiogenesis.¹⁷⁹⁻¹⁸⁰

Cell differentiation is influenced by matrix composition and elastic modulus of the support material, but can be dominated by a biochemical stimulus

Not only cell attachment and spreading, but also cell fate decisions are known to be influenced by the interplay of substrate mechanics and ECM ligands. This has been shown for stem and progenitor cell differentiation and in development and regeneration of functional neuronal^{159-160, 162, 181-182}, muscle^{157-159, 161-162}, liver¹⁶⁴, skin¹⁶³ or bone tissue^{158-159, 161-162}. For example, human MSC were cultured on substrates with different elastic moduli or indentation depths functionalized with ECM proteins, such as collagen I, or other chemical groups. Thereon, the cells showed rigidity-dependent differentiation, with neurogenesis and angiogenesis being enhanced on soft (modulus: 0.1 kPa to 6.5 kPa), myogenesis on intermediate (modulus: 8 kPa to 17 kPa) and osteogenesis on slightly stiffer substrates with elastic moduli in the range of 25 kPa to 80 kPa.^{159, 161-163} Adult neural stem cells (aNSC) showed differentiation into neurons on softer (modulus ~ 100-500 Pa) and into glials on harder RGD-modified substrates (modulus ~ 1000-10000 Pa).¹⁸² Furthermore, when combining growth factors with mechanically different materials, positive effects on osteogenesis^{165, 168, 170}, endothelialization, revascularization or angiogenesis^{166-167, 183} as well as the development of skin¹⁸⁴ were demonstrated. For example, the differentiation of myoblasts¹⁷⁰ or endothelial cells¹⁶⁶ as well as osteoblast¹⁶⁵, endothelial cell¹⁶⁷ and myoblast¹⁶⁵ function were mainly regulated by the elastic modulus of the substrate.

The influence of combined biochemical and mechanical stimuli on cell differentiation is complex and often crucially dependent on the biochemical stimulus. Between elastic moduli of 4 kPa to 30 kPa cholangiocyte differentiation of liver progenitor cells was observed to be mechanics-independent in presence of Col IV, but mechanics-regulated on FN-modified surfaces. Further, hepatocyte differentiation was mainly induced by the biochemical ligand and matrix composition (Fig. 5 a).¹⁶⁴ Human MSC on the other hand favored the myogenic fate on substrates with elastic moduli of 13 kPa to 17 kPa, while osteogenic fate was preferred on substrates with elastic moduli of 45 kPa to 49 kPa, if the cell-unspecific RGD peptide was used. However, in presence of the bone-specific BMP-2 peptide, differentiation towards the osteogenic fate was enhanced largely

independent of the substrate rigidity, except for very soft substrates (modulus: 0.5 kPa to 3.5 kPa). On these substrates, differentiation was inhibited due to a reorganization of the F-actin cytoskeleton (Fig. 5 b).¹⁵⁸

Figure 5 Cell differentiation dependence on mechanical properties of the substrate and the influence of biochemical ligands: Mechanics dependent cell differentiation on mechanically

consistent substrates can be influenced by surface immobilized ECM proteins (a) ECM protein or GF derived peptides (b), or GFs (c). Image a) reprinted and adapted from Kourouklis *et al.* ¹⁶⁴, Copyright (2016), with permission from Elsevier: Cholangiocyte differentiation of bipotential mouse embryonic liver cells (BMEL) was independent of the elastic modulus in presence of Col IV, but regulated by mechanical properties in presence of FN on different PAAM substrates (4 kPa and 30 kPa). Hepatocyte differentiation of BMEL was mainly independent of elastic modulus and guided by the present biochemical ligand. BMEL stained for cell nuclei (DAPI), albumin (ALB) and osteopontin (OPN). Image b) reprinted and adapted from Zouani *et al.* ¹⁵⁸, Copyright (2013), with permission from Elsevier: hMSC showed mechanics independent osteogenic differentiation in presence of osteogenic ligand BMP-2 mimetic peptide on polyacrylamide-co-acrylic acid substrates. In presence of the adhesion ligand RGD hMSC displayed differentiation dependent on the elastic modulus: myogenic fate on soft (modulus: 15.4 kPa) and osteogenic fate on slightly stiffer (modulus: 47.5 kPa) substrates. hMSC after four weeks stained for actin, osteopontin (OPN) and cell nuclei (DAPI). Image c) adapted and reprinted with permission from Crouzier *et al.* ¹⁶⁵, Copyright © 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim: Adhesion and spreading of C2C12 myoblasts is dependent on the presence of BMP-2 growth factor on soft PLL/HA films (modulus: 200 kPa), but independent of the ligand on substrates showing Young's moduli of around 360 kPa. An immunofluorescent staining of C2C12 was conducted for cell nuclei and actin.

Furthermore, chemical stimulation in the presence of specific GFs, such as adsorbed HGF ¹⁶⁷ or tethered BMP-2 ¹⁶⁵ or VEGF ¹⁶⁶ could also support these cellular responses on mechanically not favored surfaces. ^{165-167, 170} On substrates showing various elastic moduli (2.85 MPa to 5.05 MPa) the presence of a supporting growth factor, such as PDGF or BMP-2, could inhibit stiffness-dependent regulation of osteogenic and adipogenic differentiation genes of ADSC. ¹⁶⁸ In this context, it is also important how a GF is presented to cells, as studies have demonstrated that immobilized GFs, such as BMP-2, had a stronger effect on myoblast behavior, when compared to its soluble form, which was especially apparent on soft substrates (modulus: ~ 200 kPa) (Fig. 5 c). ¹⁶⁵ More detailed information on the impact of physical properties, the presentation of GFs as well as on the interplay of mechanotransduction and GF signaling on cell behavior and their microenvironments are summarized elsewhere. ^{19, 174-178}

Combining mechanical and biochemical gradients to identify synergistic effects

Due to the analysis of materials with different, but consistent mechanical properties modified with biochemical ligands conclusions of their synergistic effects on cells could be drawn. But nevertheless, most tissues do not show a definite mechanical value, but rather gradients in mechanical properties³², and even display concentration gradients of ECM components and GFs³³. Thus, gradients of elastic modulus play a special role in combination with biologically relevant ligands on a substrate, as both regulate many physiological processes, including cell migration³¹,¹⁸⁵⁻¹⁸⁹, spreading and cytoskeleton organization^{63, 185, 188, 190} as well as differentiation^{187, 191}. Generally, the combination of gradients of mechanical cues with ECM derived ligands, such as Col I^{31, 185, 187, 191}, FN¹⁸⁹⁻¹⁹¹ or RGD^{63, 186}, showed positive synergistic effects on fibroblasts^{31, 63, 188, 190}, MSC¹⁸⁷, bone-marrow stromal cells¹⁹¹, SMC¹⁸⁵, ADSC¹⁸⁹ and aortic cells¹⁸⁶. In addition to enhanced migration or spreading of MSC¹⁸⁷, SMC¹⁸⁵ or ADSC¹⁸⁹, especially fibroblast spreading was dependent of the elastic modulus and increased along the mechanical gradient (Young's modulus from 1 kPa to 240 kPa), when immobilized FN concentration was constant (Fig. 6 a).¹⁹⁰

The combination of elastic modulus gradients with biochemical ones, for example with an RGD gradient, enabled a step to better understand complex cell-matrix interactions.^{31, 63, 186, 188} Using this approach, increasing substrate rigidity and availability of a biochemical ligand gradient showed positive and synergistic effects on migration and spreading of fibroblasts^{31, 63, 188}, aortic cells¹⁸⁶ and bone-marrow stromal cells¹⁹¹. For instance, on dual gradient scaffolds (elastic modulus gradient from 5 kPa to 15 kPa combined with an orthogonal RGD density gradient from 0 mM to 0.7 mM immobilized peptide) human fibroblasts displayed increased spreading along both gradients, showing the best spreading at the stiffest area with highest RGD density. Especially, by keeping one parameter constant, cells displayed enhanced spreading along the appropriate increasing gradient (Fig. 6 b).⁶³ However, biochemical cues can also dominate and direct cell migration and differentiation, as fibroblasts migrated to or stayed at the region with low Young's modulus, but high collagen density (Fig. 6 c).^{31, 188} Independent of the elastic modulus of the substrate, bone-marrow stromal cells showed osteogenic or tenoblast differentiation in the presence of FN or Col, respectively¹⁹¹, and aortic cell infiltration was enhanced with increasing RGD concentrations¹⁸⁶. Table 3 summarizes studies combining chemical and mechanical surface modifications and the respective characterization methods as well as the biological response.

Figure 6 Combined effect of mechanical gradients and biochemical ligands on cell behavior: Image a) adopted and reprinted from Sunyer *et al.*¹⁹⁰ under CC BY license. NIH3T3 fibroblasts adhesion and spreading along a mechanical gradient (modulus: 1 kPa to 240 kPa) of a PAAM substrate was only depending on elastic modulus, regardless of the immobilized present FN functionalization. Image b) adopted and reprinted with permission from Tong *et al.*⁶³, Copyright (2016) American Chemical Society: Human fibroblasts (BJ) showed synergistically enhanced adhesion and spreading along parallel increasing mechanical properties (elastic modulus gradient from 5 kPa to 15 kPa) and RGD adhesion peptide gradients (0-0.7 mM) on PEG substrates. An immunofluorescent staining of fibroblasts was conducted for actin and cell nuclei. Image c) adapted and reprinted with permission from Hale *et al.*³¹, Copyright (2010) American Chemical Society: Adhesion and migration of Balb/c 3T3 fibroblasts is dependent of the present biochemical ligands on orthogonal mechanics (elastic modulus gradient from 31 kPa to 85 kPa) and Col I gradients of PAAM substrates. Over a period of 3 h fibroblasts migrated to or stayed at regions with high densities of the ligand.

Table 3 Overview of studies combining chemical and mechanical surface modifications and the respective characterization methods. The biological response *in vitro* is briefly stated.

Discrete substrates varying in elastic modulus combined with biochemical ligands

Substrate	Preparation method	Elastic modulus	Mechanical characterization	Immobilized molecules	Immobilization method	Chemical characterization	Cell type	Biological effect	Ref.
PAAM	polymerization with varying crosslinker concentration	10 Pa to 10 kPa	Rheology	RGD	EDC/NHS and sulfo-SMCC coupling	cell staining after adhesion	adult neural stem cells (aNSC)	Enhanced aNSC spreading and proliferation above 100 Pa, neuronal gene expression at 500 Pa, neuron (100-500 Pa), or glial (1000-10000 Pa) differentiation.	182
PAAM	different polymer-block ratios	7 Pa to 19 kPa	rheology	FN	radiolabeling assay with NHS	radioactive labelling	neuroblasts (PC12)	Stiffness-dependent increase of adhesion and neurite formation of PC12 cells.	160
PAAM	concentration dependent polymerization	0.1 kPa to 1 kPa, 8 kPa to 17 kPa, 25 kPa to 40 kPa	AFM	Col I	covalent bonding after sulfo-SANPAH activation	fluorescence	mesenchymal stem cells (hMSC)	MSC differentiation in neuronal cells on soft, myogenic cells on intermediate, osteogenic cells on stiff substrates.	159
PAAM or Collagen	different polymer-block ratios or collagen concentrations	0.5 kPa to 8.5 kPa 2 kPa and 40 kPa	rheology	Col I, FN, FBS, PDGF	Covalent bonding after sulfo-SANPAH activation	cell staining after adhesion	human foreskin fibroblasts (BR5), oncogenic Ras-transformed BR5-Ras, myofibroblasts (BR5-MF)	FBS enhanced fibroblast spreading with increasing ligand density and increasing stiffness; PDGF effect was independent of mechanics or ligand.	192
PAAM	concentration-dependent crosslinking with Irgacure 2959	4 kPa to 30 kPa	-	Col I, Col III, Col IV, FN, LM I	covalent bonding after sulfo-SANPAH activation	fluorescence	bipotential mouse embryonic liver progenitor cells (BMEL 9A1)	Hepatocyte differentiation induced by ECM composition; cholangiocyte differentiation mechanics-independent in presence of Col IV, but mechanically driven on FN.	164
PEGDA, ACPEG	Photo-initiated radical polymerization	4 kPa to 41 kPa	AFM	RGD	EDC/NHS	cell staining after adhesion	fibroblasts (L929)	Enhanced fibroblast spreading above a modulus of 20 kPa.	193

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3		PACAA	free radical polymerization	0.5 kPa to 70 kPa	dynamic mechanical analysis	RGD and BMP-2 peptide	EDC/NHS coupling	fluorescence, XPS, high resolution μ -imager (^3H -lysine)	mesenchymal stem cells (hMSC)	Mechanical stiffness-dependent and peptide-induced differentiation along the osteogenic or myogenic lineage.	158
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7		PAAM	concentration dependent polymerization	0.7 kPa to 80 kPa	tensile testing	Col I, Col IV, FN, LM I	covalent bonding after sulfo-SANPAH activation	fluorescence	mesenchymal stem cells (hMSC)	80 kPa and Col I increased osteogenic differentiation; 80 kPa and Col I or LM I, or 25 kPa and FN or Col IV induced myogenic differentiation.	161
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11		PAAM	different polymer-block ratios	5.5 kPa to 152 kPa	AFM	Col I, amino-, carboxyl-, phosphate-groups	covalent bonding after sulfo-SANPAH activation and plasma polymer deposition	XPS, water contact angle	mesenchymal stem cells (hMSC)	Enhanced adhesion on Col I and spreading on stiffer substrates; osteogenesis on 41 kPa with phosphate; myogenesis on 10-17 kPa with carboxyl on >10 kPa with carboxyl.	162
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18		PLL, HA	LbL-coating, crosslinking by sulfo-NHS and EDC	182 kPa to 213 kPa, 358 kPa to 367 kPa	AFM	BMP-2	post-diffusion and adsorption under acidic conditions	fluorescence	skeletal myoblasts (C2C12)	Immobilized BMP-2 had higher impact on C2C12, especially on soft substrates. Spreading, cytoskeleton formation and migration were enhanced.	165
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24		HA, PLL	LbL-coating, crosslinking by sulfo-NHS and EDC	200 kPa to 430 kPa	AFM, FT-IR	HGF	diffusion, physical adsorption	QCM-D	endothelial cells (HUVEC)	Increasing stiffness resulted in increased adhesion, migration, proliferation, and spreading. Function enhanced on soft substrates.	167
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29		PDMS, PAAM	varied base to crosslinker ratio; from increasing monomer and crosslinker concentration	PDMS: 0.1 kPa to 2.3 MPa PAA: 0.1 kPa to 740 kPa	microindentation	Col I, FN	covalent coupling after sulfo-SANPAH activation	immunofluorescence or immunohistochemistry	mesenchymal stem cells (hMSC) and primary human keratinocytes	Independent of PDMS mechanical properties similar keratinocyte and hMSC spreading and differentiation. PAA induced increased spreading and decreasing differentiation of keratinocytes, increasing spreading and osteogenic differentiation of hMSC, but decreased adipogenic differentiation with varying stiffness	163
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38		Col-GAG	different EDC/NHS ratios	2.85 MPa to 5.05 MPa	tensile testing	BMP-2 PDGF	benzophenone immobilization with UV	fluorescence	adipose derived stem cells (ADSC)	Substrate rigidity directed osteogenesis of ADSC. On soft substrates, GFs were needed. PDGF increased metabolic activity and proliferation, but inhibited	168
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differentiation. BMP-2 induced no effect on lineage-specific differentiation.

Substrates combining mechanical and biochemical gradients

Substrate	Preparation method	Elastic modulus	Mechanical characterization	Immobilized molecules	Immobilization method	Chemical characterization	Cell type	Biological effect	Ref.
HA/MeHA fibers	27 % to 100 % MeHA gradient, UV-crosslinking with Irgacure 2959	gradient: ~ 500 Pa to 10 kPa	AFM, FT-IR, SEM	RGD	gradient flow of two solutions, reaction of thiol-RGD and Me-HA	FT-IR, fluorescence	aortic arch explants	increased cell infiltration from low to high RGD of chick aortic arch explants	186
PAAM	time-dependent UV cross-linking	gradient: 1 kPa to 14 kPa	AFM	Col I	covalent bonding after sulfo-SANPAH activation	fluorescence	mesenchymal stem cells (hMSC)	MSC migration to stiffer part, myogenic differentiation.	187
PEG8NB, linear PEG2SH	time-, speed-, and photo-dependent thiol-ene reaction	gradient: 5 kPa to 15 kPa	tensile testing	RGD	time- and speed-dependent UV exposure	fluorescence	human fibroblasts (BJ)	Increased fibroblast spreading along both gradients; most pronounced on stiffest end with high RGD concentration.	63
PAAM	different concentrations polymerized via photo-initiation	5 kPa to 80 kPa, gradient: 1 kPa-80 kPa	AFM, uniaxial tensile testing	Col I	covalent bonding after sulfo-SANPAH activation	fluorescence	bovine aortic smooth muscle cells (VSMC)	Uniform gels: cell spreading, polarization, random motility increased with stiffness; gradients: spreading, durotaxis and cell orientation increased with stiffness, nearly all cells polarized, cell migration to stiffer end.	185
PAAM	different polymer-block concentrations	gradients: 0.1 kPa to 6.6 kPa, 2.4 kPa to 38.1 kPa, 3.2 kPa to 159.2 kPa	AFM, OCT	FN	Covalent bonding after sulfo-SANPAH activation	fluorescence (FRET)	adipose derived stem cells (ADSC) and skeletal myoblasts (C2C12)	ADSC migration to the stiffer end, increasing cell spreading with increasing stiffness, C2C12 spreading increased with stiffness.	189

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3	PAAM	Increasing the amount of crosslinker	gradient: 31.2 kPa to 79.8 kPa	AFM	Col I	addition of FITC-Col I beads	fluorescence	fibroblasts (3T3)	Fibroblasts stayed at high Col I or migrated to the higher Col I concentration.	31
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6	4-12% NuPage	different polymer-block ratios	gradient: 10 kPa to 100 kPa	indentation, compression and tension	FN, Col I	covalent bonding after sulfo-SANPAH activation	fluorescence	mesenchymal stem cells (hBMSC)	Enhanced hBMSC adhesion and tenoblast differentiation on Col I; enhanced osteogenic differentiation on FN with increasing ligand density and stiffness.	191
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12	PAAM	crosslinking with Irgacure 2959	gradient: 46.7 kPa to 126.7 kPa	AFM	Col I	chemical conjugation with NHS and SDA and photomask UV-exposure	fluorescence and FN binding assay	fibroblasts (3T3)	Fibroblasts migrated to the soft, Col I rich part, enhanced organization of the cytoskeleton.	188
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17	PAAM	time-dependent UV cross-linking with Irgacure 2959	gradient: 1 kPa to 240kPa	AFM	FN	covalent bonding after sulfo-SANPAH activation	fluorescence	fibroblasts (NIH3T3) and neuroblastoma cells (SY5Y)	Increasing spreading with increasing stiffness.	190
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Lessons learnt from combinatorial surface approaches

Combining several biochemical moieties or biochemical ligands with either topographical features or different mechanics, generally leads to synergistic enhancement in cell type specific responses, such as attachment or differentiation. It is known that adhesion molecules generally lead to enhanced cell attachment and spreading, while growth factors stimulate the expression of cell type specific functionality. Thereby, specific phenotypes or differentiation, combining several adhesion factors, could already lead to enhanced functionality.⁷⁸ Especially, combining growth factors with adhesion ligands induces enhanced functional cell response.^{80, 89, 91} The type as well as the dimension of surface topography is also decisive for cell responses.^{115-116, 126, 156, 194} While chemical adhesive ligands significantly enhanced adhesion, growth and differentiation of cells, grooved and fibrous topographies directed cell morphology, alignment and elongation along the pattern.^{125-128, 135, 142-153} Furthermore, restriction of a biochemical ligand pattern guided cell arrangement, orientation and differentiation.¹⁵⁵⁻¹⁵⁶ An interplay between mechanical properties of the substrate and the presented biochemical stimulus (integrin signaling) for modulating cellular behavior and responses could be seen. Nevertheless, the cell function could be dominated by a chemical stimulus leading to differentiation, even on a mechanically cell-unfavorable substrate.^{158, 164-165, 168} Furthermore, various studies clearly demonstrated a strong dependency of the cell response on concentration and ratio of the presented biochemical ligands.^{58, 76, 79, 85, 99} However, the exact concentration of adhesion molecules and GFs needed to induce a desired cell response is often not known. Arrays and gradients provide an excellent method to gain knowledge on cell fate decisions in response to biomolecule functionalization.^{66, 84, 86, 92-97, 103} Nevertheless, a clear need for further work to better understand the dependence of combinations, concentrations and ratios of biomolecules, of surface topographies and of mechanical properties of the support material can be seen, not only to steer cell behavior but also to recreate transition zones of tissues with a natural gradient.

3. Challenges in fabrication and characterization of combinatorial surface functionalized biomaterials

Material functionalization with various biochemical groups

Control over surface properties becomes challenging when combining various biochemical groups on one substrate. Castellano *et al.* immobilized combinations of YIGSR, RGDS and REDV on cobalt-chrome substrates *via* chloropropyltriethoxysilane silanization and subsequent conjugation of sulfur with chlorine.⁷¹ Notably, this directionally controlled peptide conjugation ensured the availability of the peptide's adhesion sequence for cellular attachment and was used for all different peptides and also combinations thereof. While the functionalized substrates were thoroughly characterized via X-ray photoelectron spectroscopy (XPS), surface charge measurement and quartz-crystal-microbalance (QCM-d), none of these methods was able to distinguish between different peptides. Hence, the ratio of immobilized peptides on the surface is, due to the unspecific immobilization and characterization methods, unknown and the reported synergistic effect from peptide combination RGDS/YIGSR on endothelial cell proliferation cannot be correlated to an exact peptide ratio.

This example nicely demonstrates the challenges concerning fabrication and characterization of functionalized biomaterials. Methods for fabrication and characterization of materials functionalized with various biochemical groups are therefore divided here as follows: a) random immobilization and molecule-unspecific characterization, b) random immobilization and specific characterization, c) specific immobilization and unspecific characterization and d) molecule specific immobilization and specific characterization.

Unspecific immobilization methods encompass only one tethering route for all molecules at the same time, with concentration or ratio being only loosely controlled by the composition of the biomolecule solution used for the modification process. These strategies are often based on carbodiimide/n-hydroxysuccinimide chemistry.^{74, 87, 92} They are well established and assure effective molecule immobilization, but the lack of specificity bears the risk of compromising the bioavailability, since several amino acids of a biological compound might be able to conjugate.¹⁹⁵

Specific immobilization strategies are mostly based on click chemistry, which is available as thiol-ene reaction, strain-promoted alkyne-azide cycloaddition (SPAAC), oxime ligation and copper-catalyzed alkyne-azide cycloaddition (CuAAC).^{93, 97, 102, 196} In these cases, the different biomolecules can be modified to contain one of the click chemistry partners (e.g. alkyne) and the surface to present the respective other one (e.g. azide) allowing a specific reaction only between the targeted partners. However, while most of these approaches are cytocompatible, copper-catalyzed chemistry should be avoided in biomedical applications due to its toxicity.¹⁹⁷ Another specific immobilization method of tethering FN and VEGF has recently been published by Moulisova *et al.*⁸⁹ They exploited the different surface chemistry and charge of poly(ethyl acrylate) (PEA) and poly(methyl acrylate) (PMA) films to steer the conformation of FN adsorbed to the surface. By doing so, they achieved a globular conformation of FN on PMA and a fibril structure on PEA, with the latter structure exposing both the integrin binding site RGD and the VEGF binding site of FN.⁸⁹ This biomimetic presentation of both functionalities ensured their bioavailability.

Special challenges however arise when aiming to fabricate parallel, reverse or orthogonal gradients of two different biomolecules on the surface. There are no standard methods established yet, but a range of different strategies have been described. For instance, Ma *et al.* used a confined channel diffusion method and combined thiol-ene and SPAAC click chemistry to create a dual orthogonal peptide gradient.^{93, 95} Guan *et al.* also used two different click chemistries to achieve a dual reverse gradient by exploiting a temperature gradient for controlled peptide immobilization.⁹⁷ Other simpler methods to create gradients are based on time- controlled incubation of the sample in the molecule solution, which can be done by dipping with a linear motion drive¹⁰⁰ or by filling a container with a syringe pump¹⁰¹. Here, the same conjugation chemistry can be used for all immobilized molecules. Furthermore, gradient photomasks can be used for UV-mediated molecule immobilization to achieve parallel gradients.^{86, 104}

Detection and quantification of specific molecules in a mixture of molecules on a surface is challenging, and only few characterization methods are currently available. Methods including XPS, Fourier-transform infrared spectroscopy (FTIR), atomic force microscopy (AFM) and water contact angle measurement (WCA) are very commonly used for materials chemistry characterization.^{71, 82, 86, 95, 102} These methods are generally not suitable to detect different biological molecules, because of the chemical similarity of individual functional groups. The same

accounts for biochemical methods, such as bicinchoninic acid (BCA) assay⁸⁵ or fluorescamine assay^{82, 87}. Although these methods specifically detect amino acids and thus biomolecules, they cannot differentiate between them. Hence, the actual amount of the different molecules present at the surface is not known and can only be estimated from the composition of the molecule solution. Thereby, control over the surface chemistry is limited, and discriminating effects from different molecules or concentrations on cell behavior become challenging, if not impossible. On the other hand, enzyme-linked immunosorbent assay (ELISA) is very specific for the identification of single proteins and growth factors. An ELISA has typical detection limits of 0.01 ng per sample and allowed quantification of absolute amounts of molecules as well as analysis of bioavailability of the immobilized molecule and thus information about the conformation and directionality.^{79, 84, 89} However, so far, this method is only available for whole proteins and growth factors, but not in a commercial manner for peptides. Furthermore, it is not suitable for gradient characterization.¹⁹⁸ Amino acid analysis after hydrolyzation of molecules from the surface and subsequent analysis by liquid chromatography allowed revealing characteristic amino acids from different peptides.⁷³⁻⁷⁴ This method allows quantitative detection in a pmol/cm² range. Direct labelling of biomolecules with fluorescent tags with different emission wavelengths presents a more versatile, but less quantitative method.^{97, 103-104} While this approach was used for many molecules, such as custom-synthesized peptides, and was suitable for gradient characterization¹⁹⁸, difficulties can arise from background fluorescence, detection limits and possible quenching of the fluorophore. Immunostaining of proteins such as FN or VN with nanoparticle tagged anti-bodies allowed specific and quantitative detection of the protein with AFM characterization.¹⁹⁹ Time-of-flight Secondary Ion Mass Spectrometry (ToF-SIMS) is a promising method for characterization of multi-component surfaces and gradients.²⁰⁰ Its high sensitivity and specificity for different chemical groups allows to distinguish footprints of individual amino acids and gives information about the orientation of the immobilized molecules. However, absolute quantification of chemical groups is not possible.²⁰⁰⁻²⁰³

Topographical or mechanical modification with subsequent chemical functionalization

When combining topographical modification or modification of mechanical properties with biochemical functionalization, the modification steps are typically performed sequentially. Thus, control over the surface properties is possible, because mechanical and topographical

functionalization use inherently different fabrication steps than biochemical ones. Surface topography on the one hand can be produced by a wide variety of methods, including different lithographical approaches^{122, 126, 156}, microcontact printing¹⁵⁵, multiphoton excited photochemistry¹²¹, particle adsorption^{111, 124}, sandblasting¹¹⁹, different etching techniques^{116, 126, 130, 133}, laser ablation methods¹²⁹, mold casting methods^{120, 126-127, 135-137}, embossing^{132, 134} or electrospinning^{142, 147, 204-205}. Modification of mechanical properties on the other hand is usually achieved by employing mechanically diverse polymer substrates that can be generated for example from polyethylene glycol (PEG)^{63, 157}, PDMS¹⁶³, collagen-GAG¹⁶⁸ or hyaluronic acid¹⁸⁶ combined with poly-L-lysine^{165, 167} or poly(acrylamide-co-acrylic)acid¹⁵⁸ by varying the appropriate cross linker amount. A commonly used substrate for varying mechanical properties is polyacrylamide (PAAM)^{159, 164, 166, 187-190, 192}, where distinct mechanical properties can be obtained by varying the amount of acrylamide or bi-acrylamide (B-AA)^{159, 166, 189, 192}, by time-dependent UV crosslinking with a photo initiator, such as Irgacure 2959¹⁸⁷⁻¹⁸⁸, or a combination of both^{164, 190}.

While surface topographies are characterized most frequently by atomic force microscopy (AFM)^{111, 115-116, 124, 126-127, 130, 134} and scanning electron microscopy (SEM)^{50, 119-120, 122, 128, 147, 206}, mechanical properties of biomaterials (surface and bulk properties) are commonly determined by AFM^{159, 185, 188-189}, mechanical tensile testing^{63, 157, 161, 168, 185, 191}, uniaxial compression measurements by dynamic mechanical analysis¹⁵⁸, micro-indentation tests¹⁶³ or rheology^{160, 182}. Profilometry or confocal 3D optical surface metrology allow quantification of surface roughness, similarly like AFM.²⁰⁷⁻²⁰⁹

Successful single protein or peptide functionalization can be confirmed by XPS^{120, 158, 162, 166}, immunostaining^{163, 168, 187, 191}, WCA measurement^{111, 126, 128, 133, 153}, ELISA^{119, 143}, analysis of fluorescent biomolecules^{127, 130, 142, 147, 150, 156}, the zeta potential of the surface¹³³, BCA assay⁵⁰, electron spectroscopy for chemical analysis (ESCA)¹³⁵ or QCM-d¹⁶⁷. However, some methods can be influenced by both, chemical and topographical or mechanical properties of the substrates. For example, water contact angles are dependent on surface chemistry (Wenzel-Model) and on roughness (Cassie-Baxter-Model), thus limiting the applicability of WCA measurements as sole method for biochemical characterization of topographically modified substrates.²¹⁰⁻²¹¹ Furthermore, the same technical limitations described above occur when developing chemical

gradients or when using more than one biochemical group for the modification of topographically/mechanically modified materials.

It is also important to consider that mechanical properties are inherently related to materials chemistry and that surface biochemical functionalization could alter surface mechanical properties.^{5, 212-215} In this context, one study compared compressive and tensile properties of un- and ECM-coated surfaces and revealed that the standard compression stiffness estimation by indentation-type AFM showed no significant differences in stiffness values due to dependency of indentation depth perpendicular indentation and the layer thickness of the protein modification. However, a tensile mechanical characterization using a biaxial stretcher showed higher resistance values of FN and Col coatings compared to uncoated regions causing direction-dependent and ligand-specific mechanical properties, which may affect mechanically driven cellular responses.²¹⁵ Another study showed that commonly used AFM-based indentation with colloidal probes exhibited the micrometer-scale stiffness of a material by deformation, while a force-distance measurement with a modified AFM tip could determine nanoscale stiffness of material crosslinks on the molecular level, which also mediates cell attachment.¹⁹³ A strong interdependence of all properties might be the reason, why studies systematically investigating the effect of altered topography and mechanical characteristics are rare.²¹⁶⁻²²²

4. Conclusion and future perspectives

Here, we summarized the systematic combination of several surface modifications, such as (I) various biochemical cues immobilized on a surface, (II) the combination of different topographical surface features functionalized with biochemical cues and (III) the combination of mechanical substrate properties modified with biochemical cues, demonstrating that combinations of these different cues generally elicit positive synergistic effects with regard to a variety of cell responses and fate decisions. Furthermore, we highlight the importance of fabrication and characterization methods that are specific for the individual modification in order to provide control over such complex systems and to ensure a correct correlation of effect and outcome.

The lack of comparability between studies on biomaterials and biological responses is one well-known challenge.²²³⁻²²⁶ Even though many reviewed studies investigated similar material systems,

the comparison of their results is challenging due to differences in chosen material characterization methods (e.g. bulk material tensile testing or surface mechanical nano-indentation), alterations in terminology, or disparities in biomolecule source (e.g. animal-derived vs. recombinant proteins).^{215, 227-230} Furthermore, many different cell types have been used to study the effects of surface properties on cell behavior, and even in reports using identical cell lines or cells from the same batch, results can fluctuate as genetic variability and diversification is common in cell lines.²³¹

In order to achieve better comparability between studies in the future, we propose that not only controlled fabrication methods are combined with quantitative characterization techniques, but also that the source of biochemical compounds or e.g. design of peptides should be, if possible, based on previous studies in order to provide better comparability.

Surface modification is a very useful step for understanding the interplay of materials with biology and for isolating specific effects. Here, in future studies we expect to see increased combinations of chemical, topographical and mechanical cues at once as well as increasing combinations of 3+ chemical signals. Especially, larger systematic studies should be conducted in order to be able to derive overall conclusions and lessons for strategic further development.

However, for tissue engineering and regenerative medicine moving towards 3D is crucial.²³²⁻²³⁴ The need for material surface functionalization to enhance cell interaction and mimicry of the natural cell niche is very much needed.²³⁵⁻²⁴⁰ However, further aspects, such as the substrate/scaffold porosity and architecture, increase the complexity.^{213, 236, 241-246}

For instance, a recent study, combining electrospinning and photolithography, showed that osteogenic differentiation of hMSC was enhanced on a grooved substrate made of composite electrospun nano-fibers.²⁴⁷ In this line some research groups are increasingly focusing on the design and development of smart, functional 3D biomaterials that actively interact with cells and respond to diverse stimuli, including pH, temperature or ionic strength.²⁴⁸ Furthermore, high throughput platforms and multiwell arrays enable rapid screening of different cellular stimuli, such as surface exposed topography or rigidity cues, simultaneously or isolated to assess their influence on cell behavior.²⁴⁹

Research on biomaterials for tissue engineering is poised to grow in the future²⁵⁰, as personalized regenerative medicine approaches using stem cells are moving towards clinical applications²⁵¹.

For scaling up the throughput of groups per experiment, a promising study developed a 3D niche

microarray system to better understand cellular responses in a variety of biochemically and physically functionalized 3D microenvironments.²⁵²

However, we also believe the development of this field depends not only on progress within tissue engineering itself, but also on parallel progress in basic biology. Basic biology will allow us to increasingly understand the exact properties of ECM – concentrations of chemical signals and their presentation, detailed structures, and mechanical properties.²⁵³⁻²⁵⁵ Therefore, efforts from both sides should be synergized in more interdisciplinary approaches to thrive for optimized mimicry of the extracellular matrix.

Already in 2009, general considerations about biomaterial complexity have been discussed e.g. by Place, Evans and Stevens¹³, who highlighted that also financial aspects limit complexity because multifunctionality decreases the probability of successful clinical translation. They suggested to seek the minimal complexity for achieving the desired functional outcome, such as a particular cell type adhesion, proliferation or differentiation.¹³ We agree that a balance between increasing complexity on the road to clinical applicability and related cost-effectiveness should be aimed for. The here discussed and motivated technical and scientific advances in material modification and characterization may pave the way for clinical translation of true biomimetic biomaterial concepts in the future.

We strongly believe that multifunctional surface modification approaches will gain increasing relevance, but we cannot emphasize enough that the choice of appropriate methods is of paramount importance for their success and chances of successful clinical translation.

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Abbreviations

3D	three dimensional
AA	acrylic acid
ACLF	anterior cruciate ligament fibroblasts
ACPEG	carboxy poly(ethylene glycol) acrylate
AFM	atomic force microscopy
ALB	albumin
ALP	alkaline phosphatase
aNSC	adult neural stem cells
AoSMCs	aortic smooth muscle cells
APTES	3-aminopropyltriethoxysilane
ASCs	adipose stem cells
B-AA	bi-acrylamide
BAEC	bovine aortic endothelial cells
BCA	bicinchoninic acid
BCMn	block co-polymer micelle nanolithography
BCP	biphasic calcium phosphate
bFGF	basic-fibroblast growth factor
BMP	bone morphogenic protein
BMEL	bipotent mouse embryonic liver cells
BMSC	bone marrow stem cells
BSA	bovine serum albumin
BSP	bone sialoprotein
C2C12	murine myoblast cell line
Col	collagen
CPTES	chloropropyltriethoxysilane
CuAAC	copper catalyzed alkyne-azide cycloaddition
DMPA	2,2-dimethoxy-2-phenylacetophenone
DP-MSC	dental pulp derived mesenchymal stem cells
DRIE	deep reactive ion etching
ECM	extracellular matrix

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3	EDC/NHS	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride/N-succinic
4		anhydride
5		
6	EDX	energy dispersive X-ray analysis
7	EGF	epidermal growth factor
8	ELISA	enzyme-linked immunosorbent assay
9	ESCA	electron spectroscopy for chemical analysis
10	FE-SEM	field emission-scanning electron microscopy
11	FITC	fluoresceinisothiocyanat
12	FN	fibronectin
13	FTIR	fourier transform infrared spectroscopy
14	GAG	glycosaminoglycan
15	GF	growth factor
16	HA	hyaluronic acid
17	hADSC	human adipose derived stem cells
18	HAP	hydroxyapatite
19	hBMSC	human bone marrow derived stem cells
20	HCEC	human corneal epithelial cells
21	HDT	hexadecanethiol
22	HGFI	Class I hydrophobin
23	HMDS	hexamethyldisilazane
24	hMSC	human mesenchymal stem cells
25	HPMEC	human pulmonary microvascular endothelial cells
26	HUVEC	human umbilical cord vein endothelial cells
27	IGF	insulin-like growth factor
28	IRRAS	infrared reflection-absorption spectroscopy
29	KQAGDV	Lys-Gln-Ala-Gly-Asp-Val
30	KRSR	Lys-Arg-Ser-Lys
31	LbL	layer-by-layer
32	LM	laminin
33	MBA	N,N'-methylenbisacrylamid
34	MBS	m-maleimidobenzoyl-N-hydroxysuccinimide ester
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MEF	mouse embryonic fibroblasts
mESC	mouse embryonic stem cell
MSC	mesenchymal stem cell
NGF	neuronal growth factor
ODTS	octadecyltrichlorosilane
OPN	osteopontin
PAA	poly(acrylic acid)
PAAM	poly(acrylamide)
PACAA	poly(acrylamide-co-acrylic acid)
PC	poly(carbonate)
PC12 cell	neuronal cell line
PCL	poly(ϵ -caprolactone)
PDGF	platelet derived growth factor
PDMS	poly(dimethylsiloxane)
PEA	poly(ethyl acrylate)
PEG	poly(ethylene glycol)
PEGDA	PEG-diacrylate
PEI	poly(ethylene imine)
PET	poly(ethylene terephthalate)
pGMA	poly(glycidyl methacrylate)
PLA	polylactic acid
PLCL	poly(L-lactide-co-caprolactone)
PLGA	poly(lactic-co-glycolic acid)
PLLA	poly(L-lactic acid)
PLLCL	poly(L-lactic acid-co- ϵ -caprolactone)
PMA	poly(methyl acrylate)
PS	poly(styrene)
PU	poly(urethane)
PUA	polyurethane acrylate
PVDMA	2-vinyl-4,4-dimethylazlactone
QCM-d	quartz crystal microbalance - dissipation

QK	VEGF mimicking peptide (KLTWQELYQLKYKGI)
RCO	rat calvarial osteoblasts
REDV	Arg-Glu-Asp-Val
RGD	Arg-Gly-Asp
SAM	self-assembled monolayer
SEM	scanning electron microscopy
SMC	smooth muscle cell
SPAAC	strain promoted alkyne-azide cycloaddition
SPDP	N-succinimidyl-3-(2-pyridyldithio)propionate
Sulfo-SANPAH	sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate
Sulfo-SMCC	sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate
SVVYGLR	Ser-Val-Val-Tyr-Gly-Leu-Arg
TAMRA	5-carboxytetramethylrhodamin
ToF-SIMS	time-of-flight secondary ion mass spectrometry
UV/VIS	ultraviolet-visible spectroscopy
VEGF	vascular endothelial growth factor
WCA	water contact angle
XPS	X-ray photoelectron spectroscopy
YIGSR	Tyr-Ile-Gly-Ser-Arg
NGF	nerve growth factor

Table of content graphic

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TOC graphic

Figure 1 Schematic representing the complexity of the extracellular environment in general and the natural extracellular matrix in particular. Recreating this, combinatorial materials functionalization represents a strategy towards biomimetic materials design.

Figure 2 Combination of adhesion motifs, growth factors or both for synergistic effects on cells: a) Co-immobilization of RGD and YIGSR peptides enhanced neurite outgrowth from dorsal root ganglions (scale bar = 500 μm ; adapted with permission from Zhu et al. 78. Copyright © 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim). b) Combination of epidermal growth factor and VEGF enhanced survival of endothelial cells (scale bar = 200 μm ; adapted and reprinted from Lequoy et al. 79. Copyright (2016), with permission from Elsevier). c) Surface functionalization with BMP-2 mimetic peptide and RGD increased osteogenic marker expression (i.e. RunX2) in human bone marrow stem cells (scale bar = 50 μm ; adapted and reprinted from Bilem et al. 80. Copyright (2016), with permission from Elsevier).

Figure 3 Biochemical ligand gradients: a) Orthogonal gradient of RGD and BMP-2 peptides allowed to identify optimized combinations and ratios for osteogenic differentiation (adapted and reprinted with permission from Ma et al. 95. Copyright (2016) American Chemical Society). b) Interfacial tissues, such as tendon to bone can be replicated with reverse parallel gradients of for instance BMP-2 and FGF (Adapted and reprinted with permission from Guan et al. 97. Copyright (2016) American Chemical Society).

Figure 4 Synergistic influence of spatial surface topography (grooves) and biochemical ligands on cell behavior. Sub-micron grooves on a polystyrene (PS) surface guided alignment and elongation of C2C12 myoblasts along the grooves, while RGD functionalization directed their spreading, growth and differentiation by interaction with cellular surface receptors. The grooves displayed widths from 670 nm to 920 nm and depths of around 670 nm (Cartoon for illustration only, not for scale). Immunofluorescent staining of nuclei (DAPI) and F-actin in C2C12 myoblast was done after 24 h in culture (A, a, C, c). The scale bars in the fluorescence (A, a, C, c) and SEM (B, b, D, d) images are 25 μm and 10 μm , respectively. The arrow displays the groove direction. (Images adapted and reprinted with permission from Wang et al. 126. Copyright © 2012 Wiley Periodicals, Inc.).

Figure 5 Cell differentiation dependence on mechanical properties of the substrate and the influence of biochemical ligands: Mechanics dependent cell differentiation on mechanically consistent substrates can be influenced by surface immobilized ECM proteins (a) ECM protein or GF derived peptides (b), or GFs (c). Image a) reprinted and adapted from Kourouklis et al. 164, Copyright (2016), with permission from Elsevier: Cholangiocyte differentiation of bipotential mouse embryonic liver cells (BMEL) was independent of the elastic modulus in presence of Col IV, but regulated by mechanical properties in presence of FN on different PAAM substrates (4 kPa and 30 kPa). Hepatocyte differentiation of BMEL was mainly independent of elastic modulus and guided by the present biochemical ligand. BMEL stained for cell nuclei (DAPI), albumin (ALB) and osteopontin (OPN). Image b) reprinted and adapted from Zouani et al. 158, Copyright (2013), with permission from Elsevier: hMSC showed mechanics independent osteogenic differentiation in presence of osteogenic ligand BMP-2 mimetic peptide on polyacrylamide-co-acrylic acid substrates. In presence of the adhesion ligand RGD hMSC displayed differentiation dependent on the elastic modulus: myogenic fate on soft (modulus: 15.4 kPa) and osteogenic fate on slightly stiffer (modulus: 47.5 kPa) substrates. hMSC after four weeks stained for actin, osteopontin (OPN) and cell nuclei (DAPI). Image c) adapted and reprinted with

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3 permission from Crouzier et al. 165, Copyright © 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim:
4 Adhesion and spreading of C2C12 myoblasts is dependent on the presence of BMP-2 growth factor on soft
5 PLL/HA films (modulus: 200 kPa), but independent of the ligand on substrates showing Young's moduli of
6 around 360 kPa. An immunofluorescent staining of C2C12 was conducted for cell nuclei and actin.
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Figure 6 Combined effect of mechanical gradients and biochemical ligands on cell behavior: Image a) adopted and reprinted from Sunyer et al. 190 under CC BY license. NIH3T3 fibroblasts adhesion and spreading along a mechanical gradient (modulus: 1 kPa to 240 kPa) of a PAAM substrate was only depending on elastic modulus, regardless of the immobilized present FN functionalization. Image b) adopted and reprinted with permission from Tong et al. 63, Copyright (2016) American Chemical Society: Human fibroblasts (BJ) showed synergistically enhanced adhesion and spreading along parallel increasing mechanical properties (elastic modulus gradient from 5 kPa to 15 kPa) and RGD adhesion peptide gradients (0-0.7 mM) on PEG substrates. An immunofluorescent staining of fibroblasts was conducted for actin and cell nuclei. Image c) adapted and reprinted with permission from Hale et al. 31, Copyright (2010) American Chemical Society: Adhesion and migration of Balb/c 3T3 fibroblasts is dependent of the present biochemical ligands on orthogonal mechanics (elastic modulus gradient from 31 kPa to 85 kPa) and Col I gradients of PAAM substrates. Over a period of 3 h fibroblasts migrated to or stayed at regions with high densities of the ligand.