Cell-instructive biointerfaces with dynamic complexity

Gülistan Koçer

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CELL-INSTRUCTIVE BIOINTERFACES WITH DYNAMIC COMPLEXITY

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Prof. Dr. T.T.M. Palstra,

on account of the decision of the graduation committee,

to be publicly defended

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Chapter 1

About chemical strategies to fabricate cell-instructive biointerfaces with static and dynamic complexity*

Properly functioning cell-instructive biointerfaces are critical for healthy integration of biomedical devices in the body and serve as decisive tools for the advancement of our understanding of fundamental cell biological phenomena. We start with reviewing studies that use covalent chemistries to fabricate cell-instructive biointerfaces. This type of biointerfaces typically result in a static presentation of pre-defined cell-instructive cues. Chemically defined, but dynamic cell-instructive biointerfaces introduce spatiotemporal control over cell-instructive cues and present another type of biointerfaces, which promises a more biomimetic way to guide cell behavior. Therefore, strategies that offer control over the lateral sorting of ligands, the availability and molecular structure of bioactive ligands and strategies that offer the ability to induce physical, chemical and mechanical changes in-situ are reviewed. We pay specific attention to state-of-the-art studies on dynamic, cell-instructive 3D materials. Future work is expected to further deepen our understanding of molecular and cellular biological processes investigating cell type specific responses and the translational steps towards targeted in-vivo applications.

*Koçer, G. and Jonkheijm, P., Adv. Healthcare Mater., accepted (invited review article)

1.1. Introduction

Biomaterials in regenerative medicine have evolved from being bioinert, to only mechanically supportive and biocompatible materials and to bioactive materials that elicit and participate in biological events in their environment.^[1] The majority of the current approaches in designing bioactive biomaterials profit from progress in knowledge on how to fabricate cell-instructive biointerfaces and rely on covalent chemistries (e.g., for peptide immobilization), material synthesis and fabrication methods. These approaches typically yield static, pre-defined cell-instructive properties.^[1] In particular, chemical strategies based on, e.g., self-assembled monolayers (SAMs) have provided very important insights on cell-material interactions by revealing effects of ligand composition, density, spacing and patterning on cell behavior.^[2] Even though some aspects of cell-instructive biointerfaces were inspired by the natural extracellular environment (i.e., ligand composition and density), they lack its dynamism and, in most of the cases, its spatial complexity.^[1, 3] In fact, the native extracellular environment, which defines cell and tissue fate, has remodeling ability, is complex in composition and presents signaling molecules in a spatially and temporally regulated fashion.^[1, 4] Therefore, it is now highly recognized by biomaterials scientists that the next generation of biomaterials should consist of interfaces to cells that mimic the extracellular environment more faithfully through implementation of sufficient complexity and dynamism to instruct cells towards the desired fate.^[1, 5] It is clear that the development of this new generation of biomimetic materials requires a close collaboration between chemistry, materials science and biology where fundamental understanding of biological processes can serve as design rules. Key input for these design rules originates for example, from studies on matrix biology where there exists an increased understanding on effects of components, density, spatial anisotropy of the matrix and temporal control over the synthesis of biomolecules.^[6] Additionally, in-depth studies of cell-matrix interactions have provided input on e.g., receptor-ligand interactions, activation and clustering mechanisms of receptors such as integrins.^[7] Further input comes from impressive studies in the field of developmental biology that provide insights in e.g., phases of tissue development, temporal changes in chemical and physical effectors during morphogenesis and dynamics of cell-cell interactions.^[8] Also important are contributions made in mechanobiology research to shed light on e.g., mechanisms of force generation and sensing,^[9] while stem cell biology research gives detailed insight on e.g., multipotent progenitors, stem cell niche environments and cell type specific differentiation requirements.^[10]

Although it is evident that these research fields only collectively fuel the development of a new generation of biomaterials that is more biologically informed, progress can only be made when materials scientists provide chemical strategies to synthesize such biologically informed cell-instructive biointerfaces. With the development of new chemical approaches that are based on non-covalent interactions and stimuli-responsive elements, the generation of such cell-instructive biointerfaces will become possible.^[1, 4, 11] In this line, multiple synthetic and chemical strategies including responsive interfaces on gold, supramolecular non-covalent approaches such as host-guest systems, supported lipid membranes and stimuli-responsive interfaces have been used to chemically define dynamic biointerfaces for cells by introducing specific ligands for cell-surface receptors as well as by spatiotemporally controlling surface structure to direct cell response.^[12]

Another important aspect of the extracellular environment is its dimensionality.^[13] The most investigated arrangement are cellular responses on isotropic, planar 2D substrates. The outcomes of these studies are currently motivating the design of dynamic 3D matrices to more closely recapitulate the extracellular environment, which is a 3-dimensional (3D) environment where cells reside.^[4-5, 14]

In this Chapter, we start with an introduction section on cell-matrix and cell-cell interactions to give readers a more firm comprehension on the biological interpretation of several studies. We continue reviewing state-of-the-art chemical strategies for fabrication of both static and dynamic cell-instructive biointerfaces. In the part on static cell-instructive biointerfaces, we take account of some of the historically important studies as these studies have provided the community with important insights in critical design parameters, such as ligand spacing and density, linker lengths and non-fouling properties, which have often been implemented in the fabrication of dynamic cell-instructive biointerfaces. In the part on dynamic chemical strategies emphasis lies on the regulation of cell behavior in response to spatiotemporally controlled (biomimetic) cell-instructive cues. We then continue with discussing current strategies to generate dynamic 3D materials, which integrate developed strategies of dynamic 2D interfaces, but are now becoming increasingly important towards the development of a more realistic biomimetic microenvironments for regenerative medicine applications.

1.2. The extracellular environment

The extracellular environment is equipped with multiple physical and chemical cues that actively regulate cellular behavior with high spatial and temporal precision. This dynamic environment constantly sends signals to cells via the extracellular matrix (ECM) (see Section 1.2.1), soluble factors (see Section 1.2.1.5) and neighboring cells (see Section 1.2.2) to regulate cellular functions and cell fate choices (**Figure 1.1**).^[5, 6c]

Figure 1.1. Cell-matrix and cell-cell interactions in the extracellular microenvironment.

1.2.1. Cell-matrix interactions

Being a complex assembly of multiple fibrous and structural proteins (e.g., collagen, laminins, fibronectin and elastin), proteoglycans and specialized proteins (e.g., growth factors) the ECM plays a crucial role in morphogenesis, wound healing and patterning stem cell niches.^[6b, 6c, 7b, 10a] ECM biosynthesis and characteristics (e.g., composition, concentration and rigidity) are highly dependent on tissue type and are involved in defining the tissue organization and differentiation.^[6c]

1.2.1.1. Integrin-mediated cell-matrix adhesions

Cells recognize and integrate with their ECM mainly via well-known cell adhesion signaling, which is mainly mediated by integrin receptors.^[6, 7b-d, 10a] For other receptors involved in cell adhesion signaling the reader is referred to other places.^[15] Integrins are heterodimeric transmembrane receptors with non-covalently interacting α - and β -subunits resulting in 24 different receptors that can be cell-type specific and have different binding affinities.^[7c, 7d] Integrins are able to recognize a variety of matrix-derived ligands embedded in ECM proteins (e.g., fibronectin and laminin) while the same ligand can bind to multiple integrins, possibly due to conserved acidic peptide domains within these ligands.^[7c, 16] Integrin-ligand combinations have been classified into four groups: (1) Arg-Gly-Asp (RGD) ligands that bind to all five α V integrins, $\alpha 5\beta 1$, $\alpha 8\beta 1$ and $\alpha 11\beta 3$ integrins; (2) Leu-Asp-Val (LDV) ligands that bind to $\alpha 4\beta 1$, $\alpha 4\beta 7$, $\alpha 9\beta 1$ integrins; (3) laminin/collagen binding integrins that are formed by combination of $\alpha 1$, $\alpha 2$, $\alpha 10$ and $\alpha 11$ with $\beta 1$ subunits; and (4) laminin-specific binding integrins that are three $\beta 1$ integrins ($\alpha 3$, $\alpha 6$ and $\alpha 7$) and $\alpha 6\beta 4$ integrins.^[7c, 7d] Integrin activation requires receptor clustering to increase avidity, which increases the on-rate binding of effector molecules, and a conformational change in the receptor, leading to exposed effector binding sites.^[7c]

Matrix adhesions form a physical link between cells and ECM, giving them a structural role in cell-ECM interactions.^[6a] Furthermore, they maintain a bidirectional signaling (outside-in and inside-out) between the cells and the ECM. That is, next to being able to sense ECM cues (outside-in signaling), intracellular triggers regulate integrin binding and availability for specific ligands in the ECM (inside-out signaling).^[6a, 7a]

Matrix adhesions are composed of multiple effector proteins that mediate these signaling events and altogether form the “integrin adhesome” at the cytoplasmic side. These proteins, such as e.g., talin, paxillin, zyxin and vinculin, are involved in scaffolding as well as regulatory interactions that link the actin filaments to the cytoplasmic part of the integrin receptors and relay signals to other proteins residing in the interior of the cell such as e.g., focal adhesion kinase (FAK), Src family kinases, phosphatases and Rho family GTPases.^[6a, 7a] The majority of the protein-protein interactions at the adhesion sites are reversible and controlled by signaling processes such as tyrosine (de)phosphorylation.^[7a, 17] Depending on the stage of development (i.e., maturation) after formation of the matrix adhesions, the protein composition and the assembly and disassembly kinetics of the matrix adhesions change and can show variations even within the same cell (**Figure 1.2**).^[6a, 17]

Figure 1.2. Protein composition and time scales of matrix adhesions at different stages of adhesion maturation (see text for more details).

Integrin-mediated matrix adhesions include nascent adhesions, focal complexes, focal adhesions and fibrillar adhesions (Figure 1.2).^[6a, 7a] Nascent, dot-like adhesions form at the early stages of interactions and are present underneath the lamellipodia, which are flat, sheet-like extensions seen in the leading edge of migrating cells.^[6a, 7a, 18] Nascent adhesions are rich in phosphorylated paxillin (at tyrosine residues) and are short-lived, i.e., they can disappear or transform into bigger focal complexes that are also short-lived and associated with lamellipodial protrusions; and can mature to form focal adhesions.^[6a, 7a, 17, 19] Focal adhesions form at the end of actin and myosin containing stress fibers and go through force-dependent maturation, which mainly originates from the cytoskeleton. They show a significantly longer life-time and contain lower levels of phosphorylated paxillin in comparison to nascent adhesions.^[17] Vinculin was shown to be

one of the important components of focal adhesions, directly interacting with talin and actin to induce further receptor clustering, force transmission and enlargement of focal adhesions.^[6a, 7a, 17, 20] Fibrillar adhesions form under the center of the cells, generally along the matrix fibrils originating for example from fibronectin and are highly stable and more mature than focal adhesions.^[17, 19b] These adhesions contain high levels of tensin and $\beta 1$ integrins.^[18, 19b]

Forming a dynamic ECM-actin link, integrin-mediated adhesions control cell adhesion, cytoskeletal organization, cell shape, migration, transcriptional activity, survival and differentiation in response to ECM-derived inputs.^[6a] ECM-derived inputs can be categorized as chemical (i.e., adhesive proteins and associated molecules, see Section 1.2.1.2), physical (i.e., matrix topography and geometry, see Section 1.2.1.3) and mechanical cues (i.e., matrix rigidity, see Section 1.2.1.4) influencing above-mentioned cellular processes.^[6a, 6b, 7a, 9c, 21]

1.2.1.2. Chemical inputs from the ECM

Integrin-mediated adhesions are sensitive to ECM chemistry, particularly, to the type and density of adhesion proteins.^[6a] As mentioned above, integrins can be specific towards certain ligands, and focal adhesions can be regulated differently depending on a particular ECM protein.^[6a, 7d] For example, cells adhere to fibronectin and vitronectin through $\alpha 5 \beta 1$ and $\alpha v \beta 3$ integrins, respectively, and have distinct matrix adhesions and cell spreading behavior. Interestingly, cells on vitronectin spread less and have more peripheral vinculin localization compared to the cells on fibronectin.^[6a] Similarly, matrix chemistry dictates cell migration behavior as it is tightly regulated by cell adhesion processes in a spatial and temporal manner.^[6a, 19b] Cell migration has a key role in morphogenesis, developmental processes, wound healing and tissue homeostasis as well as colonization of biomaterials by cells. It is marked with an asymmetry in intracellularly generated forces that introduces a morphological polarization to cells in the direction of migration (**Figure 1.3**).^[18, 22] This asymmetry gives a clear difference between the front (i.e., the leading edge) and the rear (i.e., trailing edge) of the cells where shape, function and molecular composition of matrix adhesions significantly differ.^[19b, 22]

Figure 1.3. Polarization of a cell to form the leading and retracting edge (i.e., trailing edge) and its migration. Polarization involves the positioning of microtubule-organizing center (MTOC) and Golgi in front of the nucleus towards the leading edge.

In the front of the cell, and under lamellipodial extensions, FAK and paxillin enriched nascent adhesions and focal complexes constantly form and maintain cell motility.^[17-18, 19b, 22] In contrast, at the rear of the cells, mature focal adhesions are released and detachment takes place, which is necessary for effective migration. Cell migration is regulated by forces generated in the cytoskeleton, which can be categorized as protrusive and contractile forces. Protrusive forces are needed to extend the membrane protrusions (i.e., filopodia and lamellipodia) at the cell front and are generated by actin polymerization. Contractile forces, which result from myosin activity (actomyosin contraction), are necessary for forward cell movement and involved in retraction and adhesion detachment at the rear of the cell.^[18, 22-23] Cell migration speed is dependent on the ratio of contractile forces applied on cell-matrix adhesions to the strength of these adhesions (i.e., the degree of cell-matrix adhesiveness) in a biphasic manner.^[22] That is, an intermediate ratio of contractile forces to adhesive strength was suggested to induce the highest migration speed.^[24] This enables cells to form new adhesions at the leading edge as well as break adhesions at the trailing edge for effective migration.^[22, 24] As integrin-ligand interactions (i.e., cell-matrix adhesions) have a central role in cell migration, intuitively, chemical cues from the matrix such as adhesive ligand density

(e.g., protein concentration on the surface) and ligand affinity to integrins, next to integrin expression levels, become critical parameters controlling this behavior.^[24]

1.2.1.3. Physical inputs from the ECM

Signals originating from physical interactions with ECM (as a result of ECM geometry and topography) equally have profound effects in directing cell fate.^[6a, 9c, 13] ECM geometry as the regulator of cell shape and extent of spreading is one such signal that is known to be important.^[6a, 9c] As such, constraining cell spreading by using artificial adhesive protein patterns in defined geometry (shape), size and density, could induce cell death whereas allowing effective cell spreading could support cell survival and growth.^[25] Controlling cell shape by pre-defining the geometry of spreading area was also shown to affect the lineage commitment of mesenchymal stem cells (MSCs) with effectively spreading cells on large fibronectin islands going through osteogenic differentiation while round cells on smaller islands became adipocytes.^[26] In addition, cells are able to sense their physical environment via matrix topography.^[7a, 9c, 27] Matrix topography is involved in shaping tissue architecture and plays an essential role in cellular and tissue organization.^[9c] Topographical features in matrix can be present at different length scales (i.e., from nano- to microscale) and shape (e.g., fibers) inducing local (i.e., at focal adhesions) and global (i.e., cell spreading and shape) changes in cell behavior. Changes in topographical features at the scale of single cell size (i.e., microscale) may influence the degree of cell spreading and give signals associated to contact guidance such as during cell migration along a fibrillar matrix.^[27-28] As such, microscale topographies can present constraints for cell migration or give directionality, steering cells towards a certain path.^[23, 28] On the other hand, matrix nanotopography presents features at the scale of single integrin adhesions, and these type of topographies could act more locally and impact integrin clustering.^[9c, 27] This in turn could induce changes in the number and distribution of focal adhesions affecting integrin-mediated signaling, cell adhesion, cytoskeletal organization and downstream processes.^[9c, 27] Therefore, matrix topography that is the result of matrix composition appears to have a more complex role in cell and tissue responses apart from defining the tissue architecture.^[9c, 21]

1.2.1.4. Mechanical inputs from the ECM

Cells in native tissues also shape their phenotype in response to mechanical cues from the matrix, e.g., as a result of matrix rigidity.^[9c, 29] ECM rigidity depends on matrix composition, density and cross-linking.^[30] Cells probe matrix rigidity by pulling on the matrix and sensing the resistivity of the matrix to this pulling. These cell generated pulling forces originate from actomyosin contractility and are transmitted to the matrix through mechanosensitive integrin-mediated adhesions.^[9a, 31] Subsequently, the resistance of the matrix to these pulling forces feeds back as an opposite force applied on the same adhesions. This force coupling and ECM sensing is transduced into biochemical signals in a process called mechanotransduction.^[9a, 29] Multiple force-dependent molecular events occur at integrin adhesions to relay signals associated with mechanotransduction.^[9a, 31] For example, it is suggested that increasing force transmission results in higher integrin activation, regulates recruitment and phosphorylation of adaptor proteins and leads to enhanced vinculin localization. This leads to focal adhesion enlargement and stabilization.^[9a, 20a, 31-32] Force sensing also influences adhesion-linked actin cytoskeleton and its arrangement, hence results in cell shape changes.^[31]

Since matrix rigidity is defined as the resistance to deformation by cell generated pulling forces, one can expect that on soft matrices cells experience low forces (i.e., traction forces) at matrix adhesions, which leads to less mature focal adhesions, in contrast to stiff matrices.^[6a, 9a, 31] Hence, clearly, there is a dynamic sensing mechanism where the cells' own contractile activity works on ECM and receives feedbacks depending on the mechanical properties of the matrix. This sensing mechanism eventually induces signaling events and transcriptional activities to control cell shape, cell migration, proliferation and differentiation.^[30-31]

One can also perceive that ligand binding properties (i.e., affinity and flexibility) can control focal adhesion maturation in a similar way as in stiffness sensing.^[9a, 9b, 24, 33]

1.2.1.5. Synergistic integrin-growth factor signaling in the ECM

Given the diversity of the components in the natural ECM, the interactions go beyond integrin-mediated adhesive processes. It has been known for long that ECM proteins are able to bind growth factors, immobilizing them for recognition by their cell surface

receptors. It is also suggested that a cross-talk between integrin and growth factor receptors exists, which can induce a synergistic signaling inside the cell. This synergy possibly occurs either by membrane proximal interactions of the receptors or by generating a cooperative network of signal transduction pathways inside the cell.^[6b, 10a] These cooperative interactions appear to be involved in the maintenance of the stem cell niche and developmental processes, pointing out a more complex role of ECM in cellular and tissue behavior.^[6b, 6c, 10a]

Clearly, ECM is complex and dynamic, but converges information to control cell fate. In this complex environment, cells do not only receive signals from ECM, but also synthesize new proteins and remodel ECM through integrin-mediated adhesions and by degrading it. In turn, cells respond to these changes, pointing out a bidirectional interaction between the cells and their ECM.^[6c, 13, 16]

1.2.2. Cell-cell interactions

Cells receive signals also from neighboring cells during tissue morphogenesis, development and remodeling.^[5, 8a, 34] These signals are transmitted mainly via the cadherin family of cell surface receptors. Cadherins control events such as cell recognition and sorting, boundary formation and maintenance, dynamic cell movements and cell polarity.^[8a, 34-35] Other receptors involved in cell-cell interactions are described elsewhere.^[36] Cadherins mediate Ca^{2+} -dependent homophilic cell-cell adhesions and are categorized as classical cadherins, protocadherins and atypical cadherins. Classical cadherins are present in all solid tissues and form the major class of cadherins that control the cell-cell interactions at adherens junctions (AJs) (Figure 1.1).^[8a, 35a, 37] They are single-pass transmembrane proteins with five extracellular (EC) domains and further classified as type I and type II classical cadherins. Type I classical cadherins have conserved His-Ala-Val (HAV) tripeptide sequences at the EC1 domain, which is the most distal domain, and include epithelial (E) and neuronal (N) cadherins. This tripeptide motif does not exist in the EC1 domain of type II cadherins, which include vascular endothelial (VE) cadherins. The EC1 domain is known to be important for the specific recognition and strong homotypic cell-cell binding via classical cadherins.^[8a, 35a] Cadherins are connected to the cell interior through their cytoplasmic domains that interact with mediator proteins such as p120, β -catenin and α -catenin. These proteins regulate the link between cadherin adhesions and the cytoskeleton in a highly controlled and dynamic manner.^[8a, 37] Furthermore, they mediate the activation of signaling molecules such as Rho family small GTPases (e.g., Rho, Rac and Cdc42) and induce signal transduction to control cell

growth, survival and differentiation. In turn cell-cell contact formation and stability is tightly regulated by the action of these signaling molecules.^[8a, 35a, 38]

There are three different stages in the formation of new cell-cell adhesions by cadherins: initiation, expansion and stabilization. In the initiation phase, cells probe their environment by extending protrusions (i.e., lamellipodia and filopodia) and search for neighboring cells to make contacts.^[35a, 39] When the first homophilic cadherin adhesions are formed, a very short-lived peak of Rac1 activity (i.e., involved in very early contact formation and downregulated at developing cell-cell adhesions) is seen at the new adhesions. Here, actin polymerization is induced by actin nucleation factors such as the Arp2/3 complex to form a branched actin array. This is followed by an increase in RhoA activity, which converts the branched actin arrays to contractile actomyosin networks parallel to the cell membrane. During this expansion phase, which is dependent on actin polymerization and actomyosin generated forces, the cell-cell junctions grow. Finally, after the expansion phase, as cell membrane activity and actin turnover is reduced; the cell-cell junctions are stabilized.^[8a, 35a, 37, 40] Although the full mechanism of signaling events is still under investigation, it is clear that a proper regulation and fine tuning of small GTPase activity is essential as well as the interactions with the actin and myosin network.^[35a, 37] As such, sustained activity of Rac1 would prevent maturation of cell-cell contacts while excessive RhoA activity and actomyosin tension could destabilize the junctions.^[35a, 41] In fact, junctional actin dynamics has an important role not only during formation but also remodeling of the cadherin adhesions. Among the mediator proteins, α -catenin, which interacts with β -catenin and actin filaments, appears to have a central role in these processes. It is known that α -catenin recruits actin binding proteins to the cytoplasmic side of cadherins and prevents actin branching during the expansion of adhesions.^[35a, 37] Cadherin adhesions also interact with microtubules via p120 mediator proteins. Therefore, a two-way interaction between the cytoskeleton and cadherin adhesions is proposed where adhesion formation and stability is controlled by cytoskeleton and cytoskeletal rearrangements are affected by cadherin-cadherin binding.^[35a, 37]

Importantly, similar to integrin-mediated adhesions, cadherin adhesions can act as mechanosensors and respond to mechanical cues from their environment such as shear flow and cytoskeletal tension (i.e., actomyosin tension) of neighboring cells.^[41] Even though its molecular basis is not fully understood, cadherin-mediated mechanosensing is known to involve myosin II generated forces and α - and β -catenin-mediated vinculin recruitment at the cytoplasmic side. In a proposed mechanism, α -catenin behaves as a

strain gauge going through conformational changes upon application of tension at the adhesions which exposes the vinculin binding sites. However, the contributions of myosin II generated tension and vinculin recruitment appear to only partially explain mechanosensing at cadherin adhesions, in contrast to integrin-mediated adhesions. This points out other processes, possibly occurring through proteins that can bind α -catenin and actin filaments.^[41]

Taken together, both cell-ECM and cell-cell adhesion mediated processes have critical importance in modulation of cellular and tissue behavior. In this line, it is also intuitive that, with the common features of being dynamically connected to actin cytoskeleton, inducing common signaling pathways and interacting with the same adaptor proteins, integrin and cadherin-mediated adhesions are interdependent and communicate during abovementioned cellular processes.^[42]

1.3. Chemical strategies to fabricate cell-instructive biointerfaces

In this section, we present synthetic strategies, either covalent or non-covalent in nature, that have been used to fabricate cell-instructive biointerfaces (**Scheme 1.1**). We describe consequences on cell behavior of the given chemically derived biointerfaces. For studies using patterning to achieve cell-instructive biointerfaces please refer to other reviews.^[43] We review static chemistries to provide the reader with insight into what has been done historically in the field to establish critical design parameters (e.g., ligand affinity, spacing and immobilization chemistry) when fabricating cell-instructive biointerfaces as well as to understand cell-material interactions, as these studies fueled the development of dynamic systems.

Scheme 1.1. Overview of chemical strategies to fabricate cell-instructive biointerfaces.

1.3.1. Chemically defined, static cell-instructive biointerfaces

Cell-instructive biointerfaces that present ligands in static conditions to cells have been used to target cells in many occasions. The ligands that have been used are biomolecules such as integrin-binding Arg-Gly-Asp (RGD) peptides^[44] with a defined ligand affinity and density (i.e., spacing) and, in some of the highlighted studies below, even with a given spatial organization (i.e., ordered vs. disordered ligands) to direct and investigate cell

adhesion, migration and differentiation. Model systems and platforms that we highlight in this section make use of self-assembled monolayers (SAMs) mostly on gold and glass, self-assembled maleimide functionalized polystyrene-block-poly(ethylene oxide) copolymers (PS-PEO-Ma) and deposition of gold nanoparticles with di-block copolymer micelle nanolithography.^[2b, 45]

1.3.1.1. Self-assembled monolayers for static, cell-instructive biointerfaces

SAMs of alkanethiolates on gold are structurally well-defined and densely packed monolayers with a high ability to form non-fouling surfaces, which makes them highly attractive platforms for cell studies.^[45a, 46] By incubating the gold substrate with defined mixtures of oligo(ethylene glycol)-alkanethiolates, which are excellent non-fouling agents, and maleimide terminated-alkanethiolates, cell-targeting ligands can readily be introduced on the SAMs through a Michael addition reaction.^[12a, 45a, 45b] The density of the ligands is directly related to the molar ratio of maleimide groups on the surface.^[45a, 45b] Alternatively, peptide-alkanethiolate conjugates can be synthesized and directly used to fabricate the SAMs in defined mixtures with oligo(ethylene glycol)-alkanethiolates. This procedure avoids on-chip synthesis, but generally provides less control over the content on the SAM.^[46-47] On these cell-instructive biointerfaces, it was confirmed that the groups surrounding the RGD ligands critically influence how cells adhere and spread.^[46] In a study by Mrksich and co-workers, SAMs on gold were fabricated from defined mixtures of RGD-alkanethiol conjugates with various non-fouling agents such as thiolated tri-, tetra-, penta- or hexa (ethylene glycol) groups (i.e., EG₃ to EG₆) (**Figure 1.4A**). An increase in the length of the oligo(ethylene glycol) groups, resulted in a decrease in the extent of Swiss 3T3 fibroblast cell adhesion and spreading after 5h. Interestingly, this effect was most pronounced at the lowest ligand density.^[46]

Furthermore, the extents of cell adhesion and spreading on SAMs with longer oligo(ethylene glycol) groups were more sensitive to changes in ligand density and significantly decreased with decreasing ligand density, in contrast to SAMs presenting shorter oligo(ethylene glycol) groups.^[46] This dependence of cell adhesion and spreading on variations in the length of oligo(ethylene glycol) groups was explained by the change in affinities of the immobilized RGD ligands to integrin receptors. The authors suggested that the affinity was modulated by the crowding of non-fouling agents surrounding the RGD ligands.^[46] Finally, cells seeded on SAMs of EG₃ mixed with 0.5 mol% RGD showed well-defined stress fibers with strong vinculin localization in the cell periphery (Figure 1.4B). In contrast, on EG₆-presenting SAMs, cells had a diffused vinculin localization and round morphology (Figure 1.4C). Overall, these results show that the microenvironment of the cell-binding ligands influences their accessibility and flexibility. These characteristics of ligand attachment and choice of non-fouling agents are critical to direct cell response, next to the variations in ligand density.^[2a, 46]

In another study by Kato and Mrksich, the same type of SAMs were employed to point out effects on cell adhesion when comparing low (linear RGD) or high (cyclic RGD)

affinity ligands for integrin-binding.^[47] In this work, SAMs on gold were prepared that present hydroxyquinone moieties surrounded by non-fouling tri(ethylene glycol)-alkanethiols at defined molar ratios. After conversion of hydroxyquinone to benzoquinone, cyclic and linear RGD ligands were covalently attached in a Diels-Alder reaction to the surface at the same densities (**Figure 1.5A**).^[47]

Figure 1.5. (A) SAMs on gold with linear (L) or cyclic (C) RGD. (B) Vinculin localization in 3T3 fibroblasts on linear and cyclic RGD at 1 mol% ligand density (at 10h). Reproduced with permission.^[47] Copyright 2004, American Chemical Society.

3T3 fibroblasts on cyclic RGD (1 mol%) had ca. 2-fold more focal adhesions (FA) that were more evenly distributed throughout the cell (at 10h). On the other hand, on linear RGD (1 mol%), larger FA with a wider size distribution appeared that were mainly located at the cell periphery (Figure 1.5B). A mechanism was proposed wherein the high affinity ligand resulted in a higher frequency of ligand-receptor interactions (i.e., nucleation)

possibly resulting in a higher number of FAs. Next to that, in this model, the high affinity ligand also resulted in a longer lifetime of ligand-receptor complex which slowed down the growth of FAs by decreasing the effective diffusion of receptors during nucleation, leading to the formation of smaller FAs in cells. Collectively, ligand affinity is a critical molecular parameter that drives changes in cell attachment and focal adhesion formation. These type of studies on well-defined surfaces are crucial for systematic studies to understand the effects of ligand affinity, density and even patterning on cell behavior.^[47]

In a recent study, the same group reported the effect of ligand density and affinity on human mesenchymal stem cell (hMSC) differentiation.^[45b] For this purpose, SAMs were again functionalized with linear and cyclic RGD in EG₃ background. Alkaline phosphatase (ALP) (as an early osteogenic marker) staining, immunofluorescence and reverse-transcriptase PCR (RT-PCR) analyses of marker genes revealed that on cyclic RGD, osteogenic differentiation was induced at high and low ligand density. In contrast, on linear RGD, at high density, myogenic differentiation was observed while at low density a more neurogenic phenotype was seen. These observations were linked to changes in cell morphology, focal adhesion density and contractility,^[26] showing that ligand affinity and density together affect stem cell fate.

SAMs of cyclic RGD (1 mol%) were further used to investigate the dynamics of cell migration under the influence of integrin antagonists such as soluble, linear RGD.^[48] A linear increase in cell speed was seen when increasing the concentration of soluble, linear RGD up to 100 μ M, while beyond 100 μ M, cell adhesion was partly lost. The authors suggested that by introducing an integrin binding antagonist, the rate of dynamic dissociation of integrins from the immobilized ligands, at the rear of the cell, could be increased which leads to higher rates of cell migration. With this, the authors proposed a model that considers focal adhesions as polyvalent complexes with high stability, where dissociation of single integrin-peptide complexes on the surface is immediately followed by their re-association. However, introduction of a soluble ligand can destabilize these complexes by blocking re-association of integrins to immobilized ligands, therefore “unzipping” this complex and leading to its turnover. Taken together, these results have pointed out a mechanism for stimulated cell migration and a way where polyvalent interactions can control cell behavior.^[48]

SAMs on silicon substrates have also been proven as valuable model biointerfaces and they share basic features with their counterparts on gold.^[2b] On these SAMs, Gooding,

Gaus and co-workers studied the effect of average ligand spacing, by variation of ligand density, in the context of endothelial cell adhesion, mechanotransduction and cells' responsiveness to vascular endothelial growth factor (VEGF).^[49] To this end, silicon surfaces were modified with a base undecenoic acid layer to which defined ratios of a non-reactive and reactive non-fouling EG₆-type agents were immobilized. RGD ligands were covalently attached to the reactive EG₆-groups (**Figure 1.6A**).

Figure 1.6. (A) Silicon SAMs with RGD densities and spacings as tabulated. (B) Confocal images of paxillin in BAECs at indicated ligand densities. Scale bar: 20 μ m. Reproduced with permission.^[49] Copyright 2011, Elsevier.

On these surfaces, an optimum RGD ligand density (1:10⁶ RGD:EO₆) was observed for a significantly higher extent of bovine aortic endothelial cell (BAEC) adhesion. Interestingly, paxillin localization (**Figure 1.6B**) and membrane order at FAs (highly ordered domains being associated with a lower membrane fluidity)^[50] showed a strong dependence on ligand density where the highest degree for membrane order was

observed for the SAM with 44 nm spacing, similar as found on fibronectin.^[49] This result indicates that the FA organization is sensitive to nanoscale changes in ligand spacing. Furthermore, VEGF stimulation (10 min) induced a higher signaling efficiency in BAECs on SAMs with 44 nm spacing. Under VEGF stimulation, cells on SAMs with 44 nm spacing acquired a more invasive angiogenic phenotype and exhibited a higher migration speed compared to the cells on any other substrate. Altogether, these data suggest that ligand spacing strongly affects FA organization and synergistic integrin-growth factor signaling, and controls endothelial cell phenotype through their ability to migrate. Therefore, next to presenting physiological importance in understanding the mechanisms of cell behavior, these surfaces could be further exploited to engineer biomaterial interfaces in order to control endothelial cell behavior for biomedical implants.^[49]

1.3.1.2. Spatially ordered, static, cell-instructive biointerfaces

Even though the use of SAMs on gold or glass presents multiple advantages to form cell-instructive biointerfaces, they generally present ligands that are randomly distributed in the monolayer.^[49] In an attempt to arrive at chemically defined surfaces where ligands are ordered with high spatial precision, Spatz and co-workers have developed and used the so-called diblock copolymer micelle nanolithography technique.^[45c] In this technique, Au-dots containing micelles of polystyrene-*block*-poly[2-vinylpyridine(HAuCl_4)_{0.5}](PS-*b*-P[2VP(HAuCl_4)_n]) diblock copolymers are transferred to glass, and following plasma etching to remove the entire polymer, an extended area of Au nanodots arranged in a nearly perfect hexagonal pattern becomes available for cell studies.^[45c, 51] The nanodots were functionalized with thiolated cyclic RGD ligands. As the size of the dots was kept at about 8 nm, which corresponds to the size of a single integrin,^[52] a single anchoring point is created to which mostly one integrin can bind. The distance between the dots was controlled by the molecular weight of the diblock co-polymers. The surrounding areas were passivated with a non-fouling polyethylene glycol (PEG) coating. Using these surfaces, the limits of cell adhesion and focal adhesion formation were investigated as function of the spacing between RGD-functionalized dots (Au/RGD) ranging from 28 nm to 85 nm.^[45c] MC3T3 osteoblasts acquired well-defined and enhanced vinculin localization at focal adhesions on 28 and 58 nm spacings whereas the higher spacings (i.e., 73 and 85 nm) showed more disorganized vinculin and actin localization (**Figure 1.7A-B**).

Figure 1.7. (A-B) Vinculin localization (in green) in MC3T3 osteoblasts on surfaces with Au/RGD dot spacings of 58 and 73 nm, resp. (red: actin, scale bar in inset: 5 μ m). (C) Scheme for focal adhesion activation at critical ligand spacings in relation to integrin clustering. Reproduced with permission.^[45c] Copyright 2004, John Wiley and Sons.

Therefore, a model for effective cell adhesion was proposed where above a critical ligand spacing (which is ~ 70 nm), functional focal adhesions cannot form due to insufficient integrin clustering and cells show reduced adhesion, spreading and actin organization. This critical ligand spacing correlates well with the periodicity in ECM proteins such as in collagen, which is around 67 nm.^[53] Importantly, REF52-fibroblasts, 3T3-fibroblasts and B16-melanocytes revealed similar behavior indicating a universal sensing mechanism occurs during cell adhesion and, therefore, making it more prominent to the design of biointerfaces.

Interestingly, on disordered RGD arrays with spacings 58 to 100 nm, the average cell area only slightly changed and well-spread cells were still observed beyond 92 nm spacing.^[51] Also, well-defined vinculin localization appeared at spacings larger than 70 nm. These results show that disordered surfaces allow for a higher range of ligand spacings to

trigger proper cell adhesion and spreading. In order to explain their observations, the authors pointed out that on ordered arrays, global ligand spacing directly corresponds to local ligand spacings while on disordered arrays this is not the case. That is, even at global ligand spacings higher than 70 nm, local ligand spacings between nanodots on disordered arrays could be less than this critical value allowing integrin clustering, focal adhesion formation and cell spreading. This further shows the importance of local variations in ligand spacings and its impact on cell response.^[45c, 51]

Another critical parameter that is known to govern integrin-ligand interactions at the biointerface is the chemical nature and the length of the spacer between the ligand and the surface. Essentially, a spacer functions by lifting the immobilized ligand (i.e., RGD) from the surface and influences its presentation and accessibility to integrins.^[44b] In literature, different types of spacers such as Glycine (G)-based spacers have been used to engineer RGD presentation to cells where the spacer length (e.g., number of glycine residues) was shown to be one of the limiting factors for integrin activation.^[44b, 54] In a recent study by Spatz and Kessler and co-workers, effects of type and length of the spacer of RGD ligands (**Figure 1.8A**) on cell adhesion were studied on the RGD nanodot arrays using aliphatic aminohexanoic acid (Ahx), PEG-based and polyproline spacers.^[55] An important feature of aliphatic Ahx and PEG-based spacers is their high flexibility while polyproline spacers are rather rigid.^[56] Therefore, polyproline spacers present an extended conformation where the exact length can be calculated for the spacers as opposed to PEG spacers, which are not fully extended and only allow to calculate an average spacer length.^[55]

Gold dot arrays with 68 nm spacing were functionalized with different types of cyclic RGD ligands (**Figure 1.8A**) where an increase in spacer length resulted in an increase in FA density in fibroblasts as well as an increase in average cell area. Longer and more hydrophilic spacers induced more stress fiber formation. Short hydrophobic spacers induced the slowest cellular spreading rate; and for all spacers, increasing their length led to an increase in the rate of cell spreading (**Figure 1.8B**). Furthermore, ligand dimerization induced a higher and faster cell spreading as investigated for polyproline spacers (**Figure 1.8B**). Paxillin localization further revealed significant effects of spacer type and length where, in general, shorter spacers resulted in a more delayed development of FAs compared to longer spacers (**Figure 1.8B**).

A)

B)

Figure 1.8. (A) Cyclic RGDs with aliphatic (**1-2**), PEG-based (**3-5**) and polyproline spacers (**6-10**), where ligands **9-10** carry two cyclic RGDs. Thiols were used for immobilization to gold dot arrays. (B) YFP-paxillin imaged in rat embryo fibroblasts (REF52 cells) on arrays with indicated ligands. Scale bar: 20 μm . Reproduced under the terms of CC BY-NC-ND License.^[55] Copyright 2013, The Authors.

Next to resulting in a higher cell spreading rate, polyproline-modified cyclic RGD ligands also induced the largest FA in size and a stronger FA development in time. Altogether, these results showed that polyproline based spacers more efficiently initiate integrin-ligand interactions than when using PEG and alkane based spacers. This enhanced effect

seen in polyproline spacers was attributed to their more extended molecular conformation and a lower density of the ligands (presumably due to less efficient packing of hydrophilic polyproline helices), which can increase the accessibility of the cyclic RGD ligands to cells. These observations clearly point out the importance of the quality of ligand presentation through control over spacer properties, with chemical precision, for proper integrin activation, clustering and subsequent maturation of FAs accompanied with effective cell spreading.^[55]

Other types of spatially defined RGD ligand systems have been used to study and direct hMSC differentiation through modulation of integrin-mediated adhesions.^[45d, 57] A case in point, by Cooper-White and co-workers, are patterns made by the self-assembly of poly(styrene-block-ethyleneoxide) (PS-PEO) block copolymers that result in vertically oriented cylinders with a pre-defined number of PEO chains in a matrix of PS. The lateral spacing of PEO nanodomains (chains) was controlled by altering the ratio between the PS-PEO and polystyrene (PS) homopolymer to achieve spacings of 34, 44, 50 and 62 nm. Functionalization of these surfaces was achieved by further chemical modification of the terminal alcohol in the PEO tether with RGD ligands.^[45d] On these ligand systems, hMSCs acquired a well-spread morphology with an increased cell area and well-defined stress fibers (after 4h) on 34 and 44 nm spacings while for 50 and 62 nm spacings, cells had disorganized actin and multiple filopodial extensions. Moreover, at 34 and 44 nm spacings, cells (at 24h) had distinct and a higher extent of vinculin localization associated with actin stress fibers as opposed to spacings above 50 nm. These results are in line with the observations for different cell types on RGD presenting gold arrays.^[45c, 51] Interestingly, cell migration speed exhibited a parabolic trend where it increased with increasing lateral spacings from 34 to 50 nm, but decreased again when going to 62 nm spacing. Furthermore, when cells were given the choice between osteogenic and adipogenic differentiation by supplementing them with a double induction medium, cells on 34 nm spaced ligands showed a higher osteogenic capacity (marked with ALP expression, at day 10) while cells on 62 nm spaced ligands were committed to adipogenic lineage (marked with oil deposition).^[45d] Altogether these results show that, next to ligand affinity and density, controlling RGD spacing with nanometer precision is an effective way to direct hMSC adhesion and differentiation.^[45b, 45d]

To conclude this Section, static cell-instructive biointerfaces have been instrumental to demonstrate that choices in ligand type, density and spacing direct cell fate on time scales that range from cell adhesion (up to few hours) to cell differentiation (up to

several days). Therefore, these biointerfaces are not only tools to study important aspects of cell biology but also inspire designs of dynamic cell-instructive biomaterials.

1.3.2. Chemically defined, dynamic cell-instructive biointerfaces

Sharing some of the fundamental design principles with static biointerfaces, cell-instructive dynamic biointerfaces potentially offer additional control over cell-ligand interactions. For example, laterally mobile ligands and ligand sorting and control over the affinity of ligand attachment to the surface, combined with temporal availability of ligands, offer additional entry to control ligand presentation.^[12a, 12b, 12d, 58] In this Section, we highlight supramolecular strategies (e.g., lipid membranes and host-guest chemistry) and strategies that use stimuli-responsive systems to dynamically instruct cell behavior on 2D systems. Furthermore, we highlight the use of supramolecular and stimuli-responsive hydrogels towards the development of 3D dynamic biomaterials.^[4, 14a]

1.3.2.1. Supported lipid membranes for dynamic, cell-instructive biointerfaces

Cell-instructive supported lipid bilayers

Supported lipid bilayers (SLBs) are cell membrane mimetic supramolecular entities that can easily form on hydrophilic solid supports (e.g., clean activated glass) and retain physicochemical properties of the cell membrane (e.g., phase behavior), making them powerful cell-instructive surface models.^[12d, 59] The physicochemical properties of SLBs are defined by their base lipid content. The head group of base lipids determines the surface chemistry of bare SLBs, e.g., zwitterionic head groups such as phosphatidylcholine (PC) form a protein and cell repellent (i.e., non-fouling) surface.^[12d, 60] The alkyl tail of base lipids determines the phase behavior of SLBs due to the characteristic (gel-to-fluid) transition temperature of these lipids. SLBs that are formed using low melting temperature lipids (e.g., 1,2-dioleoyl-sn-glycero-3-phosphocholine, DOPC, $T_m = -20^\circ\text{C}$) present mobile, hence laterally dynamic, ligands, while SLBs of high melting temperature lipids (e.g., 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, DPPC, $T_m = 41^\circ\text{C}$) present immobile, hence laterally static, ligands at physiological temperatures.^[12d, 58b, 61] In this Section, examples are discussed to illustrate that these differences in lateral dynamics of ligands affect ligand-receptor interactions, the degree of receptor clustering and the activation of subsequent signaling pathways.^[58d, 62] SLBs can be functionalized in multiple ways such as by using peptide amphiphiles that are directly incorporated in SLBs, using histidine (His) tagged ligands that complex with Ni^{2+} -NTA (nitriloacetic acid) head groups of lipids in SLBs or using biotin-avidin interactions to

introduce biotinylated ligands on avidin-functionalized SLBs.^[12d] Alternatively, full length proteins can be covalently immobilized onto SLBs via activated carboxylic acid-modified lipids and cysteine-capped peptides can be reacted with maleimide-modified lipids.^[62e, 63] Other methods to introduce ligands to SLBs rely on electrostatic interactions between charged lipids and oppositely charged ligands or some studies use charged SLBs to directly interact with cell membranes.^[64] The many ways to introduce ligands to SLBs and the variability in physicochemical properties have rendered SLBs as versatile biomimetic platforms.^[62a, 62b, 65]

In one study, Sheetz and co-workers used SLBs, physically separated by nano-sized barriers, to reveal early events (in seconds to minutes) in integrin-signaling.^[62c] Fluidic DOPC SLBs were functionalized with cyclic RGD using biotin-neutravidin interactions yielding ligand densities of ca. 1750 ± 400 molecules/ μm^2 (**Figure 1.9A**). Integrin-RGD interactions resulted in the formation of sub-micron sized, circular, clusters where colocalization of integrin- β_3 and RGD ligands increased during the first 200 sec of adhesion. At these initial phases of cluster formation, multiple cytoplasmic adaptor proteins, including paxillin, talin and FAK, were recruited to the cluster site in a force-independent manner, as inhibition of acto-myosin generated forces^[66] did not prevent their recruitment at the clusters. Interestingly, formation of integrin-RGD clusters caused remodeling of the actin network and stimulated local actin polymerization where actin filaments grew from the early clusters. These actin-enriched early clusters (i.e., nascent adhesions) were initially observed to move laterally towards each other and then pile up against the metal lines (nascent adhesions marked with YFP-paxillin, **Figure 1.9B**), which efficiently blocked their lateral movement, in a process dependent on myosin generated contractile forces. These results pointed out that the SLB barriers not only acted as spatial confinements for the activated integrin clusters but also acted as force generation sites, by passively counteracting the transport forces applied on these clusters. Furthermore, the metal barriers activated cell spreading better compared to continuous SLBs. During spreading, the initial lamellipodial extension phase (active protrusion) was accompanied by a previously unreported long-range outward movement (i.e., towards the cell periphery) of the activated integrin clusters, accumulating at the inner side of the barriers. This outward movement was suggested to be stimulated by actin polymerization at the clusters and was dependent on the activation of Src family kinases.

Figure 1.9. (A) Nano-patterned RGD functionalized fluidic SLBs (0.4 mol% biotinylated lipids, chromium lines (100 nm wide, 5 nm high) with different spacings) interact with cells. (B) Co-localization of RGD and YFP-paxillin at the contractile clusters in time during early adhesion where they piled up against the metal barriers with 2 μm gap spacing (two right boxes, scale bar: 5 μm). Reproduced with permission.^[62c] Copyright 2011, The Authors. (C and D) Localization of Dab2-mCherry and integrin- β 3-GFP in cells seeded on RGD-glass and RGD-SLB (membrane), resp. (scale bar: 10 μm). Reproduced under the terms of Creative Commons Attribution 4.0 International License.^[62f]

After the active protrusion phase, myosin-II mediated retraction was seen to stimulate the inward (i.e., towards the center of the cell) translocation of integrin clusters, with high forces applied at these clusters, resulting in their accumulation at the outer side of the physical barriers. In this line, the activation of cell spreading (during extensile phase)

and its stabilization (during retraction phase) was directly correlated to the density of the metal barriers (i.e., decreasing gap spacing). As such, a higher density of these barriers resulted in bigger cell area after 30 min, possibly due to higher density of stable adhesion sites. Finally, vinculin localization was only seen after the inward movement of activated integrin clusters (i.e., upon contraction) showing that force generation was required for this process, in line with the role of vinculin at focal adhesions.^[20a] Taken together, mechanisms associated with reorganization and clustering of focal complexes were successfully studied on dynamic cell-instructive SLBs and would have been difficult to realize with fixed ligand configurations. The use of physical barriers in SLBs can act as confinement and force generation sites while defining the spatial organization of ligated integrins for effective cell spreading.^[12d, 62c]

In a follow-up study, the same group investigated clathrin-mediated integrin endocytosis, as an important event during cell migration and adhesion dynamics, in relation to ligand mobility and force generation at integrin-mediated adhesions.^[62f] It was observed that adaptor protein Dab2 involved in clathrin-mediated endocytosis, colocalized with activated integrin- $\beta 3$ clusters on RGD-SLBs after their formation and in time concentrated at these clusters. In contrast, Dab2 was not present at integrin- $\beta 3$ clusters in cells on RGD-glass surfaces which also induced the formation of firmer (classical) focal adhesions (Figure 1.9C-D). Also, on RGD-SLB surfaces, integrin- $\beta 3$ clusters were endocytosed via clathrin as observed by the internalization of RGD-neutravidin in time and its colocalization with integrin- $\beta 3$ in endosomes, which was not observed on RGD-glass surfaces. This work shows that recruitment of Dab2 at integrin- $\beta 3$ clusters required laterally mobile ligands resulting in endocytosis of integrin clusters and adhesion turnover. Such information on spatiotemporal regulation of integrin adhesions could be, in particular, of interest for processes involving cancer invasion where proteolytically cleaved matrix may resemble mobile ligands presented in this study.^[62f]

Figure 1.10. (A) ECM-functionalized SLB via reaction of ECM protein to an activated carboxylic acid on the SLB. (B) Fluorescence images of fibronectin (Fn)-functionalized SLBs with varying cholesterol densities and incubated with hepatocytes for 3h (scale bar: 20 μ m). Reproduced with permission.^[62e] Copyright 2017, John Wiley and Sons.

Next to using peptide functionalized SLBs, ECM protein-functionalized SLBs have also been exploited as cell culture platforms.^[63b, 63c] In a recent study, Cho and co-workers have used this type of SLBs to generate cell-instructive biomimetic surfaces where lateral reorganization of immobilized ECM proteins (fibronectin (Fn) and collagen) could be modulated by tuning the SLB fluidity (Figure 1.10A).^[62e, 68] A soft protein adlayer on SLBs was achieved based on a high energy dissipation observed in quartz crystal microbalance measurements. Although it was observed that the SLB mobility was retained upon protein functionalization, the covalently bound proteins were non-diffusible on the SLB surface. Specific adhesion of hepatocytes on these functional SLBs induced the lateral movement of ECM proteins and their local enrichment underneath the adhering cell, eventually creating a depletion zone around the cell (Figure 1.10B, Chol 0%). This lateral movement of proteins by cells was suggested to occur due to applied cellular forces resulting in a viscous drag in SLBs, and required a fluid SLB underneath the adherent cell. Addition of cholesterol in the SLBs (up to 40 mol%), interestingly, led to a decrease in protein enrichment. This is possibly due to increased resistivity of protein reorganization to pulling forces by cells, as a consequence of higher viscosity and decreased fluidity of the bilayers upon adding cholesterol to SLBs (Figure 1.10B). This finding could be useful for improving commonly used PDMS-based surfaces in forming very low rigidity interfaces for biomedical applications.^[62e]

SLBs have also served as excellent models to investigate cell-cell interactions.^[65a, 65c, 69] Given that E-cadherin in natural cell membrane has low mobility,^[70] in recent work, Groves and co-workers used E-Cadherin-functionalized SLBs (Figure 1.11A) for studying epithelial cell-cell junction formation in relation to SLB fluidity.^[62d] The physiological

density of monomeric E-Cadherin on mobile SLBs was reached ($100\text{--}700\text{ molecules}/\mu\text{m}^2$) with a diffusion coefficient of $1.6\text{ }\mu\text{m}^2/\text{s}$.^[71] When MKN-28 epithelial cells came into contact with mobile E-Cadherin-SLBs (Figure 1.11B), generally no enrichment of E-cadherins was seen underneath the cells, indicating that no junctions were formed. Only in less than 1% of counted cells, extensive enrichment and junction formation was seen on mobile SLBs, while weak or impaired junctions were not seen at all (Figure 1.11C). This means that if junction formation was successfully initiated, it went to completion. When E-Cadherin was bound to low mobility SLBs, ($D\sim 0.002\text{--}0.0036\text{ }\mu\text{m}^2/\text{s}$ ^[72]), effective cell-cell junctions were formed (Figure 1.11C), while on immobile SLBs cell-cell junctions failed to form. A moderate mobility of E-Cadherin was suggested to result in two different physical effects during junction formation: i) larger opposing forces applied against actively driven transport, which can induce force-sensitive molecular events in cells in relation to adhesion and ii) enriched localization of cadherin for longer timescales. Importantly, cell-cell junction formation on low mobility SLBs was dependent on active cytoskeletal processes associated with filopodia formation and retraction as well as actomyosin activity.^[66a, 73] Furthermore, it was shown that the intracellular domain of E-Cadherin had a critical role in junction formation on low mobility SLBs. As a result, the authors suggested that E-Cadherin on the filopodial surface engages with SLB-bound E-Cadherin to form the intermediate cadherin-cadherin bond, resulting in an active nucleation process. In turn, molecules with low mobility tend to enhance the local E-Cadherin concentration under the retracting filopodia (Figure 1.11B). This process is impaired for highly mobile E-Cadherin at the physiological protein densities, possibly due to fast dissipation of molecules. Consequently, local enrichment in concentration leads to clustering of cadherin-cadherin bonds, which results in stable cell-cell junctions (Figure 1.11B-C).^[62d] Collectively, this study points out the significance of SLBs for exploring the effect of the lateral mobility of ligands on native cellular processes and revealing previously unreported mechanisms involved in E-Cadherin junction formation and regulation.^[62d, 69]

Figure 1.11. (A) Functionalization of SLB with dye-labeled extracellular domain of E-Cadherin using Ni²⁺-NTA- His₁₂ chelation (4 mol% NTA-lipid). (B) E-Cadherin junction formation on SLBs. (C) Fluorescence time series images of MKN-28 epithelial cells on mobile (fluid) and low mobility (partially fluid) E-Cadherin-SLBs. Left: bright field mode. Color bars show fold enhancement in surface density of E-Cadherin (scale bars: 5 μ m). Reproduced with permission.^[62d] Copyright 2015, The Authors.

Modelling cell-cell interactions on SLBs has also been used to direct skeletal tissue derived progenitor cell fate in the context of N-Cadherin mediated adhesions. This type of adhesions are known to be important during mesenchymal condensation, chondrogenesis as well as bone formation.^[74] In a study by Knothe-Tate and co-workers, DOPC SLBs were functionalized with the recombinant extracellular domain of human N-Cadherin using His-tag technology.^[74a] On N-cadherin SLBs, primary adult periosteum

derived cells (PDCs) adhered to SLBs by acquiring a round morphology and at high cell seeding density (35,000 cells/cm²) cell aggregation was much more enhanced than at low cell seeding density (5,000 cells/cm²). PDCs remained in suspension at both cell seeding densities on control SLBs. Gene expression profiles indicated that on N-Cadherin SLBs, at high cell seeding densities, cells were directed towards a pre-mesenchymal-condensation state while on control SLBs, at high cell seeding densities, cells were directed towards osteochondral lineage.

SLBs have also proven as suitable platforms to probe cell-cell interactions in the context of EphA2 receptor tyrosine kinase signaling, which is involved in multiple cancer types.^[75] In a study by Salaita, Groves and co-workers, the efficiency of EphA2 signaling was investigated in response to the lateral organization of receptor-ligand complexes on SLBs.^[58d] Ephrin-A1 ligands were attached through biotin-avidin interactions to SLBs in array format and used to target juxta-positioned EphA2 receptors on mammary epithelial cells (**Figure 1.12A**).^[58d] Ligand density was adjusted to be 800 ± 200 molecules/ μm^2 in the range of the density of EphA2 receptors that was detected on the surface of a representative breast cancer cell line (MDA-MB-231 cells). On continuous fluidic SLBs, EphA2-ephrin A1 micro-clusters (as formed underneath the cells within 15 min of contact) went into a radial movement and concentrate in the center of the cells in a time dependent manner (**Figure 1.12B**). The inward movement of these clusters was accompanied with an extensive actin reorganization^[76] and required actomyosin contractility. Besides, EphA2-ephrin-A1 clusters co-localized with the zones of the highest tyrosine phosphorylation throughout the cell body. High phosphorylation levels at EphA2-ephrinA1 clusters were seen regardless of the presence and extent of ephrin-A1 ligand confinement in the SLB (as achieved by using nano-sized physical barriers at defined grid spacings, **Figure 1.12B**), showing that EphA2 was locally activated by ligand binding. On continuous SLBs, ADAM10, a metalloproteinase involved in Eph receptor signaling, showed a strong co-localization with EphA2-ephrin-A1 clusters after 1h (**Figure 1.12B**). However, ADAM10 recruitment and co-localization with EphA2-ephrin-A1 clusters diminished upon decreasing the size of the clusters by applying smaller grid spacings in the SLBs (**Figure 1.12B**). These results were explained both by spatial receptor organization and mechanical regulations in receptor-ligand interactions as these barriers in the SLB also apply opposing forces, albeit passively, to receptor clusters as mentioned above.^[62c] Therefore, cellular response on the barrier SLBs can be related to spatio-mechanical characteristics of the microenvironment of cells upon interaction with ephrin-A1 ligands.

This study shows again the strength of using SLBs as biointerfaces with tunable lateral mobility of ligands for the investigation of cell signaling events in a highly biomimetic context.^[58d] Due to their ease of fabrication, SLBs can be readily combined with other patterning tools to generate hybrid surfaces that enable the presentation of multiple ligands in different configurations (e.g., mobile and immobile).^[77]

Figure 1.12. (A) Ephrin-A1 ligands attached on either continuous fluidic SLBs or nano-patterned SLBs (chromium metal lines) (B) Localization of ADAM10 with EphA2-ephrin-A1 complexes in MDA-MB-231 cells on patterned SLBs exhibiting different spatial organization of ephrin-A1 ligand (scale bar: 10 μm). Reproduced with permission.^[58d] Copyright 2010, The American Association for the Advancement of Science.

To conclude this part of the Chapter, SLBs introduce many advantages and opportunities to generate highly biomimetic platforms. SLBs have been successfully used to explore fundamental biological processes as well as to generate cell-instructive biointerfaces that can be exploited not only as smart biomaterial coatings to induce desired (stem) cell fate but also as platforms for cellular diagnostics.^[78]

Cell-instructive supported lipid monolayers

Supported lipid monolayers (SLMs) form directly when the alkyl tail of lipids come into contact with an alkylated surface, such as on octadecyltrichlorosilane (OTS) coated substrates (**Figure 1.13A**).^[79] SLMs can have different physical characteristics than SLBs as they are structurally distinct.^[80] For example, SLMs can have a fluid phase well below the melting temperature (T_m) of the lipid in contrast to SLBs where the onset of lateral mobility corresponds well to their bulk T_m .^[80b] Therefore, in general, SLBs have more predictable physical properties and are also structurally closer to the cell membrane. While SLBs enable incorporation of membrane proteins by maintaining their function, SLMs seem easier to prepare on various surfaces.^[81]

In one study, Miller and co-workers used RGD-modified SLMs to probe the effect of the fluidity of the SLM on hematopoietic cell adhesion (**Figure 1.13A**).^[79] Cyclic RGD, $\alpha_4\beta_1$ integrin binding cyclic Leu-Asp-Val (LDV) ligands,^[7d] and fibronectin C/H II heparin-binding domain (HBD) ligands were also attached to DOPC and DPPC SLMs as these sequences are known to induce hematopoietic stem and progenitor cell adhesion.^[82] On immobile DPPC SLMs, more hematopoietic cells adhered at higher peptide densities until a plateau was reached where RGD and LDV ligands induced the highest extent of cell adhesion. Less cell adhesion occurred on DOPC-based SLMs, where RGD induced the highest extent of cell adhesion. On DPPC SLMs, $\alpha_5\beta_1$ integrin acquired a more punctuated distribution in the cell body while on DOPC SLMs it mainly localized in the periphery of the cells (**Figure 1.13B-C**). The results show the effects of SLM fluidity and ligand type on hematopoietic cell adhesion.

In another study, Sackmann and co-workers, investigated endothelial cell adhesion on SLMs made of non-fouling tetraethylene glycol terminated lipids into which cyclic RGD-functionalized lipids were uniformly mixed in (1 to 10 mol%).^[81b] On control SLMs cells remained round while on RGD-SLM, cells adhered and spread. In summary, although much less employed as cell-instructive layers compared to their counterpart SLBs, SLMs can also act as a suitable model biointerface where chemical and physical properties can be tuned and ligands can be inserted to direct cell responses.

Figure 1.13. (A) SLMs with cyclic RGD ligands conjugated to 1 mol% succinic acid-dipalmitoyl glycerol (su-DPG) lipids. Integrin $\alpha_5\beta_1$ distribution in THP-1 cells. (B) DPPC and (C) DOPC SLMs with one cell on each surface (scale bar: 2 μm , $t=3\text{h}$). Reproduced with permission.^[79] Copyright 2009, American Chemical Society.

1.3.2.2. Cell-instructive, stimuli-responsive biointerfaces

Dynamic biointerfaces can also be formed using for example host-guest interactions where affinities between the host and ligand conjugated guest molecules can be effectively controlled by molecular structure.^[12a, 58c, 83] In one study, Stupp and co-workers engrafted β -cyclodextrin (β -CD) host molecules on a non-fouling alginate background.^[83a] A linear RGD peptide was modified with naphthyl and adamantane groups as guest moieties for β -CD (**Figure 1.14A**). 3T3 fibroblast cells adhered and spread effectively with well-defined stress fibers (3h), which is indicative of strong integrin-mediated cell adhesion. On only β -CD-alginate or when using negative control peptides, cells adhered but remained round and small (**Figure 1.14B**, control), showing the specificity of RGD-integrin interactions on bioactive non-covalent surfaces. When comparing the effect of guest affinity on cell spreading, it was seen that adamantane-RGDs induced a significantly higher cell spreading and higher degree of actin stress fiber formation than naphthyl-RGDs peptides on the surface (**Figure 1.14B**). This observation

was attributed to two possible reasons: i) a higher number of adamantane-RGDS ligands was present than that of naphthyl-RGDS ligands on the surface, due to the higher affinity of adamantane-RGDS for β -CD and ii) the host-guest complex for adamantane-RGDS had a higher average life time both of which can influence ligand display to cells. Furthermore, in an attempt to use the dynamics of the system, cells were first allowed to spread for 3h on surfaces in the presence of low affinity naphthyl-RGDS. Subsequent addition of 10 μ M of higher affinity adamantane-RGES (a negative control, non-integrin binding, peptide) to solution resulted in a decreased cell area and a lower percentage of cells that had well-defined stress fibers (after 3h). These results indicate that the bioactivity in β -CD-alginate was dynamically switched from cell adherent to non-adherent by replacing RGDS with RGES ligands. Altogether, these results showed that host-guest chemistry is a powerful tool to design dynamic ligand presentation to cells.^[83a]

A)

B)

Figure 1.14. (A) β -CD-alginate interacts with naphthyl and adamantane (ada)-RGD. (B) Fluorescent images of 3T3 fibroblast cells after 3h on control (only β -CD-alginate surface), β -CD surfaces incubated with 10 μ M naphthyl-RGDS and ada-RGDS. Magnified images show actin stress fibers in detail (green: actin, blue: nucleus, scale bar: 10 μ m). Reproduced with permission.^[83a] Copyright 2013, John Wiley and Sons.

Jonkheijm and co-workers have explored supramolecular cucurbit[8]uril (CB[8])-based host-guest systems to display RGD ligands to cells. This supramolecular system was used to probe cell generated adhesive forces and compared these forces to the forces generated on more traditional, static surfaces where RGD ligands were covalently immobilized.^[58c] A cysteine bearing RGD peptide was directly conjugated to the maleimide groups on a SAM to prepare static surfaces. A thiolated methyl viologen (MV²⁺), which can bind to CB[8] as the first guest, was conjugated to the maleimide groups on the surface to prepare dynamic surfaces. Then, a naphthyl-modified RGD (Np-RGD) was introduced as the second guest to bind to the binary complex of MV²⁺/CB[8] on the surface, resulting in a ternary complex (**Figure 1.15A**). C2C12 myoblast cells adhered to and spread on both surfaces to a similar extent with well-defined focal adhesions and associated actin stress fibers (**Figure 1.15B**). Single cell-adhesion force measurements were performed (**Figure 1.15A**)^[84] and showed that in terms of maximum adhesion force, detachment distance and total work required for cell detachment, there was no significant difference between both types of surfaces. These results pointed out that even though dynamic non-covalent interactions at the molecular level involve relatively weaker forces (pN level) compared to covalent interactions (nN level),^[85] cell adhesion strengths were at similar levels on these surfaces. The observation on similar force requirements for cell detachment for covalent and non-covalent surfaces indicated that the weakest interaction on both surfaces is between RGD and integrins, which was also reported to result in pN level forces.^[86] Furthermore, on both surfaces, the number of RGD-integrin interactions per cell area were at similar levels, which remained the same over a longer period (up to 5h). This clearly showed that non-covalent surfaces remained relatively stable even though a certain extent of non-covalently bound RGD ligands could dissociate from the surface given the nature of interactions. This stability of cell adhesion on non-covalent surfaces was explained by a relatively high Np-RGD density calculated on these surfaces (ca. 61 pmol/cm²) compared to sufficient densities to induce integrin saturation^[87] as well as possible increased effective Np-RGD concentration underneath adherent cells, which would promote binding of this molecule to CB[8] on the surface. Collectively, these results showed that non-covalent surfaces could perform as well as traditional covalent systems in promoting and sustaining cell adhesion and spreading, enabling a highly versatile and chemically controlled way to generate new dynamic cell-instructive biointerfaces.^[58c]

Figure 1.15. (A) Schematic presentation of the FluidFM system to study single cell adhesion forces on supramolecular SAMs (see text). (B) Fluorescent images of C2C12 myoblasts on covalent and non-covalent surfaces after 1h (red: actin, blue: nucleus, and green: vinculin, scale bar: 50 μm). Reproduced with permission.^[58c] Copyright 2017, American Chemical Society.

This type of CB[8] based host-guest systems also hold great potential to be employed as stimuli-responsive biointerfaces for spatiotemporal control of cell behavior, by carefully designing respective guest molecules which can respond to different stimulus such as light or electrochemistry (Figure 1.16A).^[12a] For example, when applying a suitable electrical potential, RGD ligand releases from the SAM signified by the observation that more than 90% of the cells became rounded and detached (Figure 1.16B-C).^[83c] The strategy was applied to electrode arrays where cell release was seen to specifically occur on the stimulated electrode and where actin staining also confirmed the specificity of cell adhesion on these gold lines. The ability to modulate ligand presentation both spatially and temporally, using a redox stimulus on these supramolecular surfaces holds great importance for generation of new biomaterial interfaces by allowing control over biological events at (sub)cellular level.^[83c]

Figure 1.16. (A) CB[8] based electro-responsive host-guest system for release of cells. (B and C) Bright field images of C2C12 cells before and after electrochemical activation at -0.5 V (vs. Ag/AgCl) for 200 s; white dots: to show the same observation area before and after stimulation, scale bar: 100 μm). Reproduced with permission.^[83c] Copyright 2012, John Wiley and Sons. (E) Dual responsive β -CD/azo-REDV system. (F) Confocal fluorescent images of cells on β -CD/azo-REDV surfaces, before and after treatment with 10 μM amantadine and UV light for 5 min (red: actin, green: vinculin and blue: actin, scale bar: 25 μm). Reproduced with permission.^[88] Copyright 2016, John Wiley and Sons.

Recently, Gao and co-workers presented a dual responsive system using the competition between a higher and lower affinity guest molecule for complexation as well as light as

an external stimuli to change the binding affinity of light-responsive azobenzene as guest molecule of β -CD (Figure 1.16D).^[88] In order to probe dynamic control of cell adhesion and migration, cells were first allowed to spread on azobenzene-modified Arg-Glu-Asp-Val (REDV) ligands on surfaces for 4h where they showed robust actin stress fiber formation with well-defined focal adhesions (Figure 1.16E). Addition of competing amantadine to the cell culture medium resulted, as expected, in a decrease in cell area and focal adhesion formation as well as increased cell migration speed. Next, when UV light (~ 365 nm, 5 min) was used to switch azobenzene from the *trans*-to-*cis* isomer, which leads to a significant decrease in affinity and release of azo-REDV from the β -CD surface, cell migration speed increased, cell area decreased and cells lost their well-defined actin stress fibers while having reduced vinculin localization at focal contacts. These results reveal a switch in cell behavior and further shows the versatility of the approach, which enables the use of multiple triggers simultaneously (Figure 1.16E).^[88]

Dynamic systems have also been realized for static ligands where chemical modification of the specific ligand or modification of the groups surrounding the ligands can introduce stimuli-responsiveness, and hence dynamics to the system.^[2b] For example, light-responsive azobenzene-modified RGD ligands were covalently immobilized to SAMs; and *trans*-to-*cis* conformational changes of azobenzene molecules in the molecular backbone of RGD ligands reduced the availability of RGD ligands to integrin binding and thereby prevented cell adhesion in a potentially reversible manner.^[89] Furthermore, switchable surfaces were also achieved using electro-responsive SAMs that either conceal or expose RGD ligands upon electrochemical stimulus.^[90] These chemical strategies to form cell-instructive biointerfaces demonstrated dynamic control over cell adhesion.

Next to these strategies, Del Campo and co-workers have extensively explored photoactivatable cell-instructive surfaces for controlled and selective cell adhesion and migration.^[58a, 91] In their system, a caged, non-integrin binding, cyclic RGD ligand is immobilized. Upon light exposure, the caging molecule is cleaved from RGD and makes the uncaged RGD ligand available for integrin binding (**Figure 1.17A**).^[58a, 91a] In one study, this system was used with a photopatterning approach to spatiotemporally control migration of human umbilical vein endothelial cells (HUVECs) towards defined areas (see Figure 1.17B for experimental steps).^[58a] Here, the patterns of inert peptide (as achieved in the first exposure step) served to visualize in the free areas for cell migration, which became available upon local activation of the peptide (Figure 1.17B-C). Photoactivated cell migration on these surfaces was compared with a traditional wound healing assay

where a scratch generates a gap for migration. Photoactivated migration resulted in a more uniform cell front with higher cell viability and a faster gap-closure, which exhibited less variability (Figure 1.17C). Migration experiments over constrained geometries, subcellular photoactivation as well as migration over increasing non-adhesive gap sizes could be addressed in a flexible way using this technique. It was shown that both single and collective migration of cells was strongly dependent on the size of the space available for migration. Overall, the spatiotemporal photocontrol over ligand availability offers a very flexible way to trigger dynamic cell adhesion and to investigate cell migration in well-defined assays.^[58a]

This system was further explored for non-invasive in vivo activation of cell adhesion on implanted materials.^[91b] Inert, caged RGD^[58a, 91a] conjugated to non-fouling poly(ethylene glycol) di-acrylate (PEGDA) hydrogels, which were subcutaneously implanted, became cell-adhesive upon transdermal UV-light exposure immediately after wound closure where (after 1 day) a high extent of cell adhesion with a uniform distribution of cells on the surfaces could be seen.^[92] In contrast, on non-UV-activated, caged RGD-hydrogels very few cells adhered after implantation. Most of the adherent cells on the uncaged RGD-hydrogels were neutrophils while the remainder of them were macrophages, indicating the activation of an acute immune response to these hydrogels (uncaged RGD-hydrogels). Interestingly, when uncaging of RGD was delayed till 7 days post-implantation, the thickness of fibrous capsules around the respective hydrogel decreased, showing that chronic immune response could be suppressed by controlling the presentation of ligands in time.^[93] Finally, the capacity to induce in vivo vascularization was studied by using protease-degradable hydrogels, which incorporated vascular endothelial growth factor (VEGF) and caged RGD ligands. Only UV-induced uncaged RGD-hydrogels resulted in the formation of tubular structures, which were positive for endothelial and smooth muscle cells and resembled functional blood vessels. Collectively, this study showed that light-controlled presentation of adhesive ligands could be effectively used for temporal control of biomaterial-cell interactions as well as biomaterial fate in vivo.^[91b]

Figure 1.17. (A) Caged RGD ligand and release of the caging group. (B) Photo-activation of cell migration: 1) light exposure to give adhesive RGD, 2) cell seeding, 3) cell adhesion and monolayer formation in light-exposed areas, 4) light exposure to activate cell migration and 5) cell migration towards available bioactive areas. (C) Photo-activated cell migration at 0, 10 and 16 h after light exposure. Reproduced with permission.^[58a] Copyright 2013, John Wiley and Sons.

Yousaf and co-workers have developed a reaction scheme to change the conformation of RGD ligands including two redox steps and two chemoselective reactions (**Figure 1.18A**).^[94] Alkyne-modified RGD was immobilized via the Huisgen azide-alkyne cycloaddition reaction yielding linear RGD on the SAM. After redox conversion of hydroquinone to benzoquinone, the N-terminus oxamine group on the RGD ligand reacted with benzoquinone yielding cyclic RGD on the SAM. A subsequent reduction step opens up the cycle yielding linear RGD again. Fibroblast cells were observed with time lapse microscopy where smaller cells that migrate faster were seen upon switching from cyclic to linear RGD. Furthermore, a higher degree of actin fiber organization and

stronger vinculin localization was observed on cyclic RGD, in line with previous results on these two different RGD configurations.^[47]

Figure 1.18. (A) Chemical strategy to induce conformational changes in RGD ligands. Reproduced with permission.^[94] Copyright 2011, American Chemical Society. (B) Induction of cell migration towards a gradient of RGD ligands (exposed to cells as a result of surface activation, see text) and in between to hydrophobic patterns (scale bar: 50 μm). Reproduced with permission.^[95] Copyright 2011, American Chemical Society.

Luo and Yousaf and co-workers further developed a combined photo/redox strategy to selectively immobilize ligands in defined areas to control cell adhesion, tissue morphing and cell migration.^[95] In this strategy, photo-protected hydroquinones on a SAM were uncaged by UV illumination and then electrochemically converted to benzoquinones,

which in turn were reactive towards oxyamine-modified RGD ligands. Adhered fibroblasts were first confined to hydrophobic patterns, and did not adhere to areas of hydroquinones. After installing RGD ligands, cells migrated from the hydrophobic patterns towards these new RGD presenting patterns (Figure 1.18B). Furthermore, when a gradient of RGD ligands was formed, cells were seen to move along the gradient (Figure 1.18B). Cells migrated faster towards lower instead of higher ligand densities. In addition, cell migration was more rapid on steep gradients, when compared to shallow gradients, irrespective of when going from high to low or low to high ligand density. Altogether, control over ligand presentation and patterning with a dual stimulus-responsive nature provides a versatile way to generate dynamic cell-instructive biointerfaces for guided cell adhesion and migration.^[95] An additional feature of using redox chemistry for coupling ligands to the surface is that real-time monitoring using cyclic voltammetry provides precise control over ligand density.^[96]

1.3.2.3. Cell-instructive, stimuli-responsive topographical biointerfaces

Size (from micron to nanoscale), arrangement and geometry of surface topography of biomaterials critically influence cell behavior as revealed by many studies exploiting pre-defined, static topographies.^[27, 97] More recently, changeable surface topographies have been fabricated and explored for controlling cell adhesion, polarization and migration.^[12b, 12c, 98] In one study, Kirschner, Anseth and co-workers used PEG-based hydrogels conjugated with photo-labile groups for making various topographies using masks and through photodegradation of the hydrogel in light-exposed areas (Figure 1.19A, i).^[98] The depth of topographical features were kept at ca. 1 μm in order to allow cell spreading along the features without a physical constraint. Cells were first seeded on top of a smooth hydrogel (2D cell culture, functionalized with fibronectin) and then exposed to a cytocompatible illumination dose of 250s. Anisotropic ($\infty:1$) channels were created first and cells responded to this change by significantly increasing their aspect ratio. Subsequently, squares were created by a second light exposure and changes in cells' aspect ratio and morphology were observed (Figure 1.19A, ii). Netti and co-workers have used light-responsive azobenzene conjugated polymers (azopolymers) for spatiotemporal control over topographical changes to direct cell spreading and alignment.^[12c] A confocal laser beam (linear polarized light of 514 nm, with exposure times of 30 or 45s, at 400 Hz scan speed) was used for alternating *trans-cis-trans* isomerization of azobenzene molecules to induce local changes in the mobility of polymer molecules and subsequent mass transport, where the *trans*-isomer yielded a more dense polymer.^[99] In this way, lines (with 3 μm periodicity and 400 nm height) and

grids ($3\ \mu\text{m} \times 3\ \mu\text{m}$) were generated in the presence of cells, which were seeded and allowed to spread on an initially flat (i.e., non-patterned) surface. Initially, NIH3T3 fibroblasts were randomly oriented (i.e., no preference for alignment direction) on the flat surface, but migrated over the patterns and co-aligned to the in-situ fabricated lines 5h after dynamic pattern inscription. Quantification of mean cell elongation on line patterns (as measured from 0h to 6h after dynamic inscription) revealed that, on average, cells maintained a similar extent of elongation over time. On the other hand, when the grid pattern was dynamically inscribed, cells had a significantly lower level of elongation compared to when they were on the flat surface, already 1h after pattern inscription. However, over time, they remained randomly oriented on these patterns (average orientation of $\sim 45^\circ$). Changes in orientation and elongation were attributed to redistribution of cell area as it did not significantly change for linear and grid patterns compared to the flat surfaces. Next to real-time changes in cell behavior, multiple patterns can be fabricated simultaneously underneath cells as well as to generate reversible, on-off, patterns while maintaining cell viability. Taken together, the ability to change topography introduces new opportunities to study in situ morphological changes in cells and stem cell lineage commitment.

Figure 1.19. (A) (i) Photolabile hydrogels containing PEGdiPDA (PEG-bis-amine conjugated with a nitro-benzyl based photodegradable acrylate (PDA)). PEGdiPDA was co-polymerized with PEG monoacrylate (PEGA) to form the hydrogels. Topographies were made by mask exposure ($\lambda=365\ \text{nm}$, $10\ \text{mW}/\text{cm}^2$). (ii) hMSC morphology and spreading on topographies. Reproduced with permission.^[98] Copyright 2012, John Wiley and Sons.

1.3.2.4. 3D cell-instructive, stimuli-responsive biointerfaces

So far, the review discussed planar biointerfaces for 2D cell culture. However, to more closely mimic native 3D matrices, currently attention is given to control cell and tissue response in 3D using the knowledge gained from studies on static and dynamic cell-instructive biointerfaces. Multiple 3D biomaterial strategies have been developed based on synthetic polymers and natural ECM (e.g., proteins and polysaccharides) to tailor physical, chemical and mechanical properties of the microenvironment that surround encapsulated cells.^[102] For a general view on the development of hydrogel based systems, going from static to dynamic complexity, please refer to other extensive reviews.^[1, 102] Yet, in this section, selected examples are discussed to highlight hydrogels, which are being developed as highly biomimetic biomaterials in the context of cell-material interactions, consisting of changeable cross-links, e.g., by enzymes or light, hydrogels that consist of non-covalent or dynamic covalent bonds and hydrogels with time regulated viscoelastic properties.^[103]

In one study, Anseth and co-workers used photodegradable PEG-based hydrogels (as given in Figure 1.19A) to change by light exposure the local network density, which regulates properties such as stiffness, water content and diffusivity.^[103a] When hMSCs were encapsulated in these photodegradable PEG hydrogels, the light-induced decrease in cross-linking density resulted, locally, in spreading of previously round cells. Within these hydrogels 3D channels were generated by photodegradation, where encapsulated cells could migrate through the channels. In another system, non-degradable PEG-based hydrogels were synthesized with pendant RGD ligands tethered via a photolabile group to enable time controlled release of the ligands (**Figure 1.20A**).^[103a] Ten days after cell encapsulation, ligands were photo-released (Figure 1.20B) and cells were further cultured in these, RGD-released, hydrogels (photocleaved RGD-hydrogels, Figure 1.20B) for up to 21 days (i.e., 11 days past light exposure) and compared with control gels (PEG-only and non-illuminated (persistent) RGD-hydrogels, Figure 1.20B). Cell viability in both RGD-hydrogels remained at high levels (~84-86% at day 21) where a significant decrease in viability at day 7 (51%) was observed in PEG-only hydrogels. Nevertheless, $\alpha\text{v}\beta\text{3}$ integrin expression in cells in photocleaved RGD-hydrogels dropped most at day 21 (5% vs 98% in non-illuminated RGD-hydrogels). Interestingly, by day 21, cells that were cultured in photo-cleaved RGD-hydrogels had an increased chondrogenic differentiation capacity compared to the cells in non-illuminated RGD-hydrogels, signified by a higher glycosaminoglycan (GAG) production and Collagen II expression and a decreased CD105 expression in cells (Figure 1.20C).^[103a] These results are in line with previous observations

where RGD incorporation in PEG-based hydrogels was required for high cell viability and contributed to initiation of chondrogenesis.^[104] In addition, an increased chondrogenic capacity with time controlled release of RGD correlates well with temporal regulation of ECM during chondrogenesis, where fibronectin expression is down-regulated at later stages.^[103a, 104] These results showed that a temporal, non-invasive change in chemical properties of hydrogels could trigger differential cell fate choices. These findings are of relevance to generate advanced 3D dynamic cell-culture platforms for regenerative medicine applications.^[1]

Other types of hydrogels have cross-links that can be cleaved enzymatically e.g., by cell-secreted matrix metalloproteinases (MMPs) as extensively studied by Hubbell and co-workers.^[105] High degradation kinetics of the cross-links led to increased cellular infiltration and increased cell spreading that could provide a more easily remodeled matrix by cells which, in turn, could potentially result in better healing in vivo.^[103e] In a recent study by Chen, Burdick and co-workers, such MMP-responsive matrix degradability in 3D hydrogels was explored in the context of angiogenic sprouting.^[103f] An MMP-labile peptide (derived from the cleavage site of native collagen and referred as native collagen degradability (NCD) sequence), as the cross-linker, was introduced to the backbone of methacrylated dextran (DexMA) hydrogels, at different concentrations during polymerization (Figure 1.20D). Therefore, hydrogels of different cross-linking densities could be achieved, which also exhibited increasing stiffness with increasing cross-linker density. In order to induce cell adhesion and infiltration, they attached RGD ligands to these otherwise non-fouling DexMA hydrogels.^[103f] Cells in hydrogels with a low cross-linker density showed a higher sprout length with leading cells being open and branched with long filopodia. In contrast, in high cross-linking density hydrogels, leading cells had narrow branches and short, spiky filopodial extensions (Figure 1.20D-E). Interestingly, in hydrogels with intermediate cross-linker density, a collective multicellular sprouting was seen as a characteristic of angiogenesis. In contrast, in case of either low or high cross-linker densities, cells mainly migrated as single cells or in small groups. The observed differences between migration depths and multicellularity of invasion was shown to be mainly due to differences in hydrogel degradability, which provides different spaces for cell spreading, migration and angiogenic sprouting, rather than differences in hydrogel stiffness, which is also dependent on the cross-linker densities.

Figure 1.20. (A) RGD ligand (black) and acrylate moiety (red) linked by a photocleavable group (blue). (B) Hydrogel systems studied, at day 10 of cell culture with given conditions. (C) CD105 (green) and COLII (red) expression in hMSCs (blue: nucleus) cultured in hydrogels at day 21 (scale bars: 100 μm). Reproduced with permission.^[103a] Copyright 2009, The American Association for the Advancement of Science. (D) Two channels embedded in RGD-(MMP cleavable)-DexMA hydrogel. Upper channel has an endothelial monolayer. Lower channel (green) contains growth factor cocktail. (E) Endothelial cells infiltrated into DexMA hydrogels with varying cross-linker concentrations used during polymerization (NCD: Native collagen degradability cross-linker as the target of MMP enzyme, numbers indicate the concentration of the cross-linker peptide used during polymerization of the hydrogel, cyan: F-actin, magenta: nuclei, scale bar: 50 μm). Reproduced under the terms of Creative Commons Attribution 4.0 International License.^[103f]

In another study, Lutolf, Hubbell and co-workers used photo-caged enzyme-reactive PEG-based hydrogels.^[103d] Upon photo-uncaging, RGD-based ligands and platelet-derived growth factor-B (PDGF-BB) were enzymatically immobilized in the hydrogel. Prior to photo-uncaging, cells were encapsulated into these hydrogels that showed effective hMSC infiltration in illuminated areas.^[103d, 106]

Another strategy applies host-guest interactions to form dynamic, bioactive hydrogels.^[14a, 103b, 107] In one study, Burdick and co-workers functionalized hyaluronic acid (HA) macromers with adamantane (guest, Ad-HA) and β -CD (host, β -CD-HA) moieties that form an HA based hydrogel by host-guest complexation (Figure 1.21A). When injected into an ex vivo target site, the supramolecular host-guest hydrogels exhibited a shear-induced flow followed by rapid recovery of the hydrogel (i.e., near-instantaneous), which retained at the target site. The host-guest hydrogels remained stable for over one week with minimal morphological changes over time and a low extent of swelling. Next, in an attempt to improve the mechanical properties, retention and stability of injected hydrogels at the implant site, an additional covalent cross-linking via a Michael addition (MA) reaction (Figure 1.21B) was introduced to the hydrogel (resulting in dual cross-linked (DC) hydrogels). In order to form DC hydrogels with desired gel times for in vivo injectability (e.g., up to an hour), which can be hampered by rapid cross-linking, MA reaction kinetics was optimized to be slow enough by choosing a proper donor-acceptor pair for reaction (i.e., thiols and methacrylate groups conjugated to Ad-HA and β -CD-HA, respectively), at an optimum polymer concentration. These dual cross-linked hydrogels combine extended gel times and injectable properties, result in desired initial retention as well as increased moduli after in situ delivery.^[103b] In vivo experiments, performed with a myocardial infarct model in rodents, further showed that within 4 weeks, host-guest hydrogels almost completely degraded. In contrast, solid fragments from the dual cross-linked hydrogels were still visible, possibly due to slow hydrolysis of thioether-ester bonds formed during Michael addition reaction. The dual cross-linked hydrogels were also evaluated in a clinical relevant case of myocardial infarction (MI). After injecting both hydrogels systems into the myocardium of rats in the infarct border zone region, a desirable regulation of tissue response occurred, but the dual cross-linked hydrogels exhibited a more improved reduction in the size of the infarct. Importantly, a significantly higher level of vascular network formation was detected for the dual cross-linked hydrogels.^[103b]

Mooney and co-workers have made viscoelastic RGD-modified alginate based hydrogels with stress-relaxation properties, as a closer mimic of native tissues.^[103c, 108] Stress-

relaxation properties and viscous damping effects arose in the hydrogels by forming dynamic, ionic cross-links between anionic guluronic acid residues on alginate chains and divalent cations. Decreasing the molecular weight of alginate, increases the polymer chain mobility resulting in a faster stress-relaxation.^[103c] hMSCs were encapsulated into fast and slow-relaxing RGD-hydrogels having a similar initial elastic moduli, yet slightly lower than the optimum values for osteogenic differentiation.^[109] After two weeks in osteogenic medium, cells in fast-relaxing hydrogels had a higher osteogenic capacity concluded from an increased matrix deposition and mineralization. In vivo experiments, in a rat calvarial defect, showed that fast-relaxing (with encapsulated hMSCs) hydrogels induced a significantly higher bone formation than slow-relaxing (with encapsulated hMSCs) hydrogels (Figure 1.21C), three months after implantation. Interestingly, fast-relaxing hydrogels with and without encapsulated hMSCs induced a similar level of bone formation. Moreover, in the case of fast-relaxing hydrogels, active bone growth was observed with elongated osteoblasts in the periphery of new bone and osteoid regions rich in collagen. The ability of fast-relaxing hydrogels to induce a better bone healing was attributed to extensive remodeling of these hydrogels in the implant site where after two weeks only a fraction of these hydrogels could be detected. In contrast, slow-relaxing hydrogels remained intact in the defect area at this timescale.^[103c] Collectively, these results point out the importance of dynamically regulated physical properties of biomaterials, next to chemical and biological cues, to confer cells the ability to remodel and invade into their surrounding matrix for promoting tissue healing.^[103c]

One other strategy to introduce dynamicity to hydrogel design is to make use of dynamic covalent chemistry (DCC) where covalent bonds can be formed, broken and re-formed under equilibrium conditions.^[103g, 110] This reversible bond formation introduces shear-thinning and self-healing properties to hydrogels (similar to supramolecular hydrogels).^[103b, 103g] Besides, it enables cells to dynamically modulate their surrounding matrix for effective spreading and to remodel the surrounding microenvironment, as these bonds could be broken by cell-applied stress and re-form rapidly.^[111a, 111b] In a very recent study, Heilshorn and co-workers have combined DCC and thermo-responsive engineered proteins (i.e., elastin like protein, ELP incorporated with cell-adhesive RGD sequence) to develop a novel dual cross-linked adaptable hydrogel with improved material properties for injectable cell delivery.^[111c]

Figure 1.21. (A) Supramolecular hydrogel formed via β -cyclodextrin (CD) and adamantane (Ad) host-guest interactions. (B) Supramolecular hydrogel formation combined with chemical cross-linking between thiol and methacrylate units. Reproduced with permission.^[103b] Copyright 2014, John Wiley and Sons. (C) Bone formation capacity in fast vs. slow relaxing RGD-modified alginate hydrogels implanted in a rat calvarial defect model. Reproduced with permission.^[103c] Copyright 2016, John Wiley and Sons.

For this purpose, a mixture of hydrazine-modified ELP (ELP-HYD) and aldehyde-modified HA (HA-ALD) macromers were used (at room temperature) to induce the first gelation through the formation of dynamic covalent hydrazone bonds, resulting in ELP-HA hydrogels. Upon heating to physiological temperatures (e.g., for in-situ experiments), mechanical properties of the hydrogel improved with a higher storage modulus, due to thermal phase segregation of ELP in the hydrogel. This thermo-responsive stiffening effect was due to the formation of a secondary physical network and was enhanced with

increasing ELP-HYD concentration in the hydrogel. Importantly, double network ELP-HA hydrogels also exhibited a long term integrity compared to a control HA hydrogel with only hydrazone cross-linking (i.e., 80% ELP-HA hydrogel retention at day 9 vs. no retention).^[111c] Finally, these ELP-HA hydrogels showed rapid shear thinning and self-healing kinetics, protected encapsulated hMSCs from the potential damage during injection where multilineage differentiation capacity of these cells were maintained, which are all critical for this type of applications. These results introduce another level for incorporation of dynamicity and injectability as well as physical and mechanical stability to hydrogel formulations, next to dual cross-linked supramolecular hydrogels.^[103b, 103g, 111c] In another study, these adaptable ELP-HA hydrogels were used to overcome the limitations of conventional hydrogels where varying HA concentration, as a critical biochemical chondrogenic signal, generally results in changes in hydrogel stiffness, hence prevents decoupling and studying individual mechanical and biochemical properties on cell response. To this end, hydrogels of comparable stiffnesses (~350 Pa) and with varying HA concentrations were achieved by tuning the stoichiometric ratio of aldehyde groups to hydrazine groups on the macromers while keeping the ELP-HYD concentration constant. Increasing HA concentration induced a dose-dependent increase in cartilage marker gene expression, increased sulfated-GAG (sGAG) production by encapsulated cells as well as reduced undesired fibrocartilage phenotype, which all mark proper retention and stimulation of chondrogenic differentiation.^[112a] Interestingly, the stiffness of these ELP-HA hydrogels were lower than previously reported values, which supported chondrogenic differentiation in gelatin based hydrogels.^[112b] The stimulation of chondrogenic phenotype in soft ELP-HA hydrogels were attributed to presence of HA as a native biochemical signal, further pointing out the importance of this cue, as well as to dynamic covalent cross-links in the hydrogels, which can be modulated by cells for desired matrix remodeling and ECM deposition.^[112a] Altogether, such dynamic hydrogel systems with controllable and decoupled properties have a great potential to serve for the understanding of fundamental cell-material interactions as well as to develop new biomaterials for regenerative medicine applications.

1.4. Conclusions and future perspectives

Progressive advancements in chemical approaches for materials development have introduced a vast amount of opportunities and strategies to design and achieve biologically informed cell-instructive biointerfaces. Such chemically defined biointerfaces nowadays present complex biochemical and physical cues at biologically

relevant length scales (i.e., from subcellular level to single cell and tissue level) and time scales (i.e., from minutes to hours to study cell adhesion and migration as well as days to weeks to study differentiation and tissue response). Furthermore, these biointerfaces offer the ability for spatial and temporal control of cell-surface interactions, and embrace dynamic behavior for improved cell-surface interactions. These cell-instructive biointerfaces have served up to now as excellent *in vitro* models to unravel fundamental biological processes as well as their introduction as versatile inspirational platforms to integrate into new generations of smart biomaterials to improve the guidance of desired cell and tissue response. As such, on cell-instructive biointerfaces with static biochemical properties, from the explorative studies to map the effects of ligand microenvironment, ligand immobilization chemistry, ligand affinity, ligand density as well as the spatial organization and spacing of the ligands on surfaces, a tremendous understanding of some of the fundamental processes that occur when cells interact with interfaces have become available. Most evident are the essential parameters such as critical ligand density and spacing that govern cell adhesion, migration and differentiation. Next step is to evaluate these systems when integrated into newly designed biomaterial interfaces (both 2D and 3D systems). To this end, more complex *in vitro* experiments that target tissue specific cells to investigate individual and combinatorial presentation of defined cues as better predictors of their *in vivo* performance are needed. Subsequently, it will be important to validate their ability to induce guided tissue response through *in vivo* experiments.

Sharing some of the fundamental design principles with static biointerfaces, dynamic biointerfaces (both at 2D and 3D settings) have introduced several advantages such as: temporal control over the presentation of cell-instructive cues, non-invasive external control over these cues using stimuli-responsiveness, presenting interfaces with lateral dynamicity and bond-restructuring properties (e.g., lipid bilayers and viscoelastic physically cross-linked hydrogels) allowing cells to reorganize their microenvironment, which could be hard to achieve in static systems. These dynamic surfaces and materials that have explored both spatial and temporal regulation of cell-instructive cues, have a very high potential to create better biomimetic biointerfaces. For example, studies on laterally mobile ligands revealed previously unreported mechanisms in ligand-receptor interactions, clustering and associated signaling events. Furthermore, dynamic systems enabling extensive remodeling by cells induced better native-tissue like responses as explored in a 3D context. However, this type of biointerfaces are still at the early stages of development for the targeted (*in vivo*) applications. Despite studies concerning several aspects of cell responses (e.g., cell adhesion and spreading on non-covalent

surfaces and photo-responsive hydrogels to guide stem cell differentiation), more in depth in vitro cellular and molecular biological studies are needed to investigate the fundamental aspects of cell behavior (e.g., cellular signaling events) in response to dynamic bioinspired cues presented within these biointerfaces, both 2D and 3D. The knowledge gained through these in depth studies will reveal critical parameters governing cell response and will feed information to tailor the design rules in generating new dynamic biomaterials for in vivo applications. For this purpose, next to being able to synthesize chemically well-defined materials, experimental methods to reveal cell-material interactions in real-time as well as tools to reveal changes in material properties in the presence of cells will help to go forward. In this line, revealing time regulated changes in material properties, particularly in 3D systems, will require in situ 3D high resolution imaging techniques to characterize local effects posed by external stimuli, cells (i.e., matrix deposition and degradation) and culture environment in relevant time scales.^[113] Reporter surfaces could also give insights on cell-material interactions in a real-time and quantitative manner.^[114] Next to that, imaging of cell phenotype in 3D with high resolution will also enable to better resolve the consequences of cell-material interactions.^[115] This will in turn enable better control on the presentation of cell-instructive cues in a spatially and temporally regulated way.^[1, 5] Furthermore, high throughput as well as combinatorial approaches to investigate cell-material interactions will introduce opportunities to evaluate multiple effectors at the same time and are powered with the ability to analyze big datasets. Clearly, realizing these goals would require multidisciplinary efforts from chemists, biologists and biomedical engineers in an integrated way.^[1]

1.4.1. Thesis outline

In this thesis, chemical approaches to fabricate dynamic cell-instructive biointerfaces that are based on light-responsive liquid crystal polymer networks and supported lipid bilayers are described. In all Chapters the emphasis is put on how to exploit these biointerfaces to guide and investigate (stem) cell behavior.

In Chapter 2, light-responsive liquid crystal polymer networks are presented as new hybrid biointerfaces with micrometer scale topographical cues and changes in nanoscale roughness at the same time to direct cell migration. Next to exploring the dependence of cell migration patterns on micrometer scale topographies, dynamic regulation of cell migration is studied upon in situ temporal changes in surface nanoroughness.

In Chapter 3, supported lipid bilayers (SLBs, formed on glass supports) are functionalized with integrin targeting RGD ligand via biotin-neutravidin interactions. The dependence of hMSC adhesion, spreading and osteogenic differentiation capacity on ligand density and mobility is investigated.

In Chapters 4 and 5, lipid insertion is used as the functionalization route for SLBs on glass supports. In Chapter 4, mobile SLBs are functionalized with an RGD peptide conjugated with saturated alkyl tails of various lengths (C12:0, C14:0 and C16:0), at given surface coverages. Dependence of hMSC adhesion, spreading and focal adhesion formation in cells on the length of the alkyl tail of the lipidated RGD peptides is studied, in relation to the interaction strength of these lipidated peptides with SLBs. In Chapter 5, the emphasis is put on studying the dynamics of cell behavior (e.g., migration) on SLBs, at high ligand densities, where a dye conjugated lipidated peptide (with C16:0 tail) allows for real-time visualization of cell-peptide interactions on the surface.

In Chapter 6, a dual peptide presentation (via lipid insertion) on mobile SLBs (again on glass supports) is explored to target both integrins and cadherins on endothelial cell surface, allowing to control the complexity of cell-surface interactions while investigating a tissue type specific cell response. Next to cholesterol conjugated RGD ligands (Chol-RGD), a cholesterol conjugated (Chol) HAVDI peptide, derived from N-Cadherin, is introduced to the cells at different molar ratios of these two peptides on SLBs. Dependence of endothelial cell adhesion, spreading and N-Cadherin localization in these cells at given peptide ratios as well as when presenting the peptides individually is shown.

In the Epilogue, results on the translation of SLB coatings from glass supports to a widely accepted biomaterial (polycaprolactone, PCL) surface are described. PCL surfaces are conjugated with a cholesteryl linker to form a biomaterial supported lipid bilayer (BSLBs) that is functionalized with a bipalmitic acid-RGD peptide. hMSC adhesion and spreading as well as differentiation capacity are studied.

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1.6. References

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Chapter 2

Light-responsive hierarchically structured liquid crystal polymer networks for harnessing cell adhesion and migration*

Extracellular microenvironment is highly dynamic where spatiotemporal regulation of cell-instructive cues such as matrix topography tightly regulates cellular behavior. Recapitulating dynamic changes in stimuli-responsive materials has become an important strategy in regenerative medicine to generate biomaterials that closely mimic the natural microenvironment. Here, light-responsive liquid crystal polymer networks are used for their adaptive and programmable nature to form hybrid surfaces presenting micrometer scale topographical cues and changes in nanoscale roughness at the same time to direct cell migration. This study shows that the cell speed and migration patterns are strongly dependent on the height of the (light-responsive) micrometer scale topographies and differences in surface nanoroughness. Furthermore, switching cell migration patterns upon in situ temporal changes in surface nanoroughness, points out the ability to dynamically control cell behavior on these surfaces. Finally, the possibility is shown to form photo-switchable topographies, which is appealing for future studies where topographies can be rendered reversible on demand.

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2.1. Introduction

The extracellular matrix (ECM) continuously submits to cells tightly regulated spatio-temporal changes in chemical, physical and mechanical cues. These dynamic changes in signals to cells eventually prompt changes in cell and tissue behavior.^[1] In regenerative medicine, various strategies have been used to engineer materials to present ECM inspired cues to direct (stem) cell fate and tissue function.^[1b, 2] Notwithstanding these achievements, the majority of biomaterials that present these cues are static, with pre-defined properties that cannot submit a changing environment to cells at any specific point in time. Therefore, designing new biomaterials with the ability to change properties in time is important as such dynamic materials would mimic more closely the natural microenvironment of cells.^[1] This can be achieved by incorporating stimuli-responsive elements into materials using for example thermo-sensitivity.^[1a, 3] Control over size (from the nano to micron scale) and arrangement of topographical features as one of the matrix cues is known to have great influence on cell adhesion, spreading, migration and differentiation and tissue organization.^[2a, 4] Therefore, recapitulating topographical changes of a natural matrix in situ (i.e., in presence of cells) is highly interesting for the generation of new biomaterials. Several studies on dynamic (i.e., in situ at a specific point in time changed, therefore temporal changes, either irreversibly or reversibly) topographies have used strain-responsive buckling of plasma oxidized polymethylsiloxane (PDMS) thin films^[5] or thermally activated shape-memory polymer (i.e., ϵ -polycaprolactone (PCL))^[6] and strain-responsive liquid crystal elastomers^[7] in dynamic control of (stem) cell alignment, spreading and differentiation.

Nevertheless, in these systems, careful selection of the stimulus considering in situ experiments as well as the versatility of surface modifications in terms of feature generation are essential. Generally, light offers flexibility and remote control, non-invasiveness and physiological compatibility, i.e., when tuned in correct doses and wavelength, to generate changes in surface or bulk properties of biomaterials.^[1a, 8] In fact, light has been extensively used to pattern and control the presentation of cell adhesive ligands in vitro^[8-9] and in vivo^[10] to tune chemical and physical properties of cell encapsulated hydrogels,^[1a, 3a] to control tissue assembly and to release patterned cells from surfaces.^[11] The conceivable benefits of employing light-responsive systems are recognized and encourage utilization to advance dynamic surface topographies across larger areas.^[12] Yet, there are only a few examples where light has been used to form (re)configurable topographies in polymer systems consisting of photo-switchable molecules in order to control cell spreading and alignment while challenges regarding in

situ studies still remain.^[12-13] In a very recent example, Netti and co-workers elegantly showed in situ spatio-temporal control of topography to direct cell alignment on azopolymers.^[14] However, so far no study has documented if in situ light-induced changes (i.e., temporal changes) in surface structure effects cell migration, which is one of the essential processes in tissue remodeling and wound healing as well as in colonization of biomaterials by desired cell types for successful implantation.^[4f, 15] In this chapter, we employ the adaptive and programmable nature of light-responsive liquid crystal polymer network (LCN) coatings^[16] to achieve new spatially arranged patterned biointerfaces for in situ temporal control of surface properties to guide cell behavior. These azobenzene based LCN systems can operate in solvent free environments and present a versatile way to change surface topography both in a reversible and irreversible fashion.^[16-17] We show that these LCNs are biocompatible and can be used as bio-instructive responsive materials to control cell adhesion and migration. Furthermore, we exploit these materials to induce in situ temporal changes (permanent in this case) in surface structural properties (i.e., roughness) to control cell migration.

2.2. Results and Discussion

2.2.1. Encoding topography using light in LCNs to harness cell behavior

Scheme 2.1. Molecular structure of the materials that were used for preparing the light-responsive liquid crystal polymer (Compounds are 1: Light-responsive molecule, 2: Chiral dopant, 3-5: Nematic hosts, 6: Photo-initiator, 7: Chain stopper/inhibitor).

The preparation of the responsive LCN coatings (with components given **Scheme 2.1** and **Table 2.1**, mesogenic monomers) is given in **Figure 2.1A**. A mixture of (meth)acrylate functionalized azobenzene and liquid crystalline monomers were used to create a so-called chiral nematic phase that is subsequently aligned in plane by shear forces and then photo-polymerized (See details in experimental section). Mask illumination of the films lead to local *trans*-to-*cis* isomerization of azobenzene molecules conjugated in the network resulting in a local formation of protrusions (volume generation) in the illuminated areas yielding (microscale) topographical cues for cells (**Figure 2.2A** and **Figure 2.1B-D**).^[17] These surface topographies can be rendered permanent by modifying the starting mixture composition.^[17a]

Table 2.1. Composition of the liquid crystal mixture.

	Compound	Function
1	A3MA	Light-responsive
2	LC756	Chiral dopant
3	C6M	Nematic host
4	RM23	Nematic host
5	RM105	Nematic host
6	Irgacure 819	Photo-initiator
7	4-Methoxyphenol	Chain stopper/inhibitor

Cell adhesion and migration were studied either on surfaces with pre-defined (fixed) microscale topographical cues or on surfaces where at a specific point in time the surface structure was changed in situ. By using a mask containing a hexagonal pattern, hexagonally arranged pillars (HP) were formed. The height of the HPs was determined for cellular studies in the micrometer scale by conveniently varying the illumination dose (intensity). A series of surfaces were prepared with pillars ranging in height from 0.2 to 1.6 μm , having a diameter of 20 μm and spaced 20 μm from each other as was verified using optical profilometry (**Figure 2.1B** and **Figure 2.1C**, HP-0.2 μm , HP-0.3 μm , HP-0.5 μm , HP-1.2 μm , and HP-1.6 μm).

Figure 2.1. Encoding topography in films using light in LCNs to harness cell behavior. (A) Schematic representation of the method to fabricate HP topographies in LCN films by using a hexagonally patterned mask and (B) 3D representation of a HP surface. (C) Height profiles of the HP surfaces. (D) 3D representation of the CP surface.

Other patterns were achieved using other masks, such as circular patterns (CP) of e.g., 15 μm in width and separation and with a height of 0.3 μm (CP-0.3 μm), demonstrating the versatility of this contactless method to fabricate topographical polymeric biointerfaces (Figure 2.1D). As control surfaces to the films with microscale topographies, ‘flat’ (with respect to microscale) LCN surfaces were used that were or were not entirely illuminated (Flat_i or Flat_ni). Upon illumination the morphology of the film alternates, which is verified by a change in the absorbance properties of the polymer films (Figure 2.2B). Atomic Force Microscopy (AFM) measurements on Flat_ni and Flat_i surfaces revealed an increase in surface roughness on the illuminated areas changing from 9.9 ± 0.9 nm to 18.2 ± 0.4 nm, showing that the nanoscale topography was changed upon illumination (Figure 2.2C-D, **Figure 2.3A-B**).

Figure 2.2. Light-induced (permanent) changes in the polymer network. (A) Mechanism of light induced topography formation in cholesteric LCNs (*trans*-to-*cis* isomerization of azobenzene resulting in loss of order). (B) UV-vis measurements showing the effect of illumination on absorbance and reflectance properties of the cholesteric polymer network. Absorbance values around 365 nm shows the absorption of azobenzene molecule in the network while the values around 700 nm shows the reflection band of the cholesteric film, which narrows as a result of a decrease in the birefringence of the material after illumination. (C and D) AFM measurements on flat not-illuminated and illuminated surfaces, respectively.

It should be noted that the formation of pillars further increases the nano roughness (*vide infra*). The surfaces were hydrophobic, irrespective of illumination, with contact angles of $88 \pm 1^\circ$ and $101 \pm 4^\circ$ for Flat_{ni} and Flat_i, respectively. In order to facilitate protein adsorption and hence cell adhesion on these surfaces, all the LCN films were incubated and coated with serum proteins overnight before performing the cell experiments.

Figure 2.3. AFM morphology and elasticity map of the various regions of a HP- 0.75 μm sample given in Figure 2.6A-B (vide infra). (A) Height image of the illuminated region and (B) the non-illuminated region. Derjaguin-Müller-Toporov (DMT) effective elastic modulus maps obtained from one Intermodulation AFM (ImAFM) scan of the (C) illuminated region and (D) non-illuminated region. The color contrasts and the scale bars depict the value of the stiffness on the sample surface in GPa. The scan sizes are 20 x 20 μm^2 .

2.2.2. Cell adhesion and migration patterns on hierarchically structured surfaces

Use of established cell lines such as NIH3T3 fibroblasts is informative in revealing adhesion related cellular processes.^[4b, 14] Adhered NIH3T3 fibroblast cells and their supported growth on all the fabricated LCN surfaces (**Figure 2.4A-I**, **Videos 2.1-2.9**, **Figure 2.5**) validated the biocompatibility of these LCN films similar to polystyrene, which is commonly used for cell cultures.

Figure 2.4. (A-D) Phase contrast images of NIH3T3 cells after 1 day on HP-1.6, HP-1.2, HP-0.5, HP-0.2 μm , respectively. (E) SEM image of cells on a HP surface (with 15 μm diameter and separation, and 0.15 μm height) after 12h. (F) Phase contrast image of cells on CP-0.3 μm surface after 1 day. (G and H) SEM images of cells on CP-0.3 μm surface after 12h and (I) phase contrast image of cells after one day on tissue culture polystyrene as control surface (scale bar: 50 μm for A-D, F and I; 10 μm for E, and 100 μm for G-H).

Figure 2.5. Number of cells per surface area on tissue culture polystyrene (TCPS), Flat_ni and Flat_i surface after 4h (mean $\pm$ standard deviation, $n=2$). (Cell density after 4h was measured as $4,696 \pm 1,227$, $5,503 \pm 2430$ and $4,178 \pm 769$ cells/cm², on TCPS, Flat_ni and Flat_i surface, respectively).

From time-lapse imaging experiments performed on the films that were incubated with fibroblasts, single cell migration behavior and the mean cell migration speed (total distance covered per time of measurements) were determined (**Figure 2.6** and **Figure 2.7A**). The initial mean cell migration speed of $0.85 \mu\text{m}/\text{min}$ observed on flat LCN films significantly dropped to $0.32 \mu\text{m}/\text{min}$ when such films were illuminated entirely while qualitatively no changes in cell morphology were observed (**Figure 2.6H** and **Figure 2.7A-C**, Videos 2.1 and 2.2).

Furthermore, cell migration patterns were derived from the time-lapse images and plotted as a change in cell migration speed in intervals of 5 min (**Figure 2.8A-B**). These plots not only confirm that cells became more static on illuminated substrates but also reveal changes in migration speed that occurred when cells were observed over the course of a few hours. These changes in migration speed occurred similarly frequent, yet of a markedly lower magnitude upon illumination (**Figure 2.8A-B**). These observations can be related to the increase in nanoscale surface roughness upon illumination, as was measured using AFM ($9.9 \pm 0.9 \text{ nm}$ vs. $18.2 \pm 0.4 \text{ nm}$, for Flat_ni vs. Flat_i, respectively, **Figure 2.2C-D**).

Figure 2.6. Characterization of single cell migration behavior on LCN surfaces. (A) AFM morphology and height image of a 0.75 μm pillar and its surrounding. (B) DMT effective elastic modulus map obtained from one ImAFM scan of the 0.75 μm HP (given in Figure A). (The color contrasts and the scale bars depict the value of the stiffness on the sample surface in GPa. The scan size is 40 x 40 μm^2). Snapshots from live cell imaging for representative cells (C-D) residing on the pillars and (E) stationary

and in contact with the pillars on the flat surfaces on HP-0.3, HP-1.6, HP-1.2 topographies, respectively (black arrow: a representative stationary cell). (F-G) Representative cells that are motile over the pillars and on the flat areas on HP-1.2 μm and HP-1.6 μm surfaces, respectively (scale bar: 50 μm). (H) Single cell migration speed on different LCN surfaces. The black circles are non-classified cell categories while the blue circles (open and closed) show category 1 and 2 respectively. (ni: not illuminated, red line: the mean cell speed, mean $\pm$ standard deviation, **: $p < 0.01$ and *: $p < 0.05$). (I) Percentage of the cells on HP surfaces representing the whole cell population counted on the surfaces in both categories.

Figure 2.7. Characterization of surface topology and pillar height dependent switch in cell migration behavior. (A) Average speed of cells on both flat surfaces and cells on HP-0.3 μm surface in Category 1. Phase contrast images of cells after one day on (B) flat not illuminated, (C) flat illuminated and (D-E) HP-0.3 μm surface (scale bar: 50 μm). Time-lapse imaging for a cell that represents Category 1 on HP-0.3 μm surface (scale bar: 50 μm).

Figure 2.8. Effect of surface topography on cell migration patterns. Cell migration patterns are given as change in single cell speed at every 5 min for four representative cells on different LCN surfaces.

It is known that cells are able to sense nanoscale features down to 8 nm and distinguish the size of the features by their nanopodial extensions.^[4b, 18] Yet, studies also show that in general cell attachment is influenced by changes in mechanical properties.^[19] To gain more insight in the nanoscale topographical and mechanical properties of the pillars and surrounding areas we imaged a hierarchically structured HP-0.75 μm film using intermodulation atomic force microscopy (ImAFM) (Figure 2.3, Figure 2.6A, B and **Table 2.2**). Height and roughness on top of the pillars and in their surrounding areas were determined and compared to illuminated flat surfaces.

Table 2.2. Surface roughness at various areas of the 0.75 μm HP sample.

	Selected area	Roughness (nm)
1	Top of a pillar	55 $\pm$ 3.3
2	Area surrounding the pillar	10 $\pm$ 1.4
3	Flat illuminated area	28 $\pm$ 1.7
4	Flat non-illuminated area	8 $\pm$ 1.3

Illumination of flat films resulted in a ca. 20 nm increase in nanoscale roughness (Table 2.2). The surfaces of illuminated pillars were found to also be rougher (55 ± 3.3 nm) compared with the non-illuminated surrounding areas (10 ± 1.4 nm). These results verified that we have fabricated hybrid interfaces presenting light-induced topographical patterns with significantly increased nanoscale roughness. ImAFM inspections also reveals simultaneously nanoscale mechanical properties of the surface of the pillars and their surrounding areas (Figure 2.3C-D and Figure 2.6B). Figure 2.6B is a typical effective elastic modulus map showing an average effective elastic modulus (ca. 0.5-0.75 GPa) of the surfaces of illuminated pillars, which was found lower than on non-illuminated surrounding areas (ca. 1.2 GPa) (see experimental section for details). Even though the elastic modulus has decreased upon illumination, we believe that both illuminated and non-illuminated areas present stiff matrices to cells to a similar extent due to their high elastic modulus (in GPa) compared to elastic natural matrix (elastic modulus ranging from a few hundred Pa to a few hundred kPa).^[19-20] Forces generated by cells are in the range of 1-5 nN μm^{-2} and cells would need to pull against and deform the underlying substrate to be able to sense and respond to stiffness.^[19a, 20-21] Therefore, our hierarchical interfaces allow us to relate the effect of nanoscale roughness and micron scale topographical cues independently of the stiffness of the surfaces.

The derived mean cell migration speeds on LCN films with the HP topographical cues revealed that surfaces with 0.3 μm high pillars (HP-0.3 μm) induced a significantly lower mean cell speed of 0.23 $\mu\text{m}/\text{min}$ when compared to the HP-0.5, HP-1.2 and HP-1.6 surfaces, which had a mean cell speed of 0.87, 0.81 and 0.87 $\mu\text{m}/\text{min}$, respectively (Figure 2.6H, Video 2.4-2.7). Interestingly, the latter topographies induced cell speeds in the same range as observed on the non-illuminated flat LCN films while the cell speed observed on the HP-0.3 topography is similar to that on the illuminated flat LCN films. The mean cell speed of 0.52 $\mu\text{m}/\text{min}$ observed on HP-0.2 topographies represents an intermediate case (vide infra). As a control surface for cell adhesion and migration, a speed of 0.55 $\mu\text{m}/\text{min}$ was observed on classical tissue culture polystyrene surfaces, which lies in between the values observed for both flat LCN surfaces (Figure 2.9, Video 2.9).

Figure 2.9. Single cell speed on tissue culture polystyrene (red line represents the mean speed, mean $\pm$ standard deviation).

To more closely inspect how the HP topographies were harnessing the spatio-temporal adhesion and organization of cells on these surfaces during migration, the live cell imaging data were analyzed in more detail (see experimental section). From the analysis, two modes of cell behavior were identified; cells in category 1 were mainly stationary with mean cell speeds as low as $0.15 \mu\text{m/min}$ and these cells were mainly in contact with the pillars while having minimal contact with the areas surrounding the pillars (Figure 2.6C-E); cells in category 2 were generally mobile with mean cell speeds as high as $1.13 \mu\text{m/min}$ and these cells were having minimal contact with pillars and were mainly moving across areas surrounding the pillars (Figure 2.6F-G). Much to our surprise, the analysis shows that the majority (84%) of the cells on HP- $0.3 \mu\text{m}$ belong to static, category 1 type cells (Figure 2.6H-I), while the majority (70-87%) of cells on films with higher pillars (HP- 0.5 , 1.2 and $1.6 \mu\text{m}$) fall to mobile, category 2 type cells. These category 2 cells migrate with no significant differences on the HP- 0.5 , 1.2 and 1.6 topographies (Figure 2.6H), yet they do migrate significantly faster when compared to the category 1 cells on HP- 0.3 topographies. It is known that higher cell speed can be related to the persistence in directional movement.^[4f, 22] However, trajectory analysis of cells in category 2 on all HP surfaces did not reveal any direct correlation of cell speed with persistence and directionality (Figure 2.10). As the average speeds of the two categories correspond with the speed of the illuminated and non-illuminated flat surfaces, we tentatively assign the remarkable, static versus mobile appearance of cells to the extent that cells were in contact with a rougher surface existing on illuminated parts of the films versus their contact with a smoother surface existing on non-illuminated parts of the films. The cell migration behavior that was imaged on HP- 0.2 topographies could not be categorized

due to a lack of resolving the topography during imaging (black circles in Figure 2.6H). Yet, cells on HP-0.2 μm were spread over both pillars and non-illuminated areas indicative of cells that fall into category 2 (Figure 2.4D and Video 2.3).

Figure 2.10. Representative cell trajectories for category 2 giving the total path length in 120 min (each color represents one cell).

Most notable are the consequences for cell migration behavior when switching from flat surfaces to a HP-0.3 topography. Category 1 cells on HP-0.3 topographies were adherent and residing only on the pillars, mainly elongating over the pillars for extended periods of up to 5h (Figure 2.7D-F) in agreement with the observed contact of cellular protrusions on the rougher pillars (Figure 2.4E). In contrast, cells on the flat LCN surfaces, irrespective of illumination, were spread, but not as elongated, which is typical for cell morphologies on 2D surfaces (Figure 2.7B-C and Figure 2.4I). This clearly points towards topography guided cell morphology, which is known to influence cell motility.^[2a, 4d-f] Intriguingly, even though cells were residing on illuminated parts of the topographic films, presumably due to increased roughness, the mean cell migration speed of the category 1 type cells on HP-0.3 significantly dropped further to 0.15 $\mu\text{m}/\text{min}$ when comparing to the cell migration speed of 0.32 $\mu\text{m}/\text{min}$ on flat, illuminated films (Figure

2.7A). The migration pattern plots for HP-0.3 topographies reveal that this category of cells became even more static with lower and less frequent changes observed in cell speed (Figure 2.8B-C). In strong contrast, cells on HP-1.2 topographies yielded a dramatic shift in the cell migration pattern to a highly dynamic behavior with increasing frequencies and magnitudes of changes in cell migration speed over time (Figure 2.8D-F).

Next to the HP topographic patterned films, single cell migration speed and cell behavior were also imaged and analyzed on the circular patterns of comparable heights and spacing as the HP patterns (Video 2.8). This analysis similarly revealed the existence of two distinct cell populations with cells aligning on the patterns of $0.3\ \mu\text{m}$ in height and $15\ \mu\text{m}$ spacing. These results show that cell adhesion and migration was guided on a different surface geometry indicating that the observations are generic (Figure 2.4F-H and **Figure 2.11**). These results demonstrate also the versatility of employing light-responsive LCN films as new biointerfaces to direct cell movement and migration speed. The changes in cell migration patterns are clearly associated with synergistic cell contact guidance by the micron scale topographies and the nanoscale roughness both of which are consequences of the light-induced isomerization of the azobenzene moieties in the bulk of the film material.^[2a, 4b] Switching cell migration behavior by switching the surface structure have important implications regarding in situ dynamic experiments where cell migration could be differentially harnessed to guide cells to specific locations on the surface.

Figure 2.11. Circular patterns on LCN surfaces showing the flexibility of encoding different surface topographies with light to guide cell behavior. (A) Surface structure measurements performed using optical profilometry showing the topographical dimensions to be approximately as $15\ \mu\text{m}$ width and separation with $0.3\ \mu\text{m}$ feature height. (B) Single cell speed on these surfaces (red line represents the mean cell speed, mean $\pm$ standard deviation).

2.2.3. Spatial and temporal switches in cell migration on the same LCN surface

To explore the possibility to guide cell migration and collect these migrating cells in local spots, the following surface topographic pattern was designed. Films were prepared with flat, non-illuminated areas surrounded by HP-1.1 topographies (**Figure 2.12A-C**). Our observations on such mixed surface-structured films revealed that the cell migration speed was similar on both areas of the surface and in agreement with the speeds and trends observed on either of the individual, non-mixed surface-structured surfaces (**Figure 2.6H** and **Figure 2.12D**, **Video 2.10**). However, much to our surprise, the majority of the cells (82%, 9 out of 11 cells that were monitored for 4h) starting from the pillar area, entered the flat area and remained in this area with a spread morphology (**Figure 2.12B** and **E**). This result shows the possibility to spatially control the localization of the cell population through cell migration on these surfaces, which has important implications in colonization of cells on biomaterials to enhance their performance.^[15]

With these results in hand, consequences of in situ temporal (i.e., changes occur in time in the presence of cells) light-induced permanent surface structural changes on the cell behavior were studied, representing a first step in exploring the potential of these materials in dynamically controlling the surface topography. For this purpose, cells were seeded on flat, non-illuminated surfaces and before illumination of the substrate the typical response of cells on these surfaces was observed (**Figure 2.8A** and **Figure 2.13A**). For this type of experiments, a critical consideration is the biocompatibility of the illumination dose.^[1a] By illuminating the film from the bottom, light penetration measurements using an intensity detector (details are given in the Experimental Section) showed no UV penetration through the samples to the cells indicating that UV light was absorbed by the film. According to this measurement, the films were exposed to light of $\lambda = 390\text{--}440\text{ nm}$ for 10 min (35 mW/cm^2), which was sufficient to induce the structural changes in roughness. According to AFM measurements on this sample an increase in surface roughness from 9.0 ± 1.2 to $11.1 \pm 2\text{ nm}$ was observed upon illumination for 10 min (**Figure 2.14**), where a change in cell migration can be expected.^[4b, 4f, 18, 23] Upon illumination, cells were monitored for 12h before measuring the cell migration speed, to observe and confirm their viability after illumination. Live cell imaging data revealed that 87% of the cells remained viable within this time (**Videos 2.11** and **2.12**). Changes in cell migration speed occurred less frequent and to a smaller extent with a decrease in mean cell speed from 1.21 to $0.54\text{ }\mu\text{m/min}$ after illumination showing a shift towards a more static behavior (**Figure 2.8B** and **Figure 2.13B-C**). These observations are in accordance

with the ex situ experiments (Figure 2.7A) where speed decreased with increased roughness.

Figure 2.12. Spatial switches in cell behavior on the same LCN surface. (A) 3D representation of the mixed surface with a Flat_ni region surrounded by HP-1.1 topographies (B) Phase contrast image of NIH3T3 cells on LCN surfaces presenting a flat not illuminated area surrounded by 1.1 μm high HPs after 3 days of culture. (C) Surface structure measurements performed using optical profilometry showing the topographical dimensions to be approximately as 1.1 μm high. (D) Single cell speed measured on both areas on the surface (E) Live cell imaging for a representative cell going from the pillar area to the flat area (scale bar: 50 μm).

Figure 2.13. Temporal switches in cell behavior on the same LCN surface. (A and B) In situ switch in cell migration patterns on flat LCN surfaces as change in single cell speed at every 5 min. (C) Mean cell speed on flat not illuminated surface (before) and 12 h after illumination (10 min) of the surface (mean $\pm$ standard deviation, $n=5$ and 6 cells, respectively).

Figure 2.14. AFM measurements (A) before illumination and (B) after 10 min illumination of cholesteric polymer film (with permanent changes) used for in situ dynamic cell experiments.

2.3. Conclusions

Consequences for cell migration on light-induced topographically patterned polymer network films were studied as function of micron scale topographical height and pattern and nanoscale surface roughness on similarly stiff surfaces for cells. Cell motility patterns could be switched from static to highly mobile by increasing the height of the pillars or from dynamic to moderately static by increasing the nanoscale roughness or to highly static on a selective topographic pattern. In situ temporal experiments further demonstrated the effect of nanoscale changes. Preliminary experiments on patterned LCN surfaces showed that it is also possible to form photo-switchable (reversible) topographies on demand (**Figures 2.15 and 2.16**) at the nanoscale, which is another important step for in situ control of cell behavior. These results demonstrate a new approach in engineering bio-instructive light-responsive surface structures. Future experiments aim at in situ reversible studies as well as manipulation of microscale topographies where light responsive elements that are switchable at longer wavelengths can be used to match the volume generation properties with cytocompatible illumination doses.^[24] The freedom that is offered in this system will enable us to generate materials that are more closely recapitulating the dynamic natural microenvironment not just giving better insights in fundamental biological processes but will also enable us to enhance biomaterial performance for regenerative medicine applications.

Figure 2.15. Height differences of the samples containing 0% and 4.4% inhibitor before light induced switching. The low parts are isotropic (iso) regions, while the high parts are cholesteric (chol) pillars.

Figure 2.16. The height difference of a sample where simultaneously a cholesteric (black) and isotropic (red) part of the sample is measured on a permanent sample (A) and a reversible sample (B). After reaching a plateau, the UV lamp (365 nm) is switched off (first dip in the height) and several minutes later the blue LED (455 nm), resulting in a drop in height in both cases.

2.4. Experimental Section

2.4.1. Preparation of light-responsive liquid crystal polymers (LCNs) and topography formation

All reagents and chemicals were obtained from commercial sources unless stated otherwise and used without further purification. A3MA was synthesized by Syncom (Groningen, The Netherlands). LC756 was obtained from BASF. C6M, RM23 and RM105 were obtained from Merck. Irgacure 819 was obtained from Ciba Specialty Chemicals Inc. 4-Methoxyphenol was obtained from Sigma-Aldrich. The molecular structures are given in Scheme 1 and their function is given in Table 1. For the fabrication of the liquid crystal polymers, a mixture of A3MA (2.0 wt%), LC756 (3.2 wt%), C6M (18.3 wt%), RM23 (29.7 wt%), RM105 (40.7 wt%), Irgacure 819 (1.7 wt%) and 4-methoxyphenol (4.4 wt%) was dissolved in 4 mL tetrahydrofuran (THF), resulting in a concentration of 0.25 g monomer/mL THF.

Thin films of the light-responsive liquid crystalline polymers were prepared and attached to a quartz glass slide. The internal surface of the bottom slide of this cell was functionalized with 3-(trimethoxysilyl)propyl methacrylate to ensure covalent attachment of the liquid crystal polymer.^[25] 50 μ L of the monomer mixture in THF was placed on the functionalized surface and heated to 70°C for 10 min to evaporate the

solvent. For easy removal of the top quartz glass slide, the corresponding quartz glass slide was functionalized with a fluorinated compound (1*H*,1*H*,2*H*,2*H*-perfluorodecyl-triethoxysilane). The cell was closed by placing the top slide on the bottom slide and subsequently the slides are sheared to induce planar alignment of the liquid crystal. Samples of roughly 8 μm in thickness are achieved using this technique. The sample is allowed to cool down to room temperature before exposing to UV-light to induce polymerization. After 5 min of illumination using an Exfo Omnicure S2000 light source and a Cut-off filter (Edmund Industrial Optics Stock No. 54516) to prevent isomerization of the azobenzene moiety, the sample was heated to 90°C and illuminated an additional 10 min to ensure full polymerization of the film. The fluorinated glass slide was removed and the substrate was ready for use.

2.4.2. Forming the structures

To form a structure, a chromium oxide mask was used. The samples were heated to 60°C to pass the glass transition temperature (T_g) and illuminated for 1 h using an Exfo Omnicure S2000 light source, whereby 57% of the light intensity has a wavelength of 395-445 nm and 43% is 320-390 nm with a total intensity between 400 and 800 mW/cm^2 , depending on the desired height of the structure. Subsequently the samples were cooled to room temperature. The height of the structures depends on the initial sample thickness and illumination intensity. Flat surfaces were prepared with the same illumination parameters but in the absence of a mask. In the case of the flat non illuminated sample, the same treatment is performed, but the sample is covered to prevent light to reach the sample during the illumination step.

2.4.3. Characterization of LCN surfaces

Surface profiles were measured using a Sensofar PL μ 2300 optical profilometer in confocal mode equipped with a 50x objective. UV-Vis measurements were performed on a Perkin Elmer Lambda 750 UV-Vis-NIR spectrophotometer equipped with a 150 mm integrating sphere containing a lead sulfide (PbS) and photomultiplier tube (PMT) detector. Roughness measurements were performed either on an Asylum Research MFP-3D-Bio or NT-MDT Solver P47 Pro AFM equipped with a Bruker NCHV tip ($f_0 = 320$ kHz, $k = 42$ N/m) in non-contact/tapping mode to measure the topography (height and phase); scanning by tip. Data analysis was performed using Gwyddion software and the roughness measured as the root mean square (RMS) height variation in the measured area. ImAFM was performed on a Dimension Icon AFM (Bruker Corp.), coupled with an

external multi-frequency lock-in amplifier (Intermodulation Products AB) to analyze the nano-mechanical properties of the samples. A silicon cantilever (Bruker RTESPA-30) with a stiffness of 40 N/m and a resonance frequency (ω) of 300 kHz is also used. The resolution of all ImAFM images taken are 512 x 512 pixels. Contact angle measurements were performed on a Dataphysics OCA 30 at room temperature using deionized water as the probe liquid.

To further investigate the effect of illumination on the surface, a sample was made with 0.75 μm HP (Figures 2.6A-B and Figure 2.3). All measurements were performed on a single sample, for direct comparison. Using standard tapping mode AFM, the height and roughness of a single pillar was measured. From the topography images the roughness of the surfaces was extracted (Table 2.2). The Root Mean Square (RMS) roughness is an average of height deviations taken from the mean image data plane. Hereby, a similar trend as for the previous measurements was found as measured before (Figure 2.2C-D). To measure the Young's modulus of the material, Intermodulation AFM (ImAFM) was performed.

The working principle of ImAFM and the methodology we used to extract the nanoscale mechanical properties of these structures are as follows. ImAFM uses an external multi-frequency lock-in amplifier to excite the cantilever at two frequencies close to the resonance frequency peak, and then it record the photo-detectors response at many intermodulation products of the two drives.^[26] The ImAFM measures the amplitude dependence of the tip-surface force quadratures at each pixel of an image while scanning. Then, amplitude and phase images are collected and displayed in the Intermodulation Products software.^[27] ImAFM uses a built-in method to determine the tip-surface force from the measured tip motion. Normally this requires certain assumptions of the tip-surface force to reconstruct the measured data. Specifically it reconstructs the tip-surface force $F(d)$ as a function of cantilever deflection

$$d = z - h \quad (1)$$

Where the probe height h and the tip position z are measured from a fixed position in the fixed reference frame where the sample is at rest. Platz et al. have approximated the nonlinear tip-surface force by a polynomial of finite degree N in the cantilever deflection, d as depicted in equation 1.^[28] Here we have also used the polynomial approximation to reconstruct the tip-surface interaction forces. Once the polynomial approximation is selected to reconstruct the force curves as a function of cantilever deflection, we can select a specific force model to fit the forces. For our samples we selected the Derjaguin-

Muller-Toporov (DMT) model (equation 2) considering a spherical tip and a flat surface to fit the polynomial function as shown below: ^[29]

$$F(z) = \begin{cases} -F_{min} \frac{a_0^2}{(z+a_0)^2} & , z > 0 \\ -F_{min} + \frac{4}{3}E^* \sqrt{-Rz^3} & , z < 0 \end{cases} \quad (2)$$

In equation 2, Z is the penetration depth, R the tip radius, a_0 the adhesion range, F_{min} the depth of force minimum, and finally E^* the effective elastic modulus. Fitting this equation to the received force curve allows one to extract the effective elastic modulus.

2.4.4. Cell culture and substrate preparation for cell seeding

NIH 3T3 (ATTC[®] CRL-1658[™]) cells were cultured in basic medium (DMEM (Sigma) supplemented with 10% FBS (Sigma), 2 mM L-glutamine (Sigma), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma)) and incubated at 37°C and 5% CO₂ in a humidified environment. Prior to cell seeding LCN substrates were sterilized with 70% ethanol and incubated in basic medium overnight at 37°C to facilitate protein adsorption.

2.4.5. Characterization of cell behavior on the surface

NIH 3T3 cells were seeded at a density of 5,000 cells/cm² on the surfaces and time lapse images were taken every 5 min using Cytomate[®] live cell imaging equipment (Eindhoven, The Netherlands).

For LCN surfaces with HP topographies and circular patterns (with 15 µm width and 15 µm separation and 0.3 µm feature height), cell migration was monitored for one day and cell trajectories were derived based on 120 min frame of the cell migration starting after 4h of cell attachment. For the analysis of cell migration trajectories and cell speed, at least 8 individual cells were followed (by their nucleus and their center) while cells that were out of frame and focus (due to focus drift despite autofocusing of the camera) or were dividing were excluded. Information on the position of the cell and total path length was obtained with MTrackJ plugin ^[4d] of ImageJ (NIH) by monitoring each cell in consecutive frames. Cell migration speed was calculated simply by dividing the total length of the trajectories (total distance covered, hence not taking the directionality into account) by the time of the trajectory for each cell as one data point.

In order to further evaluate the biocompatibility of the surfaces, number of cells per surface area was calculated for tissue culture polystyrene (TCPS), flat not illuminated substrates (Flat_ni) and flat illuminated substrates (Flat_i) after 4h of attachment by counting individual cells based on two different experiments (n=2) (Figure 2.5).

Characterization of cell alignment

To characterize the mode of adhesion and migration (and organization) on surfaces with 0.3, 0.5, 1.2 and 1.6 μm high pillars, each cell was monitored for the entire time frame (120 min) to observe their behavior and categorization is reported for the total trajectory. As a result, two main categories were reported as: (1) cells that were mainly residing on top of the pillars or with a minority staying in between the pillars while having contact with the edge of the pillars (mainly stationary cells with low speeds) and (2) cells that were crossing over the pillars and also moving on the flat surface (migratory cells with higher speeds). For the 0.2 μm high HP surface no categories were derived due to the resolution limit of the pillars.

In order to derive the cell migration patterns, a change in cell speed over time (every 5 min) was calculated for the given surfaces. Briefly, the cell speed was calculated based on distances taken in every 5 min, corresponding to the time scale of the image acquisition, for 120 min, for permanent structures.

In order to study adhesion and organization on the surfaces, cells were also seeded at a density of 5,000 cells/cm²; and after 4h or one day they were fixed with 4% paraformaldehyde (PFA) and rinsed three times with phosphate buffered saline (PBS, Sigma, pH 7.4) and surfaces were imaged with phase contrast microscopy (Evos FL Cell Imaging System, Thermo Fisher Scientific).

Furthermore, scanning electron microscopy (SEM, ESEM-FEG, XL30, Philips, The Netherlands) was performed in order to study cell adhesion. Briefly, cells were fixed with 4% PFA after 12h and they were dehydrated with ethanol (ACS grade, VWR chemicals) incubation series (70, 80, 90, 96 and 100% ethanol, 30 min each) followed by 30 min incubation in hexamethyldisilazane (HMDS). Finally samples were dried in air and sputtered with gold at 30 mA for 40 s using a sputter coater (Cresington 108 Auto, Watford, England).

In order to study in situ spatial switches in cell behavior, cells were seeded at the same density on HP_Flat surfaces (Flat_ni area surrounded by 1.1 μm high hexagonally

arranged pillars, Figure 2.12). Live cell imaging experiments were performed over the time of 48h in order to investigate cell movement from the pillar areas to the flat areas and vice versa. In order to derive the population dynamics, the number of cells that went from the pillar area or at the edge and entered in the Flat_{ni} area (and if they were staying there) were calculated over the time course of 4h to give a more extended time scale for analysis. Similarly, trajectory analyses were performed to derive the cell speed on both areas using Image J (NIH) based on 4h monitoring. Video 2.10 depicts the first 36h of cell behavior on these surfaces with time lapse images taken every 20 min in order to give an overview of cell response (and due to size limitation of the videos) even though analysis on cell migration patterns were done by using images taken every 5 min. For in situ temporal (i.e., changes are induced in time by illuminating the substrate when cells are on the surfaces) switching experiments on flat surfaces (where permanent changes were induced on the surface roughness in the presence of cells), cells were seeded on a non-illuminated surface (with 4.4% inhibitor and has permanent changes upon illumination) at the same density and cultured for 1 day (Figure 2.13). Before illumination of the surface, live cell imaging was performed in order to derive cell speed and cell migration patterns in a time scale of 1h as explained above (for 5 cells, Video 2.11). After exchanging the culture medium for live cell imaging solution (Molecular Probes, Life Technologies), the sample was illuminated from the bottom continuously for 10 min at room temperature where light penetration measurements indicated that cells were exposed to 390-440 nm light (blue light) at 35 mW/cm². Then, cells were again brought to normal culture conditions and monitored with Cytomate® as explained before, in order to derive cell migration behavior (Video 2.12). In order to analyze cell migration patterns and speed after illumination, cells were monitored for 12h for their viability and then 1h trajectories of individual cells were determined as explained above (for 6 cells). In order to quantify the cell viability after sample illumination (and within this first 12h) individual cells were monitored with live cell imaging starting right after the illumination to find out if they remained viable or went through apoptosis (Video 2.12). In total 23 cells were monitored for viability up to 8h after sample illumination (to be able to count as many cells as possible before cells migrate out of frame). Extent of viability was reported as the percentage of the cells that remained viable after illumination within the first 12 h. In addition, these live cell imaging experiments gave us possibility to evaluate the morphology of the viable cells after illumination which remained as characteristic to fibroblast phenotype. Video 2.12 reveals the cell response for a duration of 24h after illumination, based on images taken every 10 min for an overview of cell response while the analysis was performed with images taken every 5 min (as stated above) to ensure a good time resolution of cell behavior.

To determine the penetration of light to the cells in the in situ experiment described in Figure 2.13, a coating on quartz was placed between the light source and the intensity detector (UV Power Puck II).

Statistics

Data are presented as mean $\pm$ standard deviation and cell migration data were analyzed using Kruskal-Wallis one way-ANOVA for statistical significance. Multiple comparison tests were performed with Mann-Whitney test by adjusting α level downward according to Bonferroni correction comparing all groups of interest.

2.4.6. Studying the reversibility of light-responsive topographies on hexagonal patterned surfaces

In order to study reversibility of light-responsive topographies on LCN films, patterned polymer films were made containing hexagonal patterned cholesteric liquid crystal (CLC) pillars embedded in isotropic surrounding. Reversible and irreversible light switchable topographies were made based on the CLC mixture containing, either with 0% or 4.4% inhibitor by a 2 step polymerization. Therefore, a hexagonal patterned mask was placed directly on the non-polymerized planar aligned CLC film. The sample was polymerized for 6 s to form the cholesteric pillars. Then the sample was flipped, placed on a heating stage of 120°C and again illuminated to polymerize the isotropic matrix for 1 minute. The mask was removed using a razorblade. Initial heights in the structure of 130 nm were observed, as this is a negative print of the hexagonal chromium pattern on the mask (Figure 2.15).

To measure the topographies during illumination, Digital Holographic Microscopy (DHM, Reflection DHM® Lyncée Tec) was used. For illumination, two LED's (Thor labs, 365 nm, UV 675 mw/cm² and blue 455 nm, 57 mw/cm²) were used. The UV-illumination was performed from the bottom using an UV-mirror (Thor Labs) to direct the light to the sample. This light is not collected by the DHM, since there is a cut-off filter (<400nm) in place. The blue light illumination was performed under an angle from the top, as this part is not filtered from the DHM. The surface was monitored for 30 seconds before switching on the LEDs. When switching on both lamps, a rapid increase in the surface height difference was observed for the cholesteric pillars, while the isotropic matrix stays constant. A "plateau" was reached at roughly 600 s, where the rapid grow starts to diminish (Figure 2.16). In both cases, the height difference was roughly 90 nm. When the UV LED was switched off, in both cases a decrease in height was observed. When switching off the blue LED, a further decrease in height was observed. In case of the film

containing inhibitor a permanent topography was obtained with a height difference of roughly 55 nm (Figure 2.16A), while for the film without inhibitor the sample went back to its initial topography (Figure 2.16B).

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2.6. References

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2.7. Videos

Live cell imaging videos of NIH3T3 cells cultured on different LCN surfaces which were used for Figures 2.5, 6, 7, 8, 9, 10, 11, 12, 13 could be downloaded from (note that in the online version, the videos are listed after the first supporting PDF file):

<http://onlinelibrary.wiley.com/doi/10.1002/adma.201606407/supinfo>

Video 2.1. Flat not illuminated surface (Online: Video S1)

Video 2.2. Flat illuminated surface (4h-24h) (Online: Video S2)

Video 2.3. HP-0.2 μm (Online: Video S3)

Video 2.4. HP-0.3 μm (Online: Video S4)

Video 2.5. HP-0.5 μm (Online: Video S5)

Video 2.6. HP-1.2 μm (Online: Video S6)

Video 2.7. HP-1.6 μm (Online: Video S7)

Video 2.8. CP-0.3 μm (Online: Video S8)

Video 2.9. Tissue culture polystyrene (4h-24h) (Online: Video S9)

Video 2.10. HP_Flat (flat area surrounded by pillars) (Online: Video S10)

Video 2.11. In situ experiments-Flat surfaces before illumination (not-illuminated) (Online: Video S11)

Video 2.12. In situ experiments-Flat surfaces after 10 illumination (0-24h after illumination) (Online: Video S12)

Chapter 3

Guiding human mesenchymal stem cell adhesion and differentiation on supported lipid bilayers*

Mesenchymal stem cells (MSCs) are intensively investigated for regenerative medicine applications due to their ease of isolation and multi-lineage differentiation capacity. Hence, designing instructive microenvironments to guide MSC behavior is important for the generation of smart interfaces to enhance biomaterial performance in guiding desired tissue formation. Supported lipid bilayers (SLBs) as cell membrane mimetics can be employed as biological interfaces with easily tunable characteristics such as bio-specificity, mobility and density of pre-designed ligand molecules. Arg-Gly-Asp (RGD) ligand functionalized SLBs are explored for guiding human MSC (hMSC) adhesion and differentiation by studying the effect of changes in ligand density and mobility. Cellular and molecular analyses show that adhesion occurs through specific interactions with RGD ligands where the extent is positively correlated to changes in ligand density. Furthermore, cell area is significantly regulated by ligand density on ligand-mobile SLBs when compared to ligand-immobile SLBs. Finally, the osteogenic differentiation capacity of hMSCs is positively correlated to ligand density on ligand-mobile SLBs indicating that regulation of cell spreading is linked to cell differentiation capacity. These results demonstrate that hMSC behavior can be directed on SLBs by molecular design and presents SLBs as versatile platforms for future engineering of smart biomaterial coatings.

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3.1. Introduction

The extracellular matrix (ECM) accommodates multiple chemical and biophysical cues that can be sensed by cells through their cell surface receptors. The organization of cues has critical effects on the regulation of cell fate processes such as proliferation, adhesion, migration and differentiation.^[1] Synthetic approaches to mimic these cues has been an important strategy in the development of biomaterials for tissue engineering and regenerative medicine applications. Certain instructive properties of the ECM can be integrated in biological interfaces, such as the coating of biomaterials, by functionalizing them with for example cell-adhesive Arg-Gly-Asp (RGD) peptide ligands that are derived from fibronectin and recognized by transmembrane integrin receptors. When anchoring these RGD ligands on biological interfaces and let them interact with cells, control over molecular flexibility, binding affinity, ligand density and ligand mobility are critical to most effectively guide cells to desired responses.^[1a, 2]

Biological interfaces can be achieved by immobilizing ligands on an inert background to guide specific interactions. In addition, by carefully adjusting the ratio of biofunctional to inert units one can control the density and separation of pre-designed ligand molecules. The affinity of ligands with their receptors could be tuned by adjusting the molecular conformation of the ligands (e.g., cyclic vs. linear RGD) and further optimization of the ligand-receptor interaction could be achieved by introduction of a flexible surface tether (or spacer).^[2a, 2d, 3] Such chemically defined surfaces have been formed for example by using self-assembled monolayers of alkylthiolates on gold, passivated with inert oligo(ethylene glycol) groups while maleimide containing groups are used to covalently conjugate the peptide on the surface.^[2d, 4] Similarly, self-assembly of block co-polymers and deposition of gold nanoparticles on the surfaces (so-called block co-polymer lithography) have been employed to nano-pattern RGD ligands.^[5] These studies gave insights into the effects of ligand density, affinity and separation on cell adhesion, focal adhesion formation as well as on (stem) cell migration and differentiation.^[2d, 4-5]

These and other strategies use covalent modifications to conjugate ligands on surfaces resulting in a static (immobile) presentation of the ligands to cells. In contrast, the cellular microenvironment involves characteristically rapid, dynamic and adaptive processes where cells can recognize the local order and re-organize their environment to engage into an optimum level of interactions taking advantage of the dynamic (mobile) presentation of ligands. Supramolecular chemistry, to this end, introduces a

versatile approach to integrate responsive and dynamic ligands into biological interfaces, in order to guide cell behavior in a more biomimetic manner due to inherent dynamics of non-covalent bonds.^[2a, 6] Multiple supramolecular strategies based on for example host-guest chemistry, self-assembled peptide amphiphiles or lipid-based structures have been employed to generate dynamic biological interfaces to control cell behavior.^[6]

Supported lipid bilayers (SLBs) are cell membrane mimetic supramolecular architectures that form by vesicle fusion on hydrophilic solid supports.^[7] SLBs have multiple physicochemical characteristics that are essential for their biological performance and can easily be adjusted by controlling their chemical composition (i.e., base lipid content). The head group composition of base lipids defines the surface chemistry of bare SLBs where zwitterionic groups provide a protein and cell resistant surface, which allows for specific interactions with cells when ligands are incorporated in the SLB.^[8] Furthermore, the alkyl chain composition of base lipids defines the phase behavior of the SLB due to characteristic melting (transition) temperatures of the lipids. This phase behavior determines the mobility of ligands in the SLB and hence it can influence the consequences of the interactions of the ligands with their cellular receptors, receptor clustering and subsequent cell signaling pathways.^[9] SLBs consisting of lipids with a low melting temperature (e.g., 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)) present mobile ligands while SLBs consisting of lipids with a high melting temperature (e.g., 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)) present immobile ligands at physiological temperatures. These characteristics of biomimetic SLBs together with their ease of fabrication and patterning have provided insight in receptor-ligand interactions, cell adhesion mechanisms and cell signaling in relation to ligand mobility and clustering.^[9-10] In addition, intricate SLBs were developed with gradients of ligands for cells and with stimuli-responsive ligands to modulate cell adhesion.^[8a, 11] Despite these intriguing achievements, there are however only few examples where cell-SLB interactions have been targeted beyond initial cell adhesion events.^[9a, 10c, 10g, 10g-i] For example Evans *et al.* anchored N-cadherin ectodomains to SLBs to study the behavior of periosteum derived progenitor cells on these SLBs.^[12] A significant regulation in expression of genes involved in mesenchymal condensation was observed by them to be dependent on cell density and SLB functionalization.^[12] Another study reported the controlled neural stem cell adhesion, growth and differentiation on SLBs onto which polyelectrolyte films were deposited.^[13]

In this chapter, for the first time, RGD presenting SLBs are employed as a biological interface to study the short term (i.e., hours) cell adhesion and longer term (i.e., days) cell differentiation behavior of clinically relevant human mesenchymal stem cells (hMSCs) by investigating effects of ligand density and their mode of presentation (mobility) on cell morphology and osteogenic differentiation capacity of the cells.

3.2. Results and Discussion

3.2.1. Control over cell adhesion and spreading on SLBs

As cells approach a surface, the initial adhesion events involve the binding of integrin receptors to presented ligands (i.e., RGD ligands) on surfaces to form focal contacts. This results in cell spreading as well as maturation of focal contacts to focal adhesions (FA) under proper conditions.^[2a, 14] Specificity and initial regulation of these interactions can have important implications in long term (stem) cell behavior.^[4-5] Therefore, the short term behavior of hMSCs on our SLBs was first characterized by studying the specificity of the cell adhesion as well as the dependence of the cell adhesion on the ligand density and mobility. A schematic of the SLB system used in this chapter together with the molecular structures of the lipids and the cell-adhesive peptide (biotinylated RGD) is given in **Scheme 3.1**. In this chapter, DOPC and DPPC phosphatidylcholine (PC) were used because these lipids are abundant in eukaryotic cell membranes,^[15] have well-known physiochemical characteristics and have been widely used in SLB systems.^[8a, 16] To create a contrast in the mobility of the ligands at physiologically compatible temperatures, the choice of in particular DPPC seems optimal given its melting transition temperature (T_m), which occurs sharply for DPPC (Scheme 3.1).

Scheme 3.1. Chemical structures are given of the lipids and peptides used in this chapter. A schematic of the SLBs is given. The amount of RGD ligand on the surfaces was varied by varying the molar concentrations of biotinylated functional lipids. (DOPC: 1,2-dioleoyl-sn-glycero-3-phosphocholine, DPPC: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, biotinyl DOPE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(biotinyl), biotinyl-N-cap DOPE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl)).

The specificity of cell-SLB interactions was characterized by evaluating cell adhesion on bare SLBs, which do not present any ligands for binding. Cell adhesion on bare SLBs in serum containing medium after 17h was confirmed to be considerably lower when compared to FBS coated glass confirming the inert nature of the bare SLB (Figure 3.1A and B). It is important to note that 17h was selected based on time lapse imaging experiments on DOPC SLBs conjugated with 1 mol% biotin DOPE (1 mol% ligand density) showing that stable cell spreading was reached at 17h. Therefore, 17h allows us to study single cell behavior in order to reveal cell-surface interactions (Video 3.1).

The vinculin localization in hMSCs after 17h of culture on DOPC SLBs, which present RGD ligands linked to 1 mol% biotin DOPE in the SLB, was compared with that observed on a bare SLB and a control SLB, which presented negative control RGE peptide ligands. On the RGD presenting SLBs, vinculin localization at FAs was observed to be at the cell periphery (Figure 3.1C-D), which is in strong contrast to the bare and control SLBs where only cytoplasmic vinculin was observed (**Figure 3.2A-B** and E-F). These observations confirmed the specificity of integrin-RGD interactions during the adhesion of hMSCs on our ligand functionalized SLB interface.

While in general an optimum ligand density is expected for proper cell adhesion, FA formation and cell spreading, these cellular responses are also critically affected by the ligand mobility.^[2a, 2d, 5b, 10i, 17] Therefore, after having confirmed that cell adhesion occurred through specific RGD interactions, the extent of cell adhesion and cell morphological parameters as a function of ligand density and mobility were evaluated. To vary the RGD ligand density and mobility, fluid DOPC and non-fluid DPPC based SLBs were functionalized with different amounts of biotin DOPE (0.01, 0.5 and 1 mol %). After culturing hMSCs for 17h (in serum conditions), the number of cells per surface area (cell density) and cell shape factors were quantified using Cell Profiler software (see details in the Experimental Section).^[18] Important to note is that ligand densities higher than 1 mol% were avoided since beyond that value immobile fractions in DOPC SLBs were observed as was reported in literature for this type of functionalization.^[10b, 19]

Figure 3.1. Cell adhesion after 17h on (A) bare DOPC SLBs and (B) FBS coated glass substrates (scale bar: 40 μm). (C) Vinculin localization in hMSCs after 17h on 1 mol% biotinyl DOPE conjugated SLBs presenting GGSGRGDSG (in short RGD) peptide ligands. (D) Vinculin localization in hMSCs after 24h on 1 mol% biotinyl-N-cap-DOPE conjugated SLBs presenting RGD peptide. Vinculin is presented in green, nucleus in blue and actin in red (overlaid in C and D). Scale bars in C and D (and in insets) represent 20 μm . (E) Total number of adhered hMSCs after 17h per surface area on RGD presenting DOPC and DPPC SLBs for each condition (different mol% of biotin DOPE) and compared to a bare SLB.

Figure 3.2. Vinculin localization in control groups. (A and B) Vinculin localization in cells on control GGSGRGESG peptide presenting SLBs at 1 mol% functionalization level. Vinculin localization in cells on (C and D) FBS coated glass and (E and F) bare SLB (scale bars: 20 μm , $t = 17\text{h}$, blue: nucleus, red: actin cytoskeleton and green: vinculin).

On both RGD functionalized DOPC and DPPC SLBs, an increase in cell density was observed upon increasing the RGD ligand density due to a higher number of binding sites (Figure 3.1E and **Table 3.1**). These observations are in agreement with previous observations for adhesion of other types of cells on SLBs.^[10], 20] Remarkably, the cell density observed on RGD functionalized DOPC SLBs was higher throughout the series of varying ligand densities when compared to the series on RGD functionalized DPPC SLBs (Table 3.1), while on bare SLBs even lower numbers of cells adhered (158 cells/cm²)

(Figure 3.1E). This is the first experimental evidence that ligand mobility on SLBs modulates hMSC adhesion behavior.

Table 3.1. Cell adhesion density on functional DOPC and DPPC SLBs after 17h.

	Total number of cells/cm ²
DOPC 0.01 mol%	1,108
DOPC 0.5 mol%	1,411
DOPC 1.0 mol%	2,538
DPPC 0.01 mol%	295
DPPC 0.5 mol%	680
DPPC 1.0 mol%	1,110

Generally, the interaction between the extracellular environment with cells through integrin receptors is highly dynamic. Therefore, a surface where ligands can freely move and rearrange is suggested to increase the avidity of the interactions (that is required to achieve active clustering) and enhances the probability of binding and hence the efficiency of cell attachment.^[10a, 17, 21] Furthermore, conformational changes in integrin receptor sites upon ligand binding indicate a highly responsive activation mechanism.^[21] By providing ligand mobility on the SLB, a dynamic interface is offered to interact with cell surface integrin receptors,^[9a, 9b, 10c, 10i] which appeared beneficial to efficiently bind their RGD ligands as explained above.

Next, cell shape factors such as cell area, the degree of elongation (eccentricity, approaches 1 for highly elongated cells) and circularity (form factor, approaches 1 for highly circular cells) of hMSCs on SLB surfaces were quantified after 17h culture. The cell spreading area was significantly positively correlated with the increase in ligand density on RGD presenting DOPC SLBs ($p < 0.01$; **Figure 3.3A-C** and **G**, **Table 3.2**). On these SLBs, hMSC elongation was significantly the lowest on 0.01 mol% ligand density while there were no significant differences observed between cells on 0.5 and 1.0 mol% ligand functionalized DOPC SLBs, indicating a saturation in cell response. On the other hand,

the circularity of the cells significantly decreased upon increasing the ligand density (**Figure 3.4A** and C, Table 3.2). A similar trend for the cell area was observed on DPPC SLBs even though no significant differences as function of ligand density were seen in this case (Figure 3.3D-G). Furthermore, on 1 mol% RGD functionalized DPPC SLBs, cell elongation was significantly the highest while the circularity was the lowest ($p < 0.01$). This verifies that cell shape factors are controlled with ligand density also on DPPC SLBs (Figure 3.4B and D). Besides, at all ligand densities of RGD-functionalized DOPC SLBs a higher spreading area was observed than in the case of DPPC (for example $1,101 \pm 599$ vs. $559 \pm 288 \mu\text{m}^2$ at 1 mol% ligand density for DOPC vs. DPPC, respectively), a more elongated (eccentricity of 0.78 ± 0.17 vs. 0.67 ± 0.17 , respectively) and a less circular phenotype (form factor of 0.51 ± 0.20 vs. 0.65 ± 0.20 , respectively) (Figure 3.3 and Table 3.2) were deduced in the case of DOPC SLBs. Therefore, a change in the base lipid composition of SLBs directed both cell adhesion density and cell spreading behavior after 17h, even at the same ligand densities.

Cells translate the ligand density as ligand separation, which was found to be critical for integrin signaling and FA formation based on the studies on immobilized ligands.^[2d, 5] Therefore, the ligand spacing on our RGD functionalized SLBs was calculated as an approximation of the lipid head group separation (**Table S1**). It should be noted that due to different lipid composition (hence different lipid areas) at the same biotin-functionalization level, ligands are slightly more closely packed on DPPC SLBs than DOPC SLBs (Table S1). Figure 3.3H shows the dependency of cell spreading on ligand separation as intermolecular distance in nanometers. The plot reveals the linearity of the cell response as a function of ligand separation that was seen to be more pronounced on DOPC SLBs even though a correlation between decreasing cell area with increasing intermolecular spacing was seen for both types of SLBs.

Figure 3.3. Overview of cell morphology on RGD presenting DOPC (A-C) or DPPC (D-F) SLBs conjugated with 0.01 (A,D), 0.5 (B,E) and 1.0 (C,F) mol% biotin DOPE (scale bar: 40 μm). (G) Cell area plotted versus ligand density (expressed as molar ratio of biotin DOPE) on DOPC (black) and DPPC (red) SLBs. (H) Cell area plotted as a function of intermolecular distance derived from the lipid head group separation in SLBs (mean $\pm$ standard deviation, **: $p < 0.01$, one-way ANOVA followed by Bonferonni's multiple comparison test, $t = 17$ h).

Interestingly, we observed a more effective spreading of hMSCs on ligand functionalized DOPC SLBs, in contrast to DPPC SLBs, even for ligand separations that are below the reported critical values to induce adhesion and spreading.^[2d] This is an important observation that reflects the ability of cells to recognize the local order and reorganize their environment to allow for dynamic integrin-RGD recognition, cytoskeletal remodeling and eventual cell spreading to occur.^[2a, 22] This process can be strongly hampered on immobile surfaces, yet it can be accommodated by adopting ligands with mobile anchors.^[2a] We note however that these observations are strongly dependent on cell type, ligand presentation and time scale of studies.^[10i] We also note that rigidity (stiffness) sensing is important in cell adhesion and spreading and a stiffer surface in general leads to a higher cell spreading.^[2b, 23] DPPC SLBs are reported to be stiffer than DOPC SLBs with Young's modulus of 28.1 MPa and 19.3 MPa, respectively.^[24] Since these values are higher than the values estimated for natural microenvironments (e.g., for osteoid precursors of bone it is 25-40 kPa), we concluded that for both types of SLBs that cells will sense the same bulk rigidity and would not be able to distinguish them when considering forces generated by cells (1 to 5 nN μm^{-2}).^[25] Therefore, we hypothesize that our biological interface based on DPPC SLBs does not present sufficiently mobile ligands to the cells in order to actively organize their environment to spread.

Table 3.2. Quantification of cell shape factors on functional DOPC and DPPC SLBs.

	Cell Area [μm^2]	Form Factor	Eccentricity
DOPC 0.01 mol%	687 $\pm$ 400	0.59 $\pm$ 0.20	0.71 $\pm$ 0.18
DOPC 0.5 mol%	1,006 $\pm$ 553	0.54 $\pm$ 0.19	0.78 $\pm$ 0.17
DOPC 1.0 mol%	1,101 $\pm$ 599	0.51 $\pm$ 0.20	0.78 $\pm$ 0.17
DPPC 0.01 mol%	508 $\pm$ 240	0.74 $\pm$ 0.18	0.60 $\pm$ 0.18
DPPC 0.5 mol%	516 $\pm$ 263	0.71 $\pm$ 0.19	0.63 $\pm$ 0.18
DPPC 1.0 mol%	559 $\pm$ 288	0.65 $\pm$ 0.20	0.67 $\pm$ 0.17

Figure 3.4. Regulation of cell shape factors on SLBs. (A and B) Eccentricity of the cells as an indication of their elongation on the surfaces. (C and D) Form factor of the cells as an indication of their circularity (mean $\pm$ standard deviation, **: $p < 0.01$, one-way ANOVA followed by Bonferroni's multiple comparison test, each data point represents an individual cell, $t = 17h$).

In fact, when cell-surface interactions were time-lapse imaged at the early phases of adhesion well before cell spreading (first 3h), a stationary phenotype was observed on DPPC SLBs with 1 mol % ligand density in contrast with a motile phenotype on DOPC SLBs with the same ligand density (Video 3.2 and 3.3).

This observation was supported by trajectory analyses (**Figure 3.5**) indicating a distinct guidance of cell migration on different types of SLBs. Furthermore, on mobile ligand-functionalized DOPC SLB, even after effective spreading, cell migration was supported with cells actively probing their environment (Video 3.1). This observation aligns with the ability of dynamic cell-surface interactions on mobile SLBs supporting cell adhesion, spreading and migration.

Figure 3.5. Representative trajectories for individual cells for 150 min on DOPC and DPPC SLBs functionalized with 1 mol% biotin DOPE and presenting RGD peptide. Trajectories were calculated after 30 min of adhesion.

Notwithstanding that a more dynamic surface enhances initial interactions, force generation at the focal contacts when cells spread is also important and may be hindered due to mobile ligands in DOPC SLBs.^[26] While on our surfaces cell spreading was strongly dependent on ligand density (Figure 3.3A-C and G-H), intriguingly, cells on RGD presenting SLBs mainly had peripheral vinculin localization with qualitatively smaller FAs in polarized cells in contrast to cells on serum coated glass substrates (Figure 3.1C-D and Figure 3.2C-D). This indicates a distinct level of force generation and FA maturation occurred on our ligand functionalized SLBs. Besides, the quantified cell area and cell shape factors on serum coated glass after 17h show a higher cell spreading on serum coated glass in comparison to functional SLBs, yet on both types of surfaces cell adhesion is clearly regulated but to a different extent (**Figure 3.6**).

Figure 3.6. (A) Quantification of cell area, (B) elongation (eccentricity) and (C) circularity (form factor) of hMSCs on serum coated glass ($n > 200$, mean $\pm$ standard deviation, red line represents the mean value). Cell area was calculated as $2,787 \pm 1241 \mu\text{m}^2$, eccentricity as 0.83 ± 0.16 and form factor as 0.33 ± 0.17 .

3.2.2. Guiding hMSC differentiation on SLBs

3.2.2.1. Osteogenic differentiation capacity

Considering that we could control hMSC adhesion and spreading and focal adhesion formation on ligand functionalized SLBs, we next evaluated whether these initial molecular and cellular events guide hMSC fate towards defined lineages.^[4-5] To this end, longer term (i.e., days) cell behavior on SLBs was studied by investigating the osteogenic differentiation (bone forming) capacity of the cells. Alkaline phosphatase (ALP) activity (normalized to protein content) as a marker of the osteogenic differentiation capacity of cells on RGD functionalized DOPC SLBs showed a similar trend as seen in cell spreading where cells on 1 mol% biotin DOPE conjugated SLBs had 1.7 fold higher activity than the cells on 0.01 mol% biotin DOPE conjugated SLBs (**Figure 3.7A**). Interestingly, no trend

related to ligand density and osteogenic differentiation was observed on DPPC SLBs, where a significant regulation of cell spreading was also absent (Figure 3.3G, H and Figure 3.7B).

Figure 3.7. Fold induction in ALP activity normalized to total protein content on (A) DOPC and (B) DPPC SLBs with 0.01, 0.5 and 1 mol% biotin DOPE (for both type of SLBs, values are relative to 0.01 mol% biotin DOPE conjugation level). (C) ALP activity in cells on functional DOPC and DPPC SLBs as a function of intermolecular distance (mean $\pm$ standard deviation, $t = 10$ days).

It is important to note that on bare DOPC SLBs the signal related to ALP activity was due to a low population of cells that initially adhered through low level of non-specific interactions (Figure 3.8G and Figure 3.9A). In fact, monitoring the cells during differentiation revealed that after 3 days, the cell population on the ligand functionalized DOPC SLBs (with varying amounts of ligands of 0.01, 0.5 and 1.0 mol% biotin DOPE conjugations) was higher than on the bare SLBs (Figure 3.8A-C and G). Moreover, cells were observed in clusters on ligand functionalized DPPC SLBs while the bare DPPC SLB was apparently more cell resistant than bare DOPC SLBs at this time scale (Figure 3.8D-F and H). This also indicates that in a time scale of 3 days we still observed different extents of cell populations on the surfaces. Therefore, we concluded that by controlling the

initial cell adhesion, differentiation was guided on DOPC SLBs by varying the ligand density.

Figure 3.8. Overview of cell adhesion on SLBs at day 3 for mineralization condition. (A-C) Overview of cell adhesion on RGD presenting DOPC SLBs conjugated with 0.01, 0.5 and 1.0 mol% biotin DOPE, respectively. (D-F) Overview of cell adhesion on RGD presenting DPPC SLBs conjugated with 0.01, 0.5 and 1.0 mol% biotin DOPE, respectively. (G and H) Cell adhesion on bare DOPC and DPPC SLBs, respectively, at mineralization condition. Scale bar is 100 μm for all images.

Figure 3.9. Osteogenic differentiation of cells in control groups. (A) ALP activity normalized to protein content for control groups at day 10. (B and C) Alizarin Red S staining for cells on FBS coated glass at day 14 under mineralization (min) and basic condition (basic), respectively, (scale bar is 40 μm).

Furthermore, we compared the osteogenic differentiation of cells on DOPC and DPPC SLBs while investigating the dependency of ALP activity on ligand density expressed as the ligand intermolecular distance (Figure 3.7C). For this comparison, we evaluated the ALP activity on both types of SLBs, including few more ligand densities on DOPC SLBs. On DOPC SLBs, a relation between the ALP activity and the ligand separation was observed to be biphasic pointing out that a threshold value exists above which it seems possible to induce a specific cell behavior. Interestingly, at all ligand separations, cells on DOPC SLBs had a higher normalized ALP activity than on DPPC SLBs. It is known that multiple factors including cell-cell interactions through cell-cell contacts and paracrine effects of cell-secreted soluble factors and cell-ECM interactions through regulation of cell spreading and cell shape influence MSC differentiation.^[27] Studies show that even though immediate contact of neighboring cells is necessary to induce osteogenic differentiation of MSCs, ^[27b] very high seeding densities (as high as 25,000 cells/cm²) inhibit osteogenesis, possibly by blocking effective cell spreading whereas at low densities (as low as 1,000 cells/cm² to 3,000 cells/cm²) cell spreading and osteogenesis were supported.^[27c] On both type of RGD-functionalized SLBs and at the ligand densities used in our study, the cell densities (total number of cells per surface area) after 17 h (Figure 3.1E and Table 3.1) were in agreement with the cell density regime where cells can effectively sense matrix related cues as reported in literature.^[27a, 27c] Importantly, these initial cell numbers gave room for both cell-cell contacts and for efficient spreading given the proper conditions on functional SLBs (i.e., on RGD-functionalized DOPC SLBs). Interestingly, when DOPC SLBs with 0.01 mol% ligand density (DOPC-0.01) were

compared with DPPC SLBs with 1 mol% ligand density (DPPC-1), we see that the total number of cells per surface area is $1,108 \text{ cells/cm}^2$ and $1,110 \text{ cells/cm}^2$, respectively. Despite having a similar level of cell adhesion density after 17 h, these two surfaces appeared to regulate cell spreading and cell shape significantly differently with DOPC-0.01 having a cell spreading area of $687 \pm 400 \mu\text{m}^2$ and an elongation factor (eccentricity) of 0.71 ± 0.18 in contrast with DPPC-1 having a cell spreading area of $559 \pm 288 \mu\text{m}^2$ and eccentricity of 0.67 ± 0.17 . Accordingly, the ALP activity was observed to be higher on DOPC-0.01 surfaces when compared with DPPC-1 surfaces. This result allows to relate increased cell spreading to increased cell differentiation on SLBs.^[5a] Furthermore, our observations on DOPC SLBs are in line with previous reports where osteogenic differentiation of MSCs was enhanced in response to a decrease in lateral spacing of ligands.^[5a] Even though on our surfaces we did not evaluate focal adhesion formation at different ligand separations, the osteogenic differentiation of hMSCs on ligand functionalized DOPC SLBs could be linked to significant regulation of cell spreading and morphology. These results are in agreement with previous studies performed on fibronectin patterned polydimethylsiloxane substrates where cell shape was seen to direct hMSC differentiation with efficiently spreading cells going through osteogenic differentiation.^[27c]

3.2.2.2. Calcium deposition by cells

After establishing that ALP activity of hMSCs was correlated to the extent of ligand separation and could be linked to cell spreading on RGD functionalized DOPC SLBs, in addition we evaluated early mineralization events by staining for calcium deposition at day 14. Even though at this time point cells may not lead to an extensive mineralization, distinguishing the early stage cell behavior in this case is possible.^[28] Indeed, cells were observed to be at the onset of mineralization when comparing with cells on FBS coated glass as a reference (Figure 3.9B and C). Clusters of calcium deposits (i.e., mineralizing cells) were observed starting from 0.05 mol% ligand density on DOPC SLBs, while such deposits were negligible for cells on the lowest (0.01 mol%) ligand density DOPC SLBs (Figure 3.10).

Figure 3.10. Alizarin Red S staining at day 14 of calcium deposits to qualitatively determine the mineralization for cells cultured on (A) 0.01, (B) 0.05, (C) 0.1, (D) 0.5 and (E) 1 mol% biotin DOPE conjugated RGD presenting DOPC SLBs under mineralization conditions (black arrows indicate mineralization spots by the cells, scale bar: 40 μm).

Semi-quantification of mineralizing clusters by calculating their areas showed that SLBs conjugated with higher ligand densities resulted in larger clusters (Figure 3.10 and **Figure 3.11**). Furthermore, on DOPC SLBs with 1 mol% ligand density, a more homogenous distribution in cluster size was observed when compared to other ligand densities (Figure 3.11). These results suggest that hMSCs maintained their functional properties in terms of matrix deposition and mineralization on RGD functionalized DOPC SLBs, and in time they could develop into mature osteoblasts that seems to be guided by ligand density on these SLBs.

Figure 3.11. Semi-quantification of alizarin red staining as mean area of mineralizing clusters on functional DOPC SLBs (mean $\pm$ standard deviation, $t = 14$ days).

3.3. Conclusions

Our observations indicate that cell adhesion and morphology were regulated specifically and significantly by the ligand density and ligand mobility. Furthermore, the osteogenic differentiation capacity was related to the ligand density and could be linked to cell spreading on DOPC SLBs. This study shows that SLBs can act as suitable biomimetic interfaces to instruct stem cell behavior in the short and long term through engineering ligand presentation. Cellular responses that depend on changes in ligand mobility by way of changing the anchoring environment is a relatively unexplored phenomenon and studies are undertaken to further the molecular understanding of the effect of ligand conjugation (e.g., biotinylated vs. lipidated peptides), ligand mobility, flexibility and affinity on integrin signaling. These insights will aid the generation of smart biomimetic interfaces equipped with sufficient complexity to instruct cell and tissue function taking the advantage of ease of fabrication and presentation of biomolecules with nanometer precision.^[2d, 29]

3.4. Experimental Section

3.4.1. Peptide synthesis and purification

GGSGRGDSG and GGSGRGESG peptides were synthesized using an automatic solid phase synthesis robot (MultiSyntech, Syro II) following standard Fmoc chemistry on Rink amide resin (Multisyntech Chemicals GmbH). After the synthesis and final Fmoc deprotection step, the resin was washed with dichloromethane (DCM) and methanol and dried under vacuum. In order to conjugate biotin to the N-terminus of the peptides, a solution of *N*-hydroxysuccinimidobiotin (NHS-Biotin, ABCR, Germany) (5 molar equivalents with respect to the resin) and 1.1 molar equivalents of *N,N*-di-isopropylethylamine (DIPEA) in dimethylformamide (DMF) were added to the reaction vessels containing the peptides on the resin. The reaction was carried out for 4h at room temperature after which the resin was washed with DMF, DCM and methanol and dried under vacuum. The peptides were cleaved from the resin using a solution of trifluoroacetic acid (TFA):triisopropylsilane (TIS): H₂O (95:2.5:2.5 (v/v%)) using a microwave (40 Watts, CEM Discover Peptide Synthesizer) for 30 min and subsequently precipitated in cold diethyl ether. The crude peptide was purified using RP-HPLC (Reverse Phase High Pressure Liquid Chromatography, C18 Column, Waters, using water/acetonitrile gradient with 0.1% TFA). The purified peptides were analyzed by analytical HPLC and electrospray ionization mass spectrometry (**Figure S1 and S2**).

Figure S1. (A) Analytical HPLC trace (at $\lambda = 220$ nm) and (B) ESI-ToF spectrum of purified biotinylated GGSGRGDSG (calc. $[M] = 973.40$, found: $[M+2H^+] = 487.93$ m/z, $[M+H^+] = 974.82$ m/z).

Figure S2. (A) Analytical HPLC trace (at $\lambda = 220$ nm) and (B) ESI-ToF spectrum of purified biotinylated GGSGRGESG (calc. $[M] = 987.42$, found: $[M+2H^+] = 494.89$ m/z, $[M+H^+] = 988.85$ m/z).

3.4.2. Surface preparation and functionalization

Supported lipid bilayers (SLBs) were prepared following protocols in literature.^[19] SLB formation was achieved using large unilamellar vesicles (LUVs) onto hydrophilic glass supports. LUVs of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, $T_m = -20^\circ\text{C}$, Avanti Polar Lipids) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, $T_m = 41^\circ\text{C}$, Avanti Polar Lipids) with or without biotinylated 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (Biotin DOPE, Avanti Polar Lipids) were formed by extrusion of a

lipid suspension (multilamellar vesicles, MLVs) in MilliQ water (MQ, Millipore, 18.2 mΩ) through 100 nm polycarbonate (PC) membranes (Whatman) at least 11 times, above their melting temperature. After adjusting the desired molar ratios of the lipid components, the solvent was evaporated under a flow of N₂ and vials were exposed to vacuum in a desiccator for at least 1h. Then, the lipid cake was resuspended in MQ water in order to form a multilamellar vesicle (MLV) suspension of 1 mg/mL and this solution was left for hydration for at least 30 min above the melting temperature of the lipids after which extrusion was performed. Formation of vesicles was verified with dynamic light scattering (DLS, Microtrac, typical size: 120 ± 39 nm with polydispersity index of 0.54 for DOPC and 86 ± 41 nm with polydispersity index of 0.55 for DPPC vesicles). Vesicles were sterilized by filtering through 0.2 μm membranes and SLB formation was performed under sterile conditions and above the melting temperature of the lipids. In order to form SLBs on glass supports, glass wells of 96-well plates (Sensoplate, Greiner Bio-one) were treated with 1 M NaOH for 1h followed by removal of solution and washing 10X with sterile MQ water to clean the surfaces. After surface cleaning, 50 μL of 1X Dulbecco's modified phosphate buffered saline (DPBS, without CaCl₂ and MgCl₂) was added to each well followed by addition of 50 μL vesicle suspension (to achieve a final concentration of 0.5 mg/mL) for each composition and left to incubate for 1h. After formation of SLBs, excess vesicles were washed away with DPBS following serial dilution (13X) in order to avoid damage to the SLBs. Confocal imaging and fluorescence recovery after photobleaching (FRAP) experiments on SLBs confirmed homogenous formation of DOPC and DPPC SLBs and the fluidity of DOPC SLBs with mobile molecules in contrast to immobile molecules in DPPC SLBs (**Figure S3**). For these experiments, Texas Red conjugated 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (TR-DHPE, Molecular Probes, Thermo Fisher Scientific) lipid was introduced in DOPC and DPPC vesicles at 0.2 mol%. SLBs of these vesicles were visualized using confocal microscopy (Nikon Confocal A1 Microscope). FRAP experiments were performed using a 20X objective with corresponding stimulation settings. The mobile fraction and diffusion coefficient were derived from the FRAP data using Image J (NIH) and FRAPAnalyser (University of Luxembourg) with Nelder-Mead Simplex fitting method (diffusion circular spot).

Figure S3. SLB characterization. (A and B) Confocal images of DOPC SLBs conjugated with Texas Red (TR)-DHPE lipid (0.2 mol %) (Scale bar: 100 μm). (C) A representative recovery curve of FRAP analysis for DOPC SLBs ($t=0$ s is given as the bleach moment, bleach spot=10 μm , D : diffusion coefficient, $n=3$, fitting was performed following Nelder Mead-Simplex method with circular diffusion spot). (D and E) Confocal images of DPPC SLBs conjugated with TR-DHPE lipid (0.2 mol %) before and 300 s after stimulation showing the bleach spot (scale bar: 100 μm , bleach spot: 15 μm). (F) A representative recovery curve for DPPC SLBs (inset is zoomed image of the bleach spot on DPPC SLBs after 300 s, scale bar: 50 μm).

In order to form a link between biotin DOPE and the biotinylated peptides, SLBs were incubated with 1 $\mu\text{g}/\text{mL}$ neutravidin (Neutravidin, biotin binding protein, Molecular Probes, Thermo Fisher Scientific) solution in DPBS for 45 min (100 $\mu\text{L}/\text{well}$); excess neutravidin was washed away with serial dilutions in DPBS for 10X. Finally, peptide solutions (0.5 $\mu\text{g}/\text{mL}$ final concentration) in DPBS were added to the wells (100 $\mu\text{L}/\text{well}$) and incubated for 45 min after which the excess amount was washed away with serial dilutions in DPBS (10X). Bare SLBs of DOPC and DPPC were prepared in the same way and used as negative control surfaces. In order to correlate the degree of surface functionalization (mol% of biotin DOPE) to the spacing of presented ligands (in particular biotin GGSGRGDSG) intermolecular spacing was calculated according to literature^[10h] as given in Equation 1 and as reported in **Table S1**. Here, d is the intermolecular distance,

X_{mol} is the molar ratio of biotin DOPE while A_{lipid} corresponds to the area of DOPC and DPPC (gel state) lipids, 0.725 nm² and 0.479 nm², respectively.^[16] Therefore, we could approximate the intermolecular spacing based on the lipid head group spacing, which is dependent on the lipid composition at the same functionalization level.

$$d = \sqrt{\frac{A_{lipid}}{X_{mol}}} \quad (\text{Equation 1})$$

Table S1. Intermolecular distance on DOPC and DPPC SLBs with varying molar ratios of biotin DOPE.

Molar ratio of biotin DOPE [%]	Intermolecular distance DOPC SLBs [nm]	Intermolecular distance DPPC SLBs [nm]
0.01	85	69
0.05	38	31
0.1	27	22
0.5	12	10
1.0	9	7

3.4.3. Cell culture on SLBs

Bone marrow derived human mesenchymal stem cells (hMSCs, Poietics, Lonza) were cultured in basic medium (α MEM (Gibco) containing 10% fetal bovine serum (FBS) (Sigma) and 100 U/mL penicillin and 100 μ g/mL streptomycin (Sigma)) and incubated at 37°C and 5% CO₂ in a humidified environment up to passage 5. Prior to cell seeding, SLBs were washed with basic culture medium 5X with serial dilutions, in order to exchange DPBS with cell culture components. For adhesion and spreading experiments, cells were seeded at a density of 5,000 cells/cm² and incubated on surfaces for 17h (biotinyl DOPE) or 24h (biotinyl-N-cap-DOPE) after which they were fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature. After fixation, cells were washed 3X with PBS and left in 0.05% sodium azide in PBS (Sigma) and kept at 4 °C for immunofluorescence

analysis. For these experiments glass surfaces that were coated with FBS prior to cell seeding were used as control surfaces to monitor cell adhesion and morphology over time next to the bare SLB controls. When cells were cultured up to 24h on RGD presenting DOPC SLBs using 1 mol% biotinyl-N-cap-DOPE instead of biotinyl DOPE, a similar level of vinculin localization at the periphery of the cells was noted, indicating that cell adhesion on biotinyl-N-cap-DOPE and biotinyl DOPE are similar (Figure 3.1C-D). Therefore, cell differentiation experiments were only done using biotinyl-N-cap-DOPE. Live cell imaging experiments were performed using Cytomate® live cell imaging equipment (Eindhoven, The Netherlands). For this experiments, DOPC and DPPC SLBs conjugated with 1 mol% biotin DOPE were prepared and cells were seeded again at a density of 5,000 cells/cm². Time lapse images were recorded every 7.5 min and analyzed with Image J (NIH) to prepare the videos. Representative cell trajectories were measured after 30 min of adhesion, for 150 min in order to depict the early phase of interactions (first 3h) on the surfaces.

In order to study the osteogenic differentiation capacity of hMSCs in response to ligand density and SLB lipid composition, hMSCs were seeded at a density of 10,000 cells/cm² and left for adhesion in basic medium for 4h. At the end of 4h, SLBs were gently washed 2X with basic medium to remove non-adherent cells (leaving 100 µL in the wells), and 100 µL of 2X mineralization (osteogenic) medium was added to the corresponding wells in order to have a 1X final concentration of the differentiation components (100 nM dexamethasone (Sigma), 10 mM β-glycerophosphate (Sigma) and 0.2 mM ascorbic acid (Sigma)). Culture medium was exchanged every 2-3 days after gentle washing with basic medium. Next to the differentiation/mineralization conditions, for each SLB composition, cells in basic medium supplemented with 10 mM β-glycerophosphate and 0.2 mM ascorbic acid were used as controls for differentiation. Bare SLBs and FBS coated glass surfaces were used as additional control groups for differentiation. During the time of the differentiation experiments, cell morphology was monitored and imaged with Olympus CKX41 inverted microscope.

3.4.4. Immunofluorescence

In order to characterize cell adhesion and spreading on SLBs, actin cytoskeleton and vinculin (as a marker of focal adhesions) staining were performed. Briefly, PFA fixed cells were permeabilized in 0.5% Triton X-100 in PBS (Sigma) for 10 min at room temperature. After permeabilization, cells were incubated with 0.1% Triton X-100 and 5% (w/v) bovine serum albumin (BSA, Sigma) solution in PBS for at least 20 min to block nonspecific sites.

Afterwards, cells were incubated with a solution of AlexaFluor-568 phalloidin (Molecular Probes, Thermo Fisher Scientific, 1:100) and monoclonal anti-human vinculin-FITC antibody (anti-hVIN-1, Sigma Aldrich, 1:200) in 0.1 % Triton X-100 and 5% (w/v) BSA in PBS for 1h at room temperature in the dark. After washing 3X with PBS to remove excess molecules, cells were incubated with DAPI nuclear stain in PBS (1:1,000) for 10 min and washed 2X with PBS. Imaging was performed with an Olympus IX71 inverted microscope and OPERA high content screening system (Perkin Elmer) with corresponding objectives and filter combinations.

3.4.5. Studying osteogenic differentiation on SLBs

In order to study osteogenic differentiation capacity of cells on different surfaces, at day 10, cells were lysed with 1X lysis buffer in MQ (Reporter Lysis buffer, Promega) and stored at -80°C before analysis. Alkaline phosphatase (ALP) activity as a marker of osteogenic differentiation was quantified by incubating 10 µL of cell lysate with 40 µL CDP-Star substrate (Roche) in dark for 30 min and detecting chemiluminescence (Viktor Plate Reader, Perkin Elmer). In order to normalize the ALP activity, total protein content of the lysate was determined using the BCA (bicinchoninic acid) protein assay according to the manufacturer's instructions (Pierce BCA Protein Assay Kit, Thermo Scientific) measuring absorbance values at 562 nm (BioTek Instruments).

Furthermore, in order to detect calcium deposition (mineralization) qualitatively, at day 14, cells were fixed with 4% PFA, rinsed 3X with PBS and stained with Alizarin Red S (0.6% (w/v) in MQ water, pH 4.1-4.3), rinsed 3X and left in MQ water. Wells were imaged with Olympus IX71 inverted microscope with a color camera using Cell-D program (Olympus), using the same exposure parameters during imaging. In order to have an indication of extent of mineralization, area of calcium deposited clusters based on Alizarin Red S staining on functional DOPC SLBs were measured with Image J (NIH).

3.4.6. Data analysis

All experiments were performed in triplicates and data are presented as mean $\pm$ standard deviation. Data were analyzed by using one-way ANOVA followed by Bonferroni's multiple comparison test comparing all groups of interest (OriginPro v.9, OriginLab). Image analysis was performed using Image J (NIH) and Cell Profiler image analysis software (Broad Institute of MIT and Harvard). Quantification of cell shape factors were

performed by using Cell Profiler algorithms. For these analyses, more than 150 cells were taken into account for each condition.

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3.7. Videos

Live cell imaging videos of hMSCs cultured on different SLB surfaces which were used for Figure 3.5 could be downloaded from (note that in the online version, the videos are listed after the first supporting PDF file):

<http://onlinelibrary.wiley.com/doi/10.1002/adhm.201600862/supinfo>

Video 3.1. hMSC on DOPC SLBs functionalized with 1 mol% Biotin-RGD (Biotin-DOPE) (17h)
(Online: Video S1)

Video 3.2. hMSC on DOPC SLBs functionalized with 1 mol% Biotin-RGD (Biotin-DOPE) (3h)
(Online: Video S2)

Video 3.3. hMSC on DPPC SLBs functionalized with 1 mol% Biotin-RGD (Biotin-DOPE) (3h)
(Online: Video S3)

Chapter 4

Controlling hMSC adhesion and spreading by tuning the binding of lipid-modified peptides in supported lipid bilayers

Fluid DOPC SLBs were reversibly (i.e., with out-of-plane dynamicity) functionalized with integrin targeting lipid-modified RGD ligands via lipid insertion. By varying the chain length of the lipid chains (i.e., C12:0, C14:0 and C16:0 alkyl chains) that were conjugated to RGD ligands, the affinity (i.e., interaction strength) of the lipid-modified RGD to the SLB was tuned, where increasing the length of the alkyl chain resulted in an increase in affinity (i.e., higher association constant). We show that, on C14:0-RGD presenting SLBs and in the context of hMSC behavior, focal adhesion formation (vinculin localization) and cell spreading are strongly dependent on ligand density (i.e., surface coverage), where an optimum coverage was detected for the extent of cell spreading and vinculin localization. Furthermore, at the same ligand coverage, going from low (C12:0-RGD) to moderate (C14:0-RGD) affinity resulted in observing enhanced focal adhesion formation, cell spreading and elongation, while going from moderate (C14:0-RGD) to high (C16:0-RGD) affinity only changed the organization of focal adhesions, induced less cell spreading, but a similar cellular morphology. These results point out the critical importance of the affinity between lipid-modified RGD ligands and SLBs (hence lipid chain length) on cell behavior, while introducing these SLB biointerfaces as a highly tunable and dynamic (both lateral and out-of-plane dynamics) platform to guide hMSC behavior.

4.1. Introduction

Functionalization of surfaces with ligands that interact with cell receptors represents a synthetic substitute of ligands that reside in the extracellular matrix (ECM) and interact with cells. For example, cell-adhesive Arg-Gly-Asp (RGD) peptide ligands that are derived from fibronectin and recognized by transmembrane integrin receptors, have been used to fabricate instructive coatings on biomaterials. When anchoring these RGD ligands on surfaces and let them interact with cells, control over molecular flexibility, binding affinity, ligand density, and ligand mobility are critical to most effectively guide cells to desired responses. Such synthetic biological interfaces that mimic cell-ECM interactions have therefore been instrumental in the development of biomaterials for tissue engineering, regenerative medicine but also as tools for fundamental cell biology studies.^[1] Notwithstanding that enormous progress has been documented over the last decades in terms of integration of instructive properties of the ECM in biological interfaces, integration of the dynamic behavior of ligands, as occurring in their natural environment, on surfaces and sensed by cells, remains a formidable task.

Various methods have been described to include this dynamic behavior in interfaces.^[1] On the one hand, this dynamicity can be achieved in an “on-off” fashion by introducing responsive moieties in the ligand that are responsive towards e.g., light, voltage, pH or enzyme activity. For example, Del Campo and co-workers immobilized an inactive Arg-Gly-Asp (RGD) peptide that became bioactive upon uncaging using light of a suitable wavelength.^[2] Another sophisticated example from Mrksich and co-workers demonstrates that control over ligand detachment as well as attachment was achieved. In this case an RGD ligand was first electrochemically removed from the surface by oxidation of the O-silyl hydroquinone ether bond and subsequently was electrochemically re-attached by a Diels-Alder reaction between the obtained benzoquinone and a diene functionalized RGD peptide.^[3] Stupp reported the use of β -cyclodextrin-functionalized alginate complexed with naphthyl-modified RGD ligands. In this system the cell-adhesive properties were reversed by the addition of a competing, non-adhesive adamantyl-RGE peptide. Interestingly, this example also provides some insight into the effect of surface affinity of the bioactive ligand (K_a values of $1.6 \cdot 10^3 \text{ M}^{-1}$ and $5 \cdot 10^4 \text{ M}^{-1}$, for naphthyl and adamantyl guests, respectively) on cell response.^[4] Another intrinsically dynamic system was designed in our group, in which ternary complexes of CB[8], tryptophan-modified RGD peptides and surface-bound methyl viologen was used. Release of the RGD ligands was electrochemically achieved by reduction of methyl viologen that causes dissociation of the ternary complex.^[5] These

and other synthetic responsive strategies have provided important knowledge in engineering biointerfaces to more closely mimic the natural cellular environment (a more extended view is given in Chapter 1).^[1, 6]

This dynamic behavior can also be achieved by making use of supported lipid bilayers (SLBs), which are often used as artificial mimics of cell membranes. SLBs can be formed using naturally occurring lipids (e.g., 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, DOPC) introducing a highly biomimetic and biocompatible way to engineer biointerfaces.^[7] As the alkyl chain composition of the base lipids defines the lateral mobility of the ligands (i.e., using lipids of high melting vs. low melting temperature resulting in laterally immobile and mobile ligands, respectively), using lipids that contain zwitterionic head groups (e.g., phosphatidylcholine lipids) enables to form non-fouling surfaces that can be functionalized with specific ligands for receptor recognition and subsequent biological events.^[8] SLBs have been widely used as platforms to study membrane properties and biophysical parameters as well as to generate surface gradients;^[9] as interfaces to study receptor-ligand interactions in relation to ligand mobility, which was seen to support receptor clustering and signaling;^[10] as cell surface models to study cadherin mediated cell-cell interactions^[11] where also a certain extent of mobility was shown to be critical for proper junction formation;^[11a] to pattern surfaces, to introduce different domains of ligands^[8, 12] and to form responsive surfaces for reversible cell binding.^[13] Next to these studies, the use of SLBs has been reported for investigations of understanding cell adhesion,^[14] cell spreading and focal adhesion signaling in response to ligand density and mobility. Recently, a few studies appeared that were focused on studying stem cell behavior and fate on SLBs pointing out the importance of ligand density and lateral mobility.^[15] Although the achievements of employing SLBs as cell-instructive interfaces is intriguing and vast, it is remarkable that in all of these systems, generally the ligands were presented irreversibly for cell recognition, while the ligands in the form of lipid-modified peptides, which contained two alkyl chains in analogy to phospholipids (e.g., DOPC), were introduced in lipid vesicles that were used to prepare the bilayers.^[14a, 14b, 14d]

Studies on the interaction between lipid cellular membranes and lipid-modified proteins, have shown, for example, that lipid-modified GTPases are loosely inserted into cellular membranes when GTPases are farnesylated, while they become more stably anchored when palmitoylated.^[16] Other examples have described that ssDNA modified with cholesterol, stearyl or di-stearyl groups have different binding kinetics when inserted

into supported lipid bilayers.^[17] As such, in the case of the cholesterol, incorporation of ssDNA occurred fastest, followed by stearyl and di-stearyl anchors.^[17]

In this Chapter, we rationally design and synthesize a focused set of lipid-modified cell-adhesive RGD ligands, in which the lipid chain was varied in length to achieve differences in the affinities of the ligands to SLBs (**Scheme 4.1**). We characterized the binding affinities and coverages of the ligands to the SLBs with quartz-crystal microbalance with dissipation monitoring (QCM-D), which was combined with in situ spectroscopic ellipsometry (SE). These ligand affinities and densities were correlated to human mesenchymal stem cell (hMSC) behavior. By studying focal adhesion signaling, cell spreading and morphology as important regulators of stem cell fate,^[18] we show that on fluid DOPC SLBs, lipid-modified ligand affinity and density critically influenced stem cell behavior. The systematic study of the effects of dynamic ligand attachment on cell response demonstrates that a dynamic cell environment more closely reflects the natural extracellular matrix.

4.2. Results and Discussion

4.2.1. Introducing RGD ligands to SLBs via lipid insertion

As presented in Chapter 1, SLBs can be functionalized in multiple ways, which introduces versatility when they are employed as cell-instructive biointerfaces. In Chapter 3, we used biotin-neutravidin interactions to functionalize SLBs for cell recognition. In this Chapter, SLB functionalization was achieved via lipid insertion, where RGD ligands were conjugated to lipid anchors of different chain lengths (i.e., C12:0, C14:0 and C16:0 alkyl tails, hence resulting in lipid-modified RGD) (**Scheme 4.1**). This enables achieving tunability in ligand presentation to cells, due to differences in lipid-modified RGD-SLB interactions, which is controlled by the length of the alkyl chain conjugated to RGD peptide (see below).

Insertion of lipid-modified RGD peptides (i.e., C12:0-RGD, C14:0-RGD and C16:0-RGD) in fluid DOPC SLBs (**Figure S4**) was verified and characterized by combined quartz crystal microbalance with dissipation monitoring (QCM-D) and spectroscopic ellipsometry (SE) measurements. These measurements are summarized here and a detailed discussion can be found in the thesis of Mark Verheijden (in a collaboration with Prof. Ralf Richter).^[17] Essentially, QCM-D measures a frequency change upon binding of the lipid-modified peptides when a titration series for different concentrations is performed. Spontaneous

partitioning of the lipid chain in SLBs (as depicted in Scheme 4.1) is detected as an increase in mass at the SLB surface, which is observed as a decrease in the frequency (data not shown). Importantly, for all lipid-modified peptides presented in this Chapter, after insertion, washing with buffer restored the starting frequency of the bare SLB, indicating that these peptides were reversibly inserted and that SLB-inserted ligands would be in equilibrium with ligands in solution (Scheme 4.1). This introduces another level of dynamicity (i.e., out-of-plane), next to lateral dynamicity (i.e., in-plane) that is achieved in fluid SLBs.

Scheme 4.1. Schematic representation of possible equilibria between dissolved and inserted lipid-modified RGD peptides, together with the molecular structures of lipid-modified peptides that were studied in this Chapter (green arrow depicts lateral dynamicity, blue arrows depict the equilibrium between SLB-inserted lipid-modified peptides and lipid-modified peptides that are in solution).

However, while in QCM-D measurements hydrodynamically bound water accounts for a considerable part of the observed changes, to more reliably estimate the density of lipid-modified RGD at the surface, the dry mass of the surface bound lipid-modified peptides was determined using spectroscopic ellipsometry (SE).^[17] Using this technique the layer thickness or dry mass on the surface can be converted to a surface coverage (i.e., pmol/cm², Figure 4.1). To this end, a titration series for C12:0-RGD, C14:0-RGD and C16:0-RGD was performed using a QCM-D/SE set up and obtained peptide coverages (molecular density, as expressed in pmol/cm²), at defined concentrations are given in Figure 4.1A. Furthermore, surface coverages (%) were calculated from given molecular

densities, where 100% was defined as one lipid-modified peptide per phospholipid in the upper leaflet of the SLB (as calculated by taking DOPC lipid area into account, which is 0.725 nm^2 ^[19]) (Figure 4.1B).

Figure 4.1. Surface coverages of lipid-modified peptides in SLBs (C12:0-RGD, C14:0-RGD and C16:0-RGD) at different concentrations given for each peptide. Coverages as expressed in (A) pmol/cm^2 and (B) percent coverage (100% is defined as one lipid-modified peptide per phospholipid in the upper leaflet of the SLB). Values were derived from SE measurements which were performed in duplicates.

Next to surface coverages, from these titrations in the combined QCM-D/SE set up, association constants (K_a) for lipid-modified peptides upon binding to SLBs were derived (based on the shifts in equilibrium frequency) (Figure 4.1). It was observed that the binding affinities (i.e., K_a) of lipid-modified RGD ligands were strongly dependent on lipid chain length, and varied by 2 orders of magnitude ranging between 10^3 and 10^5 when going from C12:0 to C16:0 lipid chains. These results clearly show that the length of the lipid chain is a suitable design variable to tune the affinity of cell-adhesive RGD peptides to the SLB,^[16] which introduces important implications for guiding cell behavior.^[4]

4.2.2. Exploring the effects of lipid-modified peptide (RGD)-SLB interactions on hMSC behavior

Having shown that SLBs can be reversibly functionalized with lipid-modified RGD ligands of different binding affinities to bilayers, we investigated how these differences influence cell behavior by studying human mesenchymal stem cell (hMSC) adhesion and spreading as critical regulators of (stem) cell fate,^[18] to explore these platforms (interfaces) as dynamic biomimetic stem cell microenvironments.

4.2.2.1. Lipid-modified peptide density dependent hMSC adhesion and spreading

Upon specific integrin-RGD ligand engagement and signal activation at the cell-surface interface, focal adhesions, which are known to regulate cell adhesion and migration and marked with the localization of key regulator proteins such as vinculin, form and mature under appropriate conditions.^[20] The dynamic nature of these interactions is highly sensitive to surface related parameters including global ligand density, which affects focal adhesion formation through regulation of integrin clustering and signaling.^[1, 20a, 20c, 21] Therefore, to study surface coverage dependent cell responses, DOPC SLBs were functionalized with C14:0-RGD, which showed a moderate affinity of $K_a=8 \times 10^3 \text{ M}^{-1}$ ($K_d=1.5 \times 10^{-4} \text{ M}$) to the SLB. At defined concentrations, different initial surface coverages (18-20, 10-12, 6-8, 3-5 and 0.2-1%, **Figure 4.1** and **Table 4.1**) were obtained. Once equilibrium conditions were achieved for the peptide insertion process (after 2h incubation of the peptides with SLBs with no further washings and therefore leaving the peptide solution in cell culture medium), cells were added to the culture medium to interact with RGD ligands at given surface densities.

Table 4.1. Corresponding concentrations and percent coverages for C14:0-RGD peptide.

C14:0 RGD Concentration (μM)	Coverage range
50	18-20%
28.6	10-12%
14.3	6-8%
5.7	3-5%
1.9	0.2-1%
0	0%

hMSC adhesion was studied by investigating vinculin localization at focal adhesions after 18h, which was suitable to reveal single cell response and to study cell-surface interactions on SLBs as explained in Chapter 3.^[15b] Observations revealed that on bare (non-functional) SLBs, vinculin was cytoplasmic, while on RGD presenting (functional) SLBs, vinculin localized at the focal adhesions, in cell periphery (**Figure 4.2A-C** and **Figure 4.3**). Furthermore, on bare SLBs a lower extent of cell adhesion and an impaired cell shape compared to functional SLBs were seen (**Table 4.2**, **Figure 4.2A-C** and **Figure 4.4**).

Figure 4.2. hMSC adhesion and vinculin localization in cells on control surfaces. (A-C) On bare DOPC SLBs and (D-F) on serum coated glass substrates. (G) Quantification of cell area on control surfaces. ($t = 18\text{h}$, blue: nucleus, red: actin, green: vinculin, scale bars: $40\text{ }\mu\text{m}$, red line represents the mean value, $n \geq 400$, each data point represents one cell, mean $\pm$ standard deviation).

Figure 4.3. Lipid-modified peptide coverage dependent focal adhesion formation in hMSCs. Vinculin localization and corresponding profile plots for given focal adhesions (yellow line) in cells on DOPC SLBs functionalized with C14:0-RGD at (A) 18-20%, (B) 10-12%, (C) 6-8%, (D) 3-5%, (E) 0.2-1% coverages after 18h. (Scale bar: 20 μ m, blue: nucleus, red: actin and green: vinculin, yellow line represents the one dimensional region of interest for profile plotting in Image J).

These results confirm the non-fouling properties of non-functional SLBs and specificity of integrin-RGD interactions on functional SLBs. On serum coated glass substrates, typical spread morphology of hMSCs with well-defined focal adhesions (vinculin localization) was observed (Figure 4.2D-F).

It is important to note that in our system, as mentioned above, to achieve certain coverages, lipid-modified (lipidated) peptides were incubated at defined concentrations and kept in culture medium in the presence of cells, due to the dynamic nature of interactions between lipidated peptides and SLBs. Previous studies have shown that RGD ligands in solution can, at concentrations as high as 100 μM , compete with RGD ligands on the surface to decrease cell binding while concentrations between 250-500 μM were shown to inhibit cell binding.^[22] However, in these systems the competing RGD has generally no interaction with the surface (therefore freely available in solution at all times),^[22-23] which is in contrast to our system where peptides spontaneously insert to SLBs and therefore a different mode of interactions could be expected. On our surfaces, for C14:0-RGD functionalization, the concentration to achieve coverages of 18-20% is 50 μM (Table 4.1), which is lower than the reported values to prevent effective cell binding. Importantly, we did not observe any trend of C14:0-RGD concentration on cell density (total number of cells per surface area), which was comparable for 18-20% (50 μM) and 3-5% (5.7 μM) as 3,210 and 3,295 cells/ cm^2 , respectively and higher than bare SLBs (1,976 cells/ cm^2 , Table 4.2). Therefore, in this system, we are able to compare the effects of lipid-modified peptide surface coverage on cell behavior.

Figure 4.4. Total number of cells per surface area for each condition on DOPC SLBs functionalized with C14:0-RGD at different coverages in comparison to bare (non-functional) SLBs after 18h.

Table 4.2. Total number of cells per surface area per condition on C14:0-RGD functionalized DOPC SLBs (t=18h).

	Total number of cells per cm ²
Bare DOPC 0%	1,976
C14:0-RGD 18-20%	3,210
C14:0-RGD 10-12%	2,823
C14:0-RGD 6-8%	3,446
C14:0-RGD 3-5%	3,295
C14:0-RGD 0.2-1%	2,650

Interestingly, even though at all coverages for C14:0-RGD functionalized SLBs, peripheral vinculin localization was present (as a result of specific RGD-integrin engagement), a relatively more distinct and concentrated vinculin localization, hence a more mature focal adhesion formation with associated actin filaments^[24] was seen only at 10-12% coverage, which was supported with one dimensional profile plots of representative focal adhesions (Figure 4.3 and **Figure 4.5**). Decreasing the coverage to 6-8% resulted in generally (qualitatively) smaller, but still well-distinguishable focal adhesions at protruding lamellipodial extensions, a very similar behavior to what was seen at 3-5% coverage (Figure 4.3). On the other hand, at the lowest coverage (0.2-1%) generally a less defined (more diffuse) vinculin localization was present, again at the lamellipodial periphery, which is indicative of the leading edge of migrating cells^[20a] (Figure 4.3 and Figure 4.5). Intriguingly, increasing the coverage to 18-20% did not result in a higher focal adhesion maturation (especially in comparison to 6-8% and 3-5% coverage), which was revealed by vinculin localization and the associated plots (Figure 4.3 and Figure 4.5), pointing out an optimum coverage for C14:0-RGD to control focal adhesion maturation.

[24]

Figure 4.5. Cumulative vinculin profile plots for four representative focal adhesions in hMSCs on DOPC SLBs functionalized with C14:0-RGD at different coverages after 18h (FA: focal adhesion). For analysis at least 20 focal adhesions were taken into account, four of which are shown.

Regulation of cell spreading and morphology, as critical dynamic cellular processes, are very closely related to focal adhesion signaling and maturation,^[20a] and therefore are also affected by ligand density (and separation), as was shown before for hMSCs on covalently modified surfaces as well as RGD presenting SLBs that were functionalized using biotin-avidin interactions.^[15b, 18a] Therefore, using the Cell Profiler software, we quantified cell spreading and associated cell morphological parameters next to quantifying the cell density on the surfaces, to reveal the effects of surface coverage of C14:0-RGD ligands after 18h (**Figure 4.6** and **Figure 4.7**).^[25] In line with the observations on focal adhesion formation, quantification of cell area revealed that at 10-12% surface coverage, cells had a significantly higher spreading area ($1,934 \pm 1,036 \mu\text{m}^2$, **Figure 4.6B**, **Figure 4.7A** and **Table 4.3**) in comparison to all other coverages.

Figure 4.6. Overview of cell morphology on DOPC SLBs functionalized with C14:0-RGD at (A) 18-20%, (B) 10-12%, (C) 6-8%, (D) 3-5% and (E) 0.2-1% after 18h (Scale bar: 40 μm , blue: nucleus, red: actin).

Remarkably, at the highest coverage (18-20%) cell area was the lowest and comparable to the cell area that was seen at 0.2-1% coverage ($1,529 \pm 859$ vs. $1,599 \pm 841$ μm^2 , respectively, Figure 4.7A and Table 4.3). Furthermore, average cell spreading areas at 6-8% and 3-5% were also comparable with each other with no significant differences ($1,742 \pm 1,098$ vs. $1,723 \pm 1,019$ μm^2 , respectively, Figure 4.7A and Table 4.3) indicating a bimodal behavior in the regulation of cell area on C14:0-RGD presenting DOPC SLBs, which was closely associated with the results on vinculin localization (Figure 4.3 and Figure 4.7A). These observations are in agreement with literature where an optimum ligand density was found on self-assembled monolayers presenting covalently conjugated RGD to induce more extended endothelial spreading and focal adhesion maturation (or longer focal adhesion formation).^[26] Importantly, cell area on bare SLBs was always lower than for cells on functional SLBs ($1,136 \pm 627$ μm^2) (Figure 4.7A and Table 4.3).

Figure 4.7. Regulation of (A) cell area, (B) extent (approaches 1 for compact cells with less protrusions), (C) eccentricity (approaches 1 for a highly elongated cell) and (D) form factor (approaches 1 for a highly circular cell) of hMSCs on DOPC SLBs functionalized with C14:0-RGD at different coverages after 18h (red line represents the mean value, $n \geq 400$, each data point represents one cell, mean $\pm$ standard deviation, **: $p < 0.01$).

Table 4.3. Quantification of cell area on DOPC SLBs functionalized with C14:0-RGD (t=18h).

	Cell Area (μm^2)
Bare DOPC 0%	1,136 $\pm$ 627
C14:0-RGD 18-20%	1,529 $\pm$ 859
C14:0-RGD 10-12%	1,934 $\pm$ 1,036
C14:0-RGD 6-8%	1,742 $\pm$ 1,098
C14:0-RGD 3-5%	1,723 $\pm$ 1,019
C14:0-RGD 0.2-1%	1,599 $\pm$ 841

Furthermore, at 18-20% coverage, cells had a significantly lower degree of protrusion (represented by extent, approaches 1 for highly compact cells with less protrusions, 0.48 ± 0.16), lower degree of elongation (eccentricity, approaches 1 for highly elongated cells, 0.81 ± 0.16) as well as a higher degree of circularity (form factor, approaches 1 for highly circular cells, 0.43 ± 0.20) compared to all other coverages (Figure 4.6, Figure 4.7B-D, **Table 4.4**), which was in line with the observations on cell area. On the other hand, looking at the coverages from 10-12% to 0.2-1%, no clear trend in any of the cell shape parameters could be seen in response to surface coverage (Figure 4.6, Figure 4.7B-D, Table 4.4). However, we could see that at 10-12% coverage, where cells had a more distinct vinculin localization and had the highest degree of spreading, they were in the group of cells (together with cells at 3-5% and 0.2-1% coverage) with higher degree of protrusions (extent, 0.43 ± 0.16), that were more elongated (eccentricity, 0.83 ± 0.16) and were less circular (form factor, 0.37 ± 0.19) (Figure 4.6, Figure 4.7 and Table 4.4).

Table 4.4. Quantification of cell shape factors on DOPC SLBs functionalized with C14:0-RGD (t=18h).

	Extent	Eccentricity	Form Factor
C14:0-RGD 18-20%	0.48±0.16	0.81±0.16	0.43±0.20
C14:0-RGD 10-12%	0.43±0.16	0.83±0.16	0.37±0.19
C14:0-RGD 6-8%	0.44±0.16	0.82±0.15	0.39±0.21
C14:0-RGD 3-5%	0.41±0.15	0.84±0.15	0.35±0.18
C14:0-RGD 0.2-1%	0.43±0.15	0.83±0.15	0.37±0.18

Taken together, the results on focal adhesion formation (vinculin localization) and quantification of cell area indicate that an optimum surface density exists for C14:0-RGD to induce effective cell adhesion and spreading, which is in agreement with previous observations on covalent RGD presenting surfaces.^[27] These observations can be related to possible surface crowding of the peptides at very high coverages, which may adversely affect cell adhesion due to steric hindrance. Furthermore, a surface that reversibly presents ligands with lateral mobility, may result in a lower availability of ligands to cells. This may need relatively higher ligand densities to induce effective adhesion and spreading.^[14b, 15b, 26] Moreover, quantification of morphological parameters also demonstrated the effects of surface coverage of C14:0-RGD on cell shape as an important regulator of stem cell fate.^[18b]

4.2.2.2. Lipid-modified peptide affinity dependent cell adhesion and spreading

Knowing that we can specifically control cell adhesion and spreading on reversibly functionalized DOPC SLBs, we next studied the effects of lipidated peptide affinity to SLBs on cell behavior by introducing RGD ligands having different lipid anchors. For this purpose, next to C14:0-RGD ($K_a=8 \times 10^3 \text{ M}^{-1}$ and $K_d=1.5 \times 10^{-4} \text{ M}$), with moderate affinity, DOPC SLBs were functionalized with low affinity binding C12:0-RGD ($K_a=9.3 \times 10^2 \text{ M}^{-1}$ and $K_d=1.1 \times 10^{-3} \text{ M}$) and high affinity binding C16:0-RGD ($K_a=1.2 \times 10^5 \text{ M}^{-1}$ and $K_d=8.7 \times 10^{-6} \text{ M}$) to study focal adhesion signaling as well as cell spreading.

As from a surface point of view, affinity translates into the strength of the binding of the lipid chain to SLBs, from a cellular point of view, it can influence how cells perceive their physical environment at the level of single integrin-RGD bonds given the same ligand density (i.e., firmly vs. loosely interacting with the surface). This physical sensing is an integral part of the regulation of integrin adhesions and it affects focal adhesion maturation where vinculin acts as a scaffolding and signaling protein and transmits bidirectional forces between cells pulling on the ligands and the extracellular environment reacting to this pulling.^[20a, 28] In this respect, the higher the affinity between the peptide and the surface the higher is the binding strength (i.e., peptide is firmly interacting with the surface), and therefore higher resistance to pulling forces, which is known to increase focal adhesion maturation and cell spreading.^[24, 28a]

In order to study the effect of affinity only, we compared lipid-modified peptides at the same coverage (10-12%) that was seen to be optimal for C14:0-RGD functionalized SLBs in terms of focal adhesion formation and cell spreading (Figure 4.3, Figure 4.6 and Figure 4.7). By selecting the same coverage of the different lipid-modified peptides, a similar number of ligands could be presented to the cells. We studied vinculin localization knowing its role as a mechanosensitive protein at focal adhesions.^[24] At a higher affinity (i.e., going from C14:0-RGD to C16:0-RGD at 10-12%) cells again generally had distinct but also qualitatively longer focal adhesions (vinculin localization), which was supported with one dimensional profile plots (**Figure 4.8** and **Figure 4.9**). Remarkably, increasing the peptide affinity did not induce an obvious effect in focal adhesion maturation (i.e., more distinct and localized vinculin), but it apparently varied between a concentrated vs. elongated organization, which was revealed by cumulative profile plots of vinculin localization (Figure 4.5, Figure 4.8 and Figure 4.9). Interestingly, going to a lower affinity at the same coverage (i.e., C14:0-RGD vs. C12:0-RGD at 10-12%) resulted in qualitatively smaller, dot-like, nascent adhesions revealed by vinculin localization and corresponding profile plots at the lamellipodial extensions (Figure 4.8 and **Figure 4.10**).

Figure 4.8. Lipid-modified peptide affinity dependent focal adhesion formation in hMSCs. Vinculin localization and corresponding profile plots for given focal adhesions (yellow line) in cells on DOPC SLBs functionalized with C12:0-RGD at 0.2-1% (low) coverage, C12:0-RGD at 10-12% (high) coverage, C14:0-RGD at 0.2-1% coverage, C14:0-RGD at 10-12% coverage and C16:0-RGD at 10-12% coverage ($t = 18\text{h}$, scale bar: $20 \mu\text{m}$, blue: nucleus, red: actin and green: vinculin, yellow line represents the one dimensional region of interest for profile plotting in Image J).

Figure 4.9. Vinculin localization in hMSCs on DOPC SLBs functionalized with C16:0-RGD at (A) 10-12%, (B) 3-5% and (C) 1-3% coverages and corresponding profile plots for four representative focal adhesions ($t = 18h$, blue: nucleus, red: actin, green: vinculin, scale bars: 20 μm , FA: focal adhesion).

However, as the lipid-modified peptide affinity to the surface determines the effective concentrations to achieve certain coverages (Figure 4.1), for C12:0-RGD a relatively high concentration (210 μM) of the peptide was needed to achieve 10-12% coverage (Table 4.1 and Table 4.5). This free RGD concentration may result in prevention of cell binding and therefore focal adhesion formation as discussed above.^[4, 22-23] In order to further demonstrate the effect of peptide affinity at the surface, we also studied vinculin localization at 0.2-1% coverage (21 μM , in the concentration range that did not inhibit cell binding and spreading^[22-23]) where again dot-like nascent adhesions were seen that were less concentrated with vinculin in comparison to C14:0-RGD functionalized SLBs at the same coverage (Figure 4.8, 0.2-1% coverage for C12:0-RGD and C14:0-RGD). Therefore, the effect of RGD concentration in solution is not determining the cell response. These results point towards a different level of regulation in focal adhesion signaling and force

generation at the focal adhesions as a result of differences in lipid-modified peptide affinity to SLBs.^[20a, 24, 28a]

Figure 4.10. Vinculin localization in hMSCs on DOPC SLBs functionalized with C12:0-RGD at (A) 10-12%, (B) 2-5% and (C) 0.2-1% coverages and corresponding profile plots for four representative focal adhesions ($t = 18\text{h}$, blue: nucleus, red: actin, green: vinculin, scale bars: $20\text{ }\mu\text{m}$, FA: focal adhesion).

Table 4.5. Corresponding concentrations and percent coverages for C12:0-RGD and C16:0-RGD peptides used in this Chapter.

C12:0 RGD Concentration (μM)	Coverage range (C12:0-RGD)	C16:0 RGD Concentration (μM)	Coverage range (C16:0-RGD)
210	10-12%	2.1	10-12%
54	2-5%	1	3-5%
21	0.2-1%	0.7	1-3%

A very low level of cell spreading for low affinity C12:0-RGD functionalized SLBs, at low coverages (0.2-1%) was clearly seen, which was significantly lower than the cell area that was seen on C14:0-RGD functionalized surfaces at the same coverages ($1,127 \pm 672$ vs. $1,599 \pm 841 \mu\text{m}^2$, respectively) (Figure 4.11A and C and Figure 4.12A and Table 4.6). This observation already showed the effect of affinity on cell spreading. On SLBs that were functionalized with C12:0-RGD at 10-12% coverage, a significant increase was seen compared to a low coverage (0.2-1%) (Figure 4.11A-B, Figure 4.12A). At this condition spreading was supported although to a significantly lower extent compared to higher affinities at the same coverage (i.e., compared to C14:0-RGD and C16:0-RGD at 10-12%) (Figure 4.11B, D and E and Figure 4.12A, Table 4.6). Most intriguingly, on C14:0-RGD at the 10-12% coverage, cells had a significantly higher cell area compared to all other conditions, especially to the cells on SLBs functionalized with the high affinity peptide (C16:0-RGD), ($1,934 \pm 1036$ vs. $1,624 \pm 912 \mu\text{m}^2$ for C14:0-RGD and C16:0-RGD, respectively, at 10-12% coverage) indicating again an optimum condition in terms of peptide affinity to control cell spreading (Figure 4.11D-E, Figure 4.12A and Table 4.6). While a significant difference was seen in cell area between C14:0-RGD and C16:0-RGD functionalized SLBs at the same coverage (10-12%), there were no significant differences in regulation of cell shape factors (extent: 0.43 ± 0.16 vs. 0.44 ± 0.17 , eccentricity: 0.83 ± 0.16 vs. 0.83 ± 0.16 and form factor: 0.37 ± 0.19 vs. 0.38 ± 0.21 for C14:0-RGD and C16:0-RGD, respectively) suggesting a threshold value in the lipidated peptide affinity to induce morphological changes (Figure 4.12B-D and Table 4.6).

Figure 4.11. Overview of cell morphology on DOPC SLBs functionalized with (A and B) C12:0-RGD at 0.2-1% and at 10-12% coverage, respectively; (C and D) with C14:0-RGD at 0.2-1% and 10-12% coverage, respectively; and (E) C16:0-RGD at 10-12% coverage ($t=18h$, blue: nucleus, red: actin, scale bars: 40 μm).

Figure 4.12. Lipid-modified peptide affinity dependent cell spreading and shape. Regulation of (A) cell area, (B) extent (approaches 1 for compact cells with less protrusions), (C) eccentricity (approaches 1 for a highly elongated cell) and (D) form factor (approaches 1 for a highly circular cell) of hMSCs on DOPC SLBs functionalized with lipidated peptides of different affinities to the SLBs (at high and low coverages for C12:0-RGD (low affinity) and C14:0-RGD (moderate affinity)) after 18h (red line represents the mean value, $n \geq 400$, each data point represents one cell, mean $\pm$ standard deviation, **: $p < 0.01$).

On the other hand, cells on C12:0-RGD (low affinity) at the same coverage (10-12%) had a significantly lower protrusion formation (for example, extent of 0.43 ± 0.16 vs. 0.51 ± 0.14 ,

for C14:0-RGD and C12:0-RGD at 10-12%, respectively), were less elongated (eccentricity: 0.83 ± 0.16 vs. 0.77 ± 0.17 , respectively) and more circular (form factor: 0.37 ± 0.19 vs. 0.46 ± 0.19 , respectively) compared to higher affinity lipidated peptides (C14:0-RGD and C16:0-RGD). Finally, comparing C12:0-RGD and C14:0-RGD at low coverages (0.2-1%) further revealed significant differences in these parameters in response to lipid-modified peptide affinity with extent of 0.53 ± 0.15 vs. 0.43 ± 0.15 , eccentricity of 0.77 ± 0.16 vs. 0.83 ± 0.15 and form factor of 0.49 ± 0.20 vs. 0.37 ± 0.18 for C12:0-RGD and C14:0-RGD at 0.2-1% coverage, respectively (Figure 4.12B-D and Table 4.6).

Taken together, these results show that in response to an increase in the lipid-modified peptide affinity to SLBs from low to moderate, cells were more elongated with a higher degree of protrusions, and this response was seen to saturate at higher affinities as going from relatively moderate to high affinity did not induce further changes.

Table 4.6. Quantification of cell area and cell shape factors on DOPC SLBs functionalized with lipid-modified peptides of different affinities to SLBs (t=18h).

	Cell Area (μm^2)	Extent	Eccentricity	Form Factor
C12:0-RGD 0.2-1% $K_a = 9.3 \times 10^2 \text{ M}^{-1}$	1,127 $\pm$ 672	0.53 ± 0.15	0.77 ± 0.16	0.49 ± 0.20
C12:0-RGD 10-12% $K_a = 9.3 \times 10^2 \text{ M}^{-1}$	1,275 $\pm$ 755	0.51 ± 0.14	0.77 ± 0.17	0.46 ± 0.19
C14:0-RGD 0.2-1% $K_a = 8 \times 10^3 \text{ M}^{-1}$	1,599 $\pm$ 841	0.43 ± 0.15	0.83 ± 0.15	0.37 ± 0.18
C14:0-RGD 10-12% $K_a = 8 \times 10^3 \text{ M}^{-1}$	1,934 $\pm$ 1,036	0.43 ± 0.16	0.83 ± 0.16	0.37 ± 0.19
C16:0-RGD 10-12% $K_a = 1.2 \times 10^5 \text{ M}^{-1}$	1,624 $\pm$ 912	0.44 ± 0.17	0.83 ± 0.16	0.38 ± 0.21

Integrin adhesome is a multi-protein complex where intracellular protein-protein interactions are critical part of signal transduction at the adhesion sites.^[20a, 29] Paxillin as

a strong interaction partner of vinculin^[20b] acts as a scaffolding and signaling protein from the initial phases of integrin activation, where its phosphorylation by focal adhesion kinase/Src complex (FAK/Src) at tyrosine residues (Y31 and Y118) holds important consequences in the dynamics of focal adhesion assembly and turnover with effects on cell migration. Essentially, the presence of tyrosine phosphorylated paxillin was associated with dynamic adhesions, which can be newly formed nascent adhesions (decay time < 1 min) as well as more mature and stable focal adhesions at the cell periphery (decay time ~9 min).^[30] Therefore, to further investigate the focal adhesion signaling in hMSCs on SLBs interacting with lipid-modified RGD ligands of different affinities at defined densities, we studied the localization of phosphorylated paxillin (pPax, at Tyr (Y) 31) and its co-localization with vinculin at focal adhesions. For this purpose, DOPC SLBs were functionalized with C12:0-RGD peptide (relatively low affinity) at low coverage (0.2-1%), with C14:0-RGD (relatively moderate affinity) at low (0.2-1%) and high (10-12%) coverages and with C16:0-RGD (relatively high affinity) at high peptide coverage (10-12%).

Figure 4.13. Phosphorylated paxillin (pPaxillin, at Y31) in hMSCs on control surfaces. (A) bare DOPC SLBs and (B) serum coated glass (scale bar: 20 μm , blue: nucleus, red: actin, green: pPaxillin (at Y31)).

Observations on bare SLBs confirmed the specificity of integrin-RGD interactions as discussed before (**Figure 4.13**). On functional SLBs at all conditions, pPax localized at the cell periphery supporting the view of dynamic integrin adhesions (**Figure 4.14**).^[30]

Figure 4.14. Lipid-modified peptide affinity dependent paxillin phosphorylation (at Y31) and its co-localization with vinculin at focal adhesions. Corresponding profile plots are given for pPax (phospho paxillin at Y31) and its co-localization with vinculin as depicted in the overlay images for the given focal adhesion (yellow line) (scale bars: 20 μm , in left images blue: nucleus, green: phospho (Y31) paxillin, red: actin) (In right images yellow line represents the one dimensional region of interest for profile plotting in Image J).

pPax localization and corresponding intensity-distance plots supported the previous observations where for C14:0-RGD 10-12% a more distinct (thicker) protein localization hence (activation of) focal adhesion formation was visible while increasing the peptide affinity (C16:0-RGD) at the same coverage resulted in sharper peak intensities hence thinner, but qualitatively more elongated pPax localization at focal adhesions as seen in Figure 4.14 and **Figure 4.15**. Lowering the peptide coverage to 0.2-1% at the same affinity (for C14:0-RGD) as well as lowering the peptide affinity and coverage at the same time (going to C12:0-RGD 0.2-1%) resulted in more diffuse pPax localization and dot-like focal adhesions, respectively, in contrast to high coverage and peptide affinity conditions (Figure 4.14 and Figure 4.15).

Figure 4.15. Cumulative phosphorylated paxillin (at Y31) profile plots for four representative focal adhesions in hMSCs on DOPC SLBs functionalized with lipidated peptides of different affinity (at two different coverages for C14:0-RGD) after 18h (FA: focal adhesion). For analysis at least 20 focal adhesions were taken into account, four of which are shown.

As paxillin is known to interact with vinculin to control focal adhesion formation, we performed a co-staining of vinculin and pPax, and compared the intensity profiles to study the co-localization of these proteins. We repeated our previous observations on vinculin localization and found in this experiment a high correlation of vinculin-pPax, which was revealed by overlay images and intensity profiles (Figure 4.14). In order to derive a more quantitative measure for co-localization, the Pearson correlation coefficient (r) was calculated based on the linear fitting of pPax and vinculin intensities for the given focal adhesions (Figure 4.14). This coefficient reflects the degree of linear relationship between two components (where +1 value reflects the perfect linear correlation) and was calculated for above co-localization plots. Linear fitting revealed the r values to be 0.84, 0.95, 0.93 and 0.83 for C12:0-RGD 0.2-1%, C14:0-RGD 0.2-1%, C14:0-RGD 10-12% and C16:0-RGD 10-12%, respectively. As r values of the given comparisons were always above 0.8, for all the conditions given, we can say that pPax (Y31) and vinculin localizations were highly correlated at the focal adhesions in agreement with literature.^[20b, 29-30] These observations further demonstrated, at a phosphorylated-protein level, that on these SLBs integrin signaling is differentially regulated in response to peptide affinity as well as density.

4.3. Conclusions

Our data on focal adhesion signaling and cell spreading suggest that the peptide presentation defined by its affinity to lipid bilayer is an important parameter on SLBs presenting laterally mobile ligands at defined ligand densities.^[21] These results are especially interesting for the view on ligand accessibility and presentation on fluid SLBs, presenting laterally mobile ligands.^[14e, 21] Having lateral dynamics in our system, we clearly see a critical effect of ligand presentation (e.g., using C12:0-RGD vs. C14:0-RGD) possibly due to the reversibility of the interactions and associated binding strengths. Remarkably, while going from C12:0-RGD ($K_a=9.3 \times 10^2 \text{ M}^{-1}$) to C14:0-RGD ($K_a=8 \times 10^3 \text{ M}^{-1}$) enhanced focal adhesion formation, cell spreading and elongation, going from C14:0-RGD to C16:0-RGD ($1.2 \times 10^6 \text{ M}^{-1}$) seemed to change only organization of the focal adhesions, resulted in less cell spreading area, but similar cellular elongation and regulation of other morphological parameters. Therefore, we envision that combination of lateral dynamics and reversibility may require an optimum level of binding strength and ligand density on the surface to induce proper focal adhesion signaling and cell spreading.

4.4. Experimental Section

4.4.1. Peptide synthesis and purification

KGSGRGDSG peptide was synthesized using an automatic solid phase synthesis robot (MultiSyntech, Syro II) following standard Fmoc chemistry on Rink amide resin (Multisyntech Chemicals GmbH). After the synthesis and final Fmoc deprotection step, the resin was washed with dichloromethane (DCM) and methanol and dried under vacuum. In order to conjugate palmitic acid to the N-terminus of the peptide (hence resulting in C16:o-RGD, mono-palmitic acid-RGD), a solution of palmitic acid N-hydroxysuccinimide ester (NHS-Palmitate, Sigma) (5 molar equivalents with respect to the resin) and 1.1 molar equivalents of N,N-diisopropylethylamine (DIPEA) in dimethylformamide (DMF) were added to the reaction vessels containing the peptides on the resin. The reaction was carried out for 4h at room temperature after which the resin was washed with DMF, DCM and methanol and dried under vacuum. The peptides were cleaved from the resin using a solution of trifluoroacetic acid (TFA):triisopropylsilane (TIS): H₂O (95:2.5:2.5 (v/v%)) using a microwave (40 Watts, CEM Discover Peptide Synthesizer) for 30 min and subsequently precipitated in cold diethyl ether. The crude peptide was purified using RP-HPLC (Reverse Phase High Pressure Liquid Chromatography, C18 Column, Waters, using water/acetonitrile gradient with 0.1% TFA). The purified peptide was analyzed by electrospray ionization mass spectrometry and analytical HPLC (**Figure S1**).

Figure S1. ESI-ToF spectrum of purified C16:0-KGSGRGDSG together with its analytical HPLC trace (calc. $[M] = 1056.63$, found: $[M+2H^+] = 529.15$ m/z, $[M+H^+] = 1057.36$ m/z).

C12:0 and C14:0-RGD peptides were synthesized and purified using established methods available in our group (by Mark Verheijden) as explained elsewhere (**Figure S2** and **S3**) (explained in the thesis of Mark Verheijden).

Figure S2. ESI-ToF spectrum of purified C12:0-KGSGRGDSG (calc. $[M] = 1001.56$, found: $[M+2H^+] = 500.92$ m/z, $[M+H^+] = 1001.09$ m/z).

Figure S3. ESI-ToF spectrum of purified C14:0-KGSGRGDSG (calc. $[M] = 1029.60$, found: $[M+2H^+] = 515.31$ m/z, $[M+H^+] = 1029.70$ m/z).

4.4.2. Surface characterization of lipid-modified peptide-SLB interactions

Insertion of lipidated peptides (C12:0-RGD, C14:0-RGD and C16:0-RGD) into DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine, $T_m = -20^\circ\text{C}$, Avanti Polar Lipids) SLBs was characterized by quartz crystal microbalance with dissipation monitoring (QCM-D) (and spectroscopic ellipsometry for results explained in Figure 4.1, by Mark Verheijden) as described elsewhere (in the thesis of Mark Verheijden). It is important to note that the non-modified KGSGRGDSG peptide (bare-RGD, without lipid anchor) did not show any insertion, indicative of the absence of non-specific interaction between SLB and peptide ligands that were not modified with lipids. Additionally, no micelle formation was observed for all of the lipid-modified peptides, in the range of the concentrations used.^[17]

Figure S4. FRAP experiment using a DOPC SLB doped with 0.5 mol% of the fluorescent TopFluor-PE lipid. (A) Image recorded directly after bleaching (top) and after five minutes of recovery (bottom) (scale bar: 20 μm). (B) FRAP curve (black dots) fitted with modified Bessel functions (red line) as described by Soumpasis et al.^[37] FRAPAnalyser (University of Luxembourg) was used for the fitting to determine the diffusion constant and the degree of recovery (100% recovery with diffusion constant of $0.79 \pm 0.025 \mu\text{m}^2/\text{s}$). The first 11 data points correspond to intensity before bleaching and are not included in the model.

Fluorescence recovery after photobleaching (FRAP) was performed to characterize SLBs on glass supports as described in Chapter 3, with a Nikon A1 confocal microscope (**Figure S4**). For these experiments, TopFluor-phosphoethanolamine (TopFluor-PE, Avanti Polar Lipids) (dye conjugated) lipids were introduced in DOPC vesicles at 0.5 mol%. In all cases, SLB formation was carried out above the melting temperature of the constituent lipids.

4.4.3. Surface preparation and functionalization for cell experiments

SLB formation was achieved using the fusion of LUVs of approximately 100 nm onto hydrophilic glass supports. DOPC LUVs were sterilized by filtering through 0.2 μm membranes and SLB formation was performed under sterile conditions at room temperature. In order to form SLBs on glass supports, glass wells of 96-well plates (Sensoplate, Greiner Bio-one) were treated with 1 M NaOH for 1h followed by removal of solution and washing 10X with sterile MQ water to clean the surfaces. After surface cleaning, 50 μL of 1X Dulbecco's modified phosphate buffered saline (DPBS, without CaCl_2 and MgCl_2) was added to each well followed by addition of 50 μL vesicle suspension (with a final concentration of 0.1 mg/mL) and left to incubate for 1h. After formation of SLBs, excess vesicles were washed away with DPBS following serial dilution (10X) in order to avoid damage to the SLBs (leaving 100 μL DPBS/well). After their formation and

before incubation with lipidated peptides for bio-functionalization, SLBs were further washed 5X, again using serial dilutions, with serum free basic medium (see below) in order to add medium components in this step. Then, appropriate concentrations (stock solutions, 100 μ L/well) which were selected to achieve different coverage ranges (Figure 4.1, Table 4.1 and Table 4.5, concentrations are given in the presence of cells) of lipidated peptides in 1X DPBS were added to the SLBs and incubated at least for 2h, based on SE/QCM-D results, at room temperature in order to reach an equilibrium in the interaction of the lipidated peptides with the SLB. No further washing was performed after the peptide incubation step and surfaces were used as such for cell experiments.

4.4.4. Cell culture on SLBs

Bone marrow derived human mesenchymal stem cells (hMSCs, Poietics, Lonza) were cultured in basic medium (α MEM (Gibco) containing 10% fetal bovine serum (FBS) (Sigma) and 100 U/mL penicillin and 100 μ g/mL streptomycin (Sigma)) and incubated at 37°C and 5% CO₂ in a humidified environment up to passage 5.

For adhesion and spreading experiments, cells at p5 were seeded at a density of 5,000 cells/cm² with cell suspension adjusted to have 10% final serum concentration in wells and incubated on surfaces for 18h after which they were fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature. After fixation, cells were washed 3X with PBS and left in 0.05% sodium azide in PBS (Sigma) and kept at 4 °C for immunofluorescence analysis. As control surfaces to monitor cell adhesion and morphology over time, glass surfaces that were coated with FBS prior to cell seeding were used. As a negative control for cell adhesion, bare (non-functional) SLBs were used.

4.4.5. Immunofluorescence

In order to characterize cell adhesion and spreading on SLBs, actin cytoskeleton, vinculin and tyrosine phosphorylated paxillin (Y31) stainings were performed. Briefly, PFA fixed cells were permeabilized in 0.5% Triton X-100 in PBS (Sigma) for 10 min at room temperature. After permeabilization, cells were incubated with 0.1% Triton X-100 and 5% (w/v) bovine serum albumin (BSA, Sigma) solution in PBS for at least 20 min to block nonspecific sites. Afterwards, cells were incubated with a solution of AlexaFluor (AF)-568 phalloidin (Molecular Probes, Thermo Fisher Scientific, 1:100) and monoclonal anti-human vinculin-FITC antibody (anti-hVIN-1, Sigma Aldrich, 1:200) in 0.1 % Triton X-100 and

5% (w/v) BSA in PBS for 1h at room temperature in the dark. For experiments performed to investigate phosphorylated paxillin (phosphorylated at Y31, pPax) levels, after the blocking step, cells were incubated with rabbit-anti-human paxillin (phospho Y31) antibody (Abcam, ab32115, rabbit monoclonal antibody, 1:500) overnight at 4°C in 0.1 % Triton X-100 and 5% (w/v) BSA. Subsequently, cells were gently washed with PBS 3X (10 min each). Then, secondary antibody (AlexaFluor-647 goat-anti-rabbit IgG (H+L), Molecular Probes, Thermo Fisher Scientific, 1:500) was simultaneously incubated with AF-568 phalloidin and anti-hVIN-1 as given above in 0.1 % Triton X-100 and 5% (w/v) BSA. For pPax staining, as a background control check, cells were stained with only secondary antibody and without primary antibody; and background subtraction was performed accordingly in the fluorescence images for pPax channel. For nuclear staining in each experiment, after washing 3X with PBS to remove excess molecules, cells were incubated with DAPI in PBS (1:1,000) for 10 min and washed 2X 10 min with PBS. Imaging was performed with OPERA high content screening system (Perkin Elmer) with corresponding objectives and filter combinations.

4.4.6. Data analysis

All experiments were performed in triplicates and data are presented as mean $\pm$ standard deviation. Data were analyzed by using Kruskal-Wallis one way-ANOVA for statistical significance. Multiple comparison tests were performed with Mann-Whitney U test by adjusting α level downward according to Bonferroni correction comparing all groups of interest (OriginPro v.9, OriginLab). Image analysis was performed using Image J (NIH) and Cell Profiler image analysis software (Broad Institute of MIT and Harvard). Quantification of cell shape factors were performed by using Cell Profiler algorithms. For these analyses, more than 400 cells were taken into account for each condition. For visualization and readability purposes, the brightness of the images in the figures were adjusted when needed. However, for all the data treatment including quantification and profile plotting raw images (gray values of intensity) were used following background subtraction. Overlay images of pPax and vinculin were also prepared by merging background subtracted raw images without any pre-adjustment. In order to investigate focal adhesion profiles, profile plots of vinculin and pPax were drawn in Image J (plot profile command) with a one dimensional region of interest (line) for at least 20 focal adhesions using corresponding raw images in separate channels for vinculin and pPax. For plotting the data in figures, four representative focal adhesions were selected to depict different cases within the same condition. In order to investigate the co-

localization of vinculin and pPax at focal adhesions, pixel to pixel (for plotting in the figures distance is converted to micron) comparison of intensity values of vinculin and pPax at the same region of interest (one dimensional) was performed using, Image J and profile plotting. ^[20b] Linear fitting (OriginLab) was performed for these intensity values giving a Pearson correlation coefficient (r) which shows the degree of linear correlation between two components and is +1 for a perfect correlation. ^[20b]

4.5. Acknowledgments

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Chapter 5

Probing the effects of clustering RGD ligands in supported lipid bilayers on hMSC migration and lamellipodial dynamics

By functionalizing a fluid SLB with lipidated (i.e., lipid-modified) RGD ligands, we achieved a dynamic cell-instructive SLB system that exhibit lipidated peptide clustering (i.e., domain formation) at high ligand densities. We show that hMSC adhesion and focal adhesion formation in cells was strongly dependent on lipidated peptide-SLB interactions. Using a dye-conjugated C16:0-RGD peptide (C16:0-Rhd/RGD) we were able to monitor cell-peptide interactions and show that cells internalize the peptides at later stages of cell adhesion and spreading. Live cell imaging experiments further showed that cellular dynamics (i.e., lamellipodial dynamics and cell migration behavior) were regulated by peptide clustering and domain stability, which were strongly dependent on the type of the lipid anchor that was conjugated to RGD peptide.

5.1. Introduction

As presented in Chapter 1, multiple characteristics of cell-adhesive ligands (i.e., RGD ligands targeting integrins) such as their affinity, immobilization chemistry, density and spacing have critical effects on cell-surface interactions.^[1] Next to these, the nanoscale spatial organization of RGD ligands and their clustered presentation on the surfaces are known to affect cell adhesion and migration by directly influencing integrin clustering on the cell surface, a process known for critically affecting integrin activation and signaling.^[2] For example, using covalently immobilized RGD ligands, it was shown that clustered RGD presentation induced more cell spreading and a higher degree of focal adhesion formation compared to individual ligand presentation even at the same average overall ligand densities on a surface.^[3]

Fluid supported lipid bilayers (SLBs) that present mobile ligands introduces the ability to achieve clustered ligand presentation and hence receptor clustering on the cell surface, simply due to lateral diffusion of receptor-bound ligands underneath cells.^[4] This characteristic of the SLBs has been exploited to investigate the effect of mobility and spatial organization of ligands on SLBs in multiple biological contexts such as cell-cell interactions and ephrin signaling (for a more extensive view please refer to Chapter 1).^[4a, 5] However, in these studies observed clustering occurred only in the presence of cells. This was possibly due to low ligand densities that were used (e.g., approaching physiological receptor densities), which required cell generated forces for ligand reorganization and clustering.^[4a] In this Chapter, we functionalized fluid DOPC SLBs via lipid insertion using an RGD peptide that was conjugated with different lipid anchors (i.e., C16:0 and Cholesterol), at high densities (> 10% coverage) (**Scheme 5.1**). By using a dye-conjugated C16:0-RGD peptide (C16:0-Rhd/RGD), we show that the lipid-modified (lipidated)-RGD peptides spontaneously clustered within DOPC SLBs and they preserved their lateral mobility. Furthermore, we show that clustering properties and lateral mobility of lipidated RGD peptides strongly depended on the lipidated RGD peptide-SLB interactions, hence the lipid anchor type. Using time lapse imaging, we further followed cellular dynamics and cell-peptide interactions and were able to relate the high density of the peptides and peptide clustering with lamellipodial dynamics and cell migration. The results demonstrate that the ability to form peptide clusters on a dynamic platform is a versatile approach to tune cell dynamics.

Scheme 5.1. Scheme for the study presented in this Chapter where (lipid-modified) lipidated-RGD ligands, targeting integrins on the cell surface, cluster in SLBs at high peptide densities.

5.2. Results

5.2.1. Cell adhesion and spreading on SLBs functionalized with irreversibly inserted Cholesterol (Chol)-RGD

Next to alkyl tail conjugated RGD peptides, as presented in Chapter 4, a cholesterol conjugated RGD peptide was introduced to DOPC SLBs via lipid insertion, in order to study hMSC behavior on these surfaces.

Characterization of Chol-RGD insertion by quartz crystal microbalance with dissipation monitoring (QCM-D) as well as spectroscopic ellipsometry measurements^[6] (SE, as discussed in detail in the thesis of Mark Verheijden, in a collaboration with Prof. Ralf Richter) showed that, in contrast to alkyl tail conjugated peptides, cholesterol (Chol)-RGD peptides irreversibly inserted into SLBs, as they were not removed after washing with buffer in corresponding measurements (**Figure 5.1**). However, the peptide could specifically be removed by washing chol-RGD-functionalized SLBs with a solution of β -cyclodextrin (CD) (1 mM), as known in literature (Figure 5.1).^[7] These results demonstrate that control over ligand presentation to cells on SLBs was achieved in terms of SLB-inserted ligands that are either in equilibrium with the solution above the SLB or not.

Figure 5.1. (A) QCM-D and (B) spectroscopic ellipsometry measurements showing the insertion of Chol-RGD in DOPC SLBs (already in place at the start of the measurements) at various concentrations (F : frequency and D : dissipation, $\beta\text{-CD}$: cyclodextrin).

With these results in hand, we then studied the effect Chol-RGD coverage (i.e., ligand density) in DOPC SLBs on hMSC adhesion and spreading as well as on focal adhesion signaling in these cells. For this purpose, DOPC SLBs were functionalized with Chol-RGD at 0.1 μM and 0.5 μM peptide concentrations (leaving the peptide in solution), resulting in surface coverage ranges of 2-5% and 20-30%, respectively (as determined by combined QCM-D/SE experiments explained in Chapter 4, 100% coverage is defined as one lipidated peptide per lipid in the top leaflet, Figure 5.1B).

It was observed that, after 18h, cells on SLBs with 20-30% Chol-RGD coverage spread effectively (**Figure 5.2B**), where they had a distinct vinculin and phosphorylated paxillin (pPax) localization at focal adhesions (as markers of focal adhesion activation and maturation)^[8] (Figure 5.2D and F). Importantly, on bare DOPC SLBs, vinculin was mainly cytoplasmic and diffuse (in rounder cells, **Figure 5.3A** and C, D), which shows that cell-SLB interactions were specifically governed by the presence of Chol-RGD peptide. Furthermore, at 2-5% peptide coverage, generally nascent and qualitatively smaller focal contacts at the cell periphery were observed where cell spreading was also reduced compared to 20-30% peptide coverage (Figure 5.2A, C, E).

Figure 5.2. Overview of hMSC adhesion on DOPC SLBs incubated with (A) 0.1 μM (2-5% coverage) and (B) 0.5 μM Chol-RGD (20-30% coverage). Vinculin localization in cells on DOPC SLBs incubated with (C) 0.1 μM (2-5% coverage) and (D) 0.5 μM Chol-RGD (20-30% coverage). Phosphorylated paxillin (at Y31) localization in cells on DOPC SLBs incubated with (E) 0.1 μM (2-5% coverage) and (F) 0.5 μM Chol-RGD (20-30% coverage). (Blue: nucleus, red: actin, green: vinculin and yellow: phosphorylated (at Y31) paxillin, scale bar in A and B: 40 μm and C, D, E and F: 20 μm , $t=18\text{h}$).

Figure 5.3. Overview of hMSC adhesion on (A) bare DOPC SLBs and (B) serum-coated glass as control groups for Chol-RGD conjugated DOPC SLBs. Vinculin localization in cells on (C and D) bare DOPC SLBs and (E and F) serum-coated glass (Blue: nucleus, red: actin, green: vinculin scale bar in A and B: 40 μm and C, D, E and F: 20 μm , $t=18\text{h}$).

On functional SLBs, at both coverages, vinculin and pPax were seen to co-localize in cells, even though in this chapter we report it qualitatively (as quantitatively studied in Chapter 4 for other lipidated peptides) (Figure 5.2E-F). Altogether, these observations show that, by tuning the ligand density, hMSC behavior could be guided differentially on Chol-RGD functionalized SLBs, while pointing out the importance of ligand density on focal

adhesion activation and maturation in cells on these surfaces. As also pointed out in Chapter 3 and Chapter 4, serum-coated glass surfaces allowed us to monitor the morphology of hMSCs over time, where cells qualitatively had a more pronounced vinculin localization as well as thicker actin fibers compared to the cells on higher density, Chol-RGD-functionalized surfaces. These observations once again show a distinct level of focal adhesion sensing and regulation on functional SLBs vs. serum coated glass surfaces (Figure 5.2 and 5.3E-F).

Next, we quantified cellular morphological parameters, such as cell area, extent (approaches 1 for compact cells with less protrusions), eccentricity (approaches 1 for highly elongated cells) and form factor (approaches 1 for highly circular cells), for both coverages of Chol-RGD functionalized SLBs as well as for serum coated glass conditions, after 18h. On functional DOPC SLBs, increasing the peptide coverage from 2-5% to 20-30%, significantly increased the cell spreading area (i.e., $1,407 \pm 672$ vs. $1,747 \pm 999 \mu\text{m}^2$ for 2-5% and 20-30% peptide coverage, respectively) and resulted in cells with a significantly higher degree of protrusion formation (i.e., extent: 0.49 ± 0.16 vs. 0.44 ± 0.17 , for 2-5% and 20-30% peptide coverage, respectively) and a lower circularity (i.e., form factor: 0.45 ± 0.21 vs. 0.38 ± 0.22 for 2-5% and 20-30% peptide coverage, respectively) (Figure 5.4 and Table 5.1). On the other hand, on both functionalized SLBs, cells had a similar extent of elongation. These results were in line with the observations on focal adhesion formation in cells at both peptide coverages, and further shows that cell morphology is guided by the ligand density on these surfaces. Finally, on serum-coated glass, among all the groups, cells had the highest cell spreading area ($2,474 \pm 1,419 \mu\text{m}^2$), were the least circular (form factor: 0.34 ± 0.22), most elongated (eccentricity: 0.85 ± 0.18) and had the highest degree of protrusion formation (extent: 0.40 ± 0.18) (Figure 5.4 and Table 5.1).

Table 5.1. Quantification of cell shape factors for hMSCs on Chol-RGD conjugated DOPC SLBs and on serum-coated glass (t=18h).

	Cell area (μm^2)	Eccentricity	Extent	Form Factor
Chol-RGD 2-5% Coverage	$1,407 \pm 672$	0.80 ± 0.16	0.49 ± 0.16	0.45 ± 0.21
Chol-RGD 20-30% Coverage	$1,747 \pm 999$	0.81 ± 0.17	0.44 ± 0.17	0.38 ± 0.22
Serum-coated glass	$2,474 \pm 1,419$	0.85 ± 0.18	0.40 ± 0.18	0.34 ± 0.22

Figure 5.4. Cell shape factors for hMSCs on Chol-RGD conjugated DOPC SLBs (at two different ligand coverages) and on serum coated glass (t=18h, **: p<0.01, n ≥ 400, each data point represents one cell, red line represents the mean value, mean ± standard deviation).

To conclude this Section, here we show, by using a different lipid type, that lipidated RGD peptide-SLB interactions can be further tuned to achieve cell-instructive SLBs for guiding hMSC behavior.

5.2.2. Probing the effects of peptide-SLB interactions at high ligand densities on hMSC adhesion and spreading

In order to further investigate lipidated RGD peptide-SLB interactions and SLB properties in the presence of different lipidated RGD peptides, DOPC SLBs were incubated with various concentrations of C16:0-RGD (0.2, 2 and 10 μM) and Chol-RGD (0.2, 2 and 20 μM), and visualized with fluorescence microscopy. A dye-conjugated lipid (either with Texas Red or Bodipy, introduced to SLBs in low molar ratios, see Experimental Section) allowed to visualize SLBs and their homogeneity at defined peptide concentrations, which subsequently resulted in different surface coverages as explained above and in Chapter 4. Interestingly, for both C16:0-RGD and Chol-RGD, while at 0.2 μM peptide concentration a homogeneous SLB was observed, an increase of the peptide concentration to 2 μM resulted in the appearance of regions of higher fluorescence intensity (domains) on the SLB surface. The size and number of these domain regions was observed to further increase by increasing the peptide concentration, hence peptide coverage on the surface (Figure 5.5). According to QCM-D/SE measurements as presented in Chapter 4, for C16:0-RGD, 2 μM concentration resulted in 10-12% coverage range on the surface, which seemed to be sufficiently high to induce such an effect.

As the peptide concentration (i.e., peptide surface coverage) had a profound effect on the extent of domain formation in SLBs, we next evaluated whether domain formation is related to peptide clustering on the surface. For this purpose, a dye-conjugated C16:0-RGD peptide (with rhodamine, C16:0-Rhodamine (Rhd)/RGD) was synthesized and introduced to SLBs, allowing to directly study the interaction and properties of the lipidated-RGD peptides when they insert into SLBs. Intriguingly, when DOPC SLBs were incubated with 10 μM C16:0-Rhd/RGD, high intensity domain regions were observed when monitoring only the peptide (after washing the excess peptide in solution, red signal in Figure 5.5, bottom row, originates only from rhodamine), which could be overlaid with high intensity domain regions originating from dye-conjugated lipid in SLBs (green signal from TopFluor (Bodipy)-PE in SLBs, Figure 5.5, bottom row). Altogether, these results indicate that domain formation coincides with peptide clustering on the surface and is enhanced at increasingly high peptide coverages.

Figure 5.5. Domain formation at high peptide (ligand) densities. DOPC SLBs doped with fluorescently labelled lipids after 1h incubation with increasing concentrations of various lipidated peptides. For C16:0-Rhodamine (Rhd)/RGD case excess peptide washed away right before imaging. For C16:0-RGD and Chol-RGD, red fluorescence originates from TexasRed conjugated DHPE lipid. For C16:0-Rhd/RGD, both pictures are taken at the same location; red fluorescence originates from C16:0-Rhd/RGD and green from Topfluor-PE. The graph represents the intensity profile along the white line.

Insertion and interaction of C16:0-Rhd/RGD with DOPC SLBs was further verified and studied by QCM-D measurements, while comparing to the insertion of C16:0-RGD and Chol-RGD at the same time (Figure 5.6A-C). It was observed that in comparison to C16:0-RGD, the desorption of C16:0-Rhd/RGD from SLBs was slower and required higher flow rates during buffer wash (Figure 5.6A and C). This shows that the interaction strength of C16:0-Rhd/RGD with SLB is higher than that for C16:0-RGD peptide. In the case of Chol-RGD, again desorption was only observed when the SLBs were incubated with β -CD solution, after insertion (Figure 5.6).

Figure 5.6. QCM-D measurements to compare different lipidated peptides in terms of their interaction with DOPC SLBs (already in place at the start of the measurements) at different lipidated peptide concentrations. Insertion of (A) C16:0-RGD, (B) Chol-RGD and (C) C16-Rhd/RGD (*flow rate increased to from 100 to 150 μ L/min). (D) Molecular structures of the lipidated RGD peptides that were used in this study (Rhd: rhodamine and Chol: cholesterol).

Next, the phase behavior of DOPC SLBs at high ligand densities was evaluated with fluorescence recovery after photobleaching (FRAP) experiments. When the mobility of a domain region for C16:0-RGD (10 μ M) peptide was inspected, a similar mobility to a bare (non-functional) SLB was observed as marked with their corresponding recovery curves (Figure 5.7A). Besides, for C16:0-Rhd/RGD (10 μ M), outside of the domain region, again a similar recovery behavior was observed compared to C16:0-RGD functionalized SLBs and bare SLBs. On the other hand, within a domain area for Chol-RGD (20 μ M) functionalized

SLBs, a slower recovery (hence lower mobility) was observed. These results show that lateral dynamicity of SLBs at high ligand densities (and when they exhibit domain formation) was preserved, yet the extent of this dynamicity (mobility) was dependent on the lipid type that was conjugated to RGD peptide.

Figure 5.7. Characterization of SLBs exhibiting domain formation at high ligand densities. (A) Fluorescence recovery after photobleaching (FRAP) analysis performed for bare DOPC SLB (no peptide), for C16:0-RGD and Chol-RGD domain regions in a DOPC SLB doped with fluorescently labeled lipids (TexasRed (TR)-DHPE or TopFluor (TF)-PE) as well as FRAP analysis for a non-domain region in a DOPC SLB presenting C16:0-Rhd/RGD peptide. (B) FRAP locations for the analysis presented in A after bleaching and 5 min after recovery (scale bar: 20 μ m, Rhd: rhodamine).

In order to investigate how hMSC adhesion and spreading was affected at these high ligand densities in SLBs, DOPC SLBs were functionalized with C16:0-RGD (10 μ M), C16:0-Rhd/RGD (10 μ M) and Chol-RGD (5 μ M). After 2h incubation on the surfaces, excess peptide was washed away from the solution, prior to cell seeding. On C16:0-RGD functionalized SLBs, after 18h, we observed a qualitatively lower extent of cell spreading and rounder cells, compared to C16:0-Rhd/RGD and Chol-RGD functionalized SLBs (Figure 5.8).

Figure 5.8. Overview of hMSC adhesion and spreading on DOPC SLBs at high ligand densities. (A) Bare DOPC SLBs (pure DOPC). DOPC SLBs incubated with (B) 10 μ M C16:0-RGD, (C) 10 μ M C16:0-Rhd/RGD and (D) 5 μ M Chol-RGD. Excess peptide was washed away prior to cell seeding. (Blue: nucleus and red: actin, scale bar: 40 μ m). (Actin staining was performed with a far-red dye to exclude rhodamine signal and false colored as red in this Figure, see experimental section, $t=18$ h).

Interestingly, in cells on C16:0-RGD functionalized SLBs, mainly nascent adhesions with less defined vinculin localization was observed compared to the cells on C16:0-Rhd/RGD and Chol-RGD functionalized SLBs (Figure 5.9A-C). Among all functional SLB groups, cells on C16:0-Rhd/RGD demonstrated a stronger vinculin localization at focal adhesions (Figure 5.9B). Furthermore, we repeated our observations on bare SLBs and glass surfaces, validating once again the specificity of interactions (i.e., integrin-RGD interactions) on SLBs.

Figure 5.9. Vinculin localization in hMSCs on DOPC SLBs at high ligand densities. DOPC SLBs incubated with (A) 10 μM C16:0-RGD, (B) 10 μM C16:0-Rhd/RGD, (C) 5 μM Chol-RGD, (D) bare SLBs (pure DOPC) and (E) serum coated glass. Excess peptide was washed away prior to cell seeding. (Blue: nucleus and red: actin and green: vinculin, scale bar: 20 μm). (Actin staining was performed with a far-red dye to exclude rhodamine signal and false colored as red in this Figure, see experimental section, $t=18\text{h}$).

5.2.3. Dynamics of cell behavior on SLBs with clustered peptide presentation (at high ligand densities)

Showing that our functional SLBs, at high ligand densities, present a dynamic and bio-active surface to cells, we used live cell imaging, in an attempt to reveal dynamics of cell behavior as well as to understand cell-SLB (hence cell-peptide) interactions in real-time. For this purpose, DOPC SLBs were doped with a Topfluor-PE (dye-conjugated) lipid at low concentrations (0.5 mol%). As this lipid does not present any net charges (hence, it is neutral), it preserves the non-fouling properties of background (bare DOPC SLBs) and allows us to specifically study cell-peptide interactions while being able to monitor the

SLB properties over time (**Figure 5.10**). In fact, time lapse videos revealed minimal to no cell binding on bare DOPC-Topfluor-PE (referred as DOPC-TF) (Video 5.1) for 15h (time course of imaging).

Figure 5.10. SLB (with or without lipidated peptides) stability over time. DOPC SLBs (doped with TopFluor-PE lipid) incubated with 10 μM C16:0-RGD, 10 μM C16:0-Rhd/RGD and 5 μM Chol-RGD at 20 min and 15h:20 min after cell seeding. After 2h of incubation and before cell seeding, excess peptide was washed away. Bare SLB was included as a control (Green: TopFluor-PE, scale bar: 100 μm).

For live cell imaging experiments, C16:0-RGD (10 μM), C16:0-Rhd/RGD (10 μM) and Chol-RGD (5 μM) were inserted into DOPC-TF SLBs, in line with Section 5.2.2, i.e., before cell seeding, DOPC-TF SLB were incubated with peptides for 2h and subsequently washed to remove excess peptide. On RGD-functionalized SLBs, domain formation (as high intensity regions of green fluorescent) was again observed, albeit with different properties depending on the type of the lipid that was conjugated to RGD ligand (discussed below). For the case of C16:0-Rhd/RGD, domain regions as observed with fluorescently labeled lipid (green signal) also coincided with rhodamine (red) signal showing once again the presence of peptide clustering in SLBs and its involvement in domain formation (Figure 5.11, Video 5.2 and Video 5.3).

Importantly, real-time monitoring of SLB properties revealed that their stability (for both bare and functionalized SLBs) was preserved in the presence of cells, as monitored up to 15h (time course of imaging), marked with a homogeneous green fluorescence due to the presence of TopFluore-PE within the SLBs (Figure 5.10 and Videos 5.1-5.5).

Figure 5.11. Dynamics of peptide presentation in SLBs. Snapshots from live cell imaging at the early stages of cell adhesion on DOPC SLBs (with Topfluor-PE lipid) presenting C16:0-Rhd/RGD (0 min: 20 min after cell seeding, scale bar: 100 μ m, red: rhodamine, arrow head: a dark spot co-localized with a cell disappearing after cell moved from that spot, arrow: following a dark spot co-localized with a cell in time). Note that the red signal only originates from rhodamine. Top row shows snapshots of rhodamine channel only; bottom row shows an overlay of rhodamine channel and cells in transmission mode.

As mentioned above, introduction of C16:0-Rhd/RGD to SLBs enabled us to study and monitor cell-peptide interactions in real time, due to inherent fluorescence of this dye conjugated lipidated peptide. As revealed from time-lapse videos, already within the first 30 min after cell seeding, cell adhesion was evident on C16:0-Rhd/RGD presenting SLBs (also for other two functional surfaces, Videos 5.2-5.5 and Figure S4). On these SLBs, no obvious preference for cell adhesion was seen when comparing the domain areas and areas outside of the domains.

In these initial phases of cell adhesion (starting from 20 min after cell seeding), when we inspected only rhodamine signal (channel), we observed the appearance of dark circular spots on SLBs, which are indicative of depletion of the peptide from the surface. These dark spots appeared regardless of cells being in contact with domains or not.

Interestingly, these dark spots were co-localized with adherent cells, showing that the effect is induced by cell-peptide interactions and cell binding on SLBs (**Figure 5.11** and Video 5.3). Furthermore, when cells moved away from their original position, the dark spots had disappeared immediately, once again showing the mobility of C16:0-Rhd/RGD in SLBs and its availability for cells (Figure 5.11, arrow head). On the other hand, these spots persisted underneath the cells and in time, the intensity of these spots gradually increased even when still in contact with cells (as monitored every 30 min for 90 min), indicating peptide diffusion from surrounding areas to these regions (Figure 5.11, arrow).^[9]

Being intrigued by these observations, we investigated cell-peptide interactions at later time points (i.e., when cells were spread on the surfaces), again on C16:0-Rhd/RGD functionalized SLBs. After around 6h of cell seeding, the majority of the cells were seen to be spread on these surfaces (Video 5.2 and 5.3). When we monitored a representative spread (and stationary) cell for 9h (starting 6h after cell seeding and monitoring every 3h), interestingly, we observed red fluorescence across the cell body indicating that peptides were taken up by the cell (Video 5.3 and **Figure 5.12A**). Fixed cell imaging (after 18h) further showed the accumulation and localization of the peptides inside the cells, where the red signal (from rhodamine) was only observed in cells cultured on C16:0-Rhd/RGD (Figure 5.12B) and not on C16:0-RGD and Chol-RGD peptides. Taken together, these results clearly demonstrate that, next to the lateral dynamicity of the peptides on the surface (i.e., in-plane), there is another level of dynamics (i.e., out-of-plane) that occurred for this peptide in SLBs, as it reversibly inserted into SLBs and became available to take up by cells. It is important to note that, even though C16:0-Rhd/RGD was taken up by cells, it remained at high densities in the SLB and thus remained available for cells throughout the experiment, as marked with persistent red fluorescence signal from the SLB and the presence of domains in SLBs, even after 15h of cell experiments (Video 5.2-5.3 and Figure 5.10).

Figure 5.12. Dynamics of cell-peptide interactions and peptide take up by the cells. (A) Snapshots from live cell imaging for a representative cell exhibiting peptide accumulation in cell body, on DOPC SLBs (with Topfluor-PE lipid) presenting C16:0-Rhd/RGD (0h: 6h after cell seeding, red: rhodamine, scale bar: 20 μm). (B) Fluorescence images of hMSCs after 18h on different SLB surfaces. Note that red signal only originates from rhodamine. (Fixed cell imaging, blue: nucleus, red: rhodamine, green: actin, scale bar: 20 μm , $t=18\text{h}$).

Next to observing cell adhesion and cell-peptide interactions in real-time, studying cell migration on SLBs can give important insights on the dynamics of cell behavior on these surfaces. As we discussed in Chapter 1 and Chapter 2, cell migration is closely related to cell adhesion, where a biphasic relation between adhesion strength and migration speed is suggested.^[10] Lamellipodial extensions are important for cell migration as the driving factor at the leading edge of the cells. These extensions are powered by actin polymerization and inherently dynamic, i.e., they are transient structures that protrude and retract during migration.^[11] Therefore, we were interested in studying lamellipodial dynamics to have a further understanding on cell behavior on our functionalized SLBs, in relation to peptide clustering (i.e., domain formation).

Figure 5.13. Lamellipodial dynamics for cells where lamellipodial extensions are in contact with or outside of the domains on DOPC SLBs (with TopFluor-PE lipid) presenting C16:0-Rhd/RGD peptide. Displacement (extension or retraction of lamellipodia) is given for every 5 min for 30 min total time frame for 3 and 4 representative cells, respectively. (Green: TopFluor-PE, red arrow shows the distance between the reference point in the cells and the edge of the lamellipodial extension, scale bar: 20 μm).

For this purpose, we monitored lamellipodial extensions, in representative spread cells, for their retraction and extension relative to a reference point in cells, on C16:0-Rhd/RGD and Chol-RGD functionalized SLBs (see Experimental Section). This analysis could not be done for C16:0-RGD functionalized SLBs, as cells on these SLBs did not show effective (or stable) spreading during the time course of live cell imaging experiments and they mainly had filopodial extensions (Video 5.5). The degree of extension and retraction of lamellipodia was measured and reported in terms of displacement every 5 min (in reference to a previous frame), corresponding to the time scale of image acquisition, for 30 min. In order to understand whether peptide clustering (i.e., the presence of domains) had an effect on lamellipodial dynamics, we monitored cells having these extensions either in contact with or outside of the domains and calculated the displacements accordingly, giving a semi-quantitative measure for cell behavior. On

C16:0-Rhd/RGD presenting SLBs, when these lamellipodial extensions were in contact with domains, they had more stable changes in displacement (i.e., a similar degree of displacement compared to each preceding displacement). On the other hand, when the lamellipodial extensions were not in contact with domains, more abrupt changes and generally a higher degree of displacement was observed (**Figure 5.13**). On Chol-RGD functionalized SLBs, the degree of displacement, for the lamellipodial extensions both in contact with and outside of the domains, was at a lower degree when compared to C16:0-Rhd/RGD case. Furthermore, the difference between the lamellipodial dynamics when they were in contact with domains and outside of the domains was less pronounced in the case of Chol-RGD peptide (**Figure 5.14**).

Figure 5.14. Lamellipodial dynamics for cells where lamellipodial extensions are in contact with or outside of the domains on DOPC SLBs (with TopFluor-PE lipid) presenting Chol-RGD peptide. Displacement (extension or retraction of lamellipodial) is given for every 5 min for 30 min total time frame for 3 and 4 representative cells, respectively. (Green: TopFluor-PE, red arrow shows the distance between the reference point in the cells and the edge of the lamellipodial extension, scale bar: 20 μm).

Besides, cells on Chol-RGD presenting SLBs, had a delayed spreading (majority of cells spreading around 8h), with multiple smaller lamellipodial extensions, and in some cases with extensive filopodial protrusions, compared to the cells on C16:0-Rhd/RGD functionalized SLBs (Figure 5.13, Figure 5.14 and videos 5.2-5.4).

We then calculated the cell migration speed on SLBs functionalized with C16:0-RGD, C16:0-Rhd/RGD and Chol-RGD peptides. Cell speed was calculated (simply as total trajectory divided by the time of the trajectory with at least 10 cells taken into account per condition) based on 120 min trajectory and after 4h of cell adhesion. No significant differences in cell speed was observed when comparing C16:0-Rhd/RGD and Chol-RGD functionalized SLBs, while on C16:0-RGD, cells exhibited a significantly faster migration rate (**Figure 5.15**).

Figure 5.15. Cell speed on DOPC SLBs (with TopFluor-PE lipid) presenting C16:0-Rhd/RGD, Chol-RGD and C16:0-RGD (each data point presents one cell, $n \geq 10$, mean $\pm$ standard deviation, red line: mean value, based on 120 min trajectory, *: $p < 0.05$).

In order to better understand the relation of peptide clustering (i.e., domain formation) with both lamellipodial dynamics and cell migration speed, we calculated the size of the clusters, as domain area and perimeter at the start of the experiment (20 min after cell seeding, **Figure 5.16**). Domain area and perimeter were the largest in the case of C16:0-Rhd/RGD, with a broad size distribution within the individual domains (at least 10

domains were counted). In contrast, for C16:0-RGD, domain area and perimeter were the smallest with a very narrow size distribution (Figure 5.16).

Figure 5.16. Size of the domains in terms of their area and perimeter at the start of the live-cell imaging experiment (20 min after cell adhesion). (Each data point presents a randomly selected domain, mean $\pm$ standard deviation, red line: mean value).

In relation to this, when the stability of the domains was monitored over time, for C16:0-Rhd/RGD and C16:0-RGD relatively stable domains were observed in contrast to Chol-RGD case, where domains seemed to change in shape and size as seen from time lapse videos (Figure 5.10, Videos 5.2-5.5).

5.3. Discussion

The way that ligands are presented to cells, next to their density on the surface, has profound effects on cell-surface interactions.^[1b, 1f, 12] In this line, nanoscale spatial organization and clustered presentation of RGD ligands are known to be critical for integrin activation and subsequent focal adhesion signaling, and eventually for cell adhesion and migration.^[2a, 2d, 3] Dynamic display of ligands in SLBs introduces multiple advantages to engineer ligand presentation to cells at defined densities and spatial organization where lateral mobility (hence lateral dynamicity) of the ligands could effectively be tuned.^[4a, 5] In this Chapter, we demonstrate that fluid SLBs could be functionalized with lipidated RGD ligands at high ligand densities, via lipid insertion, where these peptides seemed to cluster spontaneously within the SLB, resulting in peptide-enriched regions, which were defined as domains. This remarkable effect was seen to be enhanced at high peptide surface coverages (i.e., higher peptide

concentration in solution) and required lipid mobility, as no clustering was observed for gel phase (immobile) SLBs (data not shown, as explained in thesis of Mark Verheijden). While the phase behavior (mobility) of SLBs was preserved also in the presence of domains for C16:0-RGD and in a non-domain region for C16:0-Rhd/RGD peptides, a drop in ligand mobility was observed for Chol-RGD domains, in line with the known effect of cholesterol on phase behavior of SLBs.^[13] Since no relation to phase separation could be made, the observed peptide clustering was attributed to steric effects related to peptide crowding and lipid anchor wedging.^[14] A more extended discussion on mechanism of domain formation is given in the thesis of Mark Verheijden.

Observations on vinculin localization in cells on SLBs revealed a strong dependence of focal adhesion formation on the type of the lipid anchor conjugated to RGD ligands or presence of rhodamine for C16:0-Rhd/RGD, at high ligand densities (i.e., high peptide concentration in solution). As such, cells on C16:0-RGD had much more diffused vinculin localization compared to the cells on C16:0-Rhd/RGD even though initially incubated at the same concentration with SLBs. However, it is important to note that before cell seeding, the excess peptide was washed away. As it also was demonstrated in QCM-D measurements, C16:0-RGD peptide was possibly removed from the surface more easily than it would be for C16:0-Rhd/RGD during washing, influencing the effective surface density presented to cells. Additionally, a higher interaction strength of C16:0-Rhd/RGD with the SLB may have contributed to the growth of focal adhesions as they are mechanosensitive ^[2c, 15] (as shown in Chapter 4 for C12:0 vs. C14:0 and C16:0-RGD), even though here we cannot exclude the effect of ligand density. Reduced peptide density on C16:0-RGD in this case can also explain the differences between the results for C16:0-RGD (at 10-12% coverage) as described in Chapter 4 and in this Chapter. Focal adhesion formation in cells on Chol-RGD at high ligand density (5 μ M peptide concentration), resembled the case when it was presented to SLBs at 0.5 μ M (20-30% coverage). Since washing the excess peptide from solution in the former case would not remove the peptide from surface, a threshold in ligand density may have existed for this peptide for focal adhesion sensing even though direct comparison of both conditions also in terms of cellular morphological parameters would be necessary. The observation that C16:0-Rhd/RGD in SLBs induced a qualitatively more pronounced vinculin localization (also compared to Chol-RGD) may suggest an enhanced diffusion of ligand bound integrin receptors during focal adhesion nucleation, which could lead to focal adhesion growth, as was observed for covalently immobilized linear (low affinity) RGD ligands.^[1e] However, more in depth analysis on integrin localization and clustering is needed to reveal the effects.

Live cell imaging experiments revealed multiple interesting observations when cells were in contact with SLBs presenting clustered peptides at high ligand densities. Firstly, the observations on the appearance of dark regions (circular spots) underneath adherent cells (when monitoring C16:0-Rhd/RGD peptide in SLBs at early stages of adhesion) prompted several questions on cell-peptide interactions, especially if peptides are taken up by cells, as they appeared as depletion regions. In fact, at later stages of cell adhesion and spreading, the uptake of peptides was clearly seen due to accumulation of rhodamine (C16:0-Rhd/RGD) signal in the cell body. We tentatively relate this to an active mechanism where cells could possibly pull the ligands from the surface upon integrin binding and/or internalize the peptide via endocytosis as was observed for RGD-SLBs functionalized via biotin-neutravidin interactions.^[16] Furthermore, real-time monitoring of a region underneath a cell at early stages of adhesion revealed a gradual recovery in fluorescence signal from the dye conjugated peptide, in contrast to a dark region that was filled with peptide immediately when a cell moved from that region. This suggested a reduced mobility of ligands underneath the cells, as was observed before for protein functionalized SLBs in contact with cells.^[9]

Secondly, monitoring the extent of lamellipodial protrusion and retraction (i.e., displacement) when cells were in contact and outside of the domains also indicated several intriguing directions on how cells sense the peptide presentation in relation to cellular dynamics.^[11] More stable changes in displacement when lamellipodial extensions were in contact with domains, compared to when they were outside of the domains on C16:0-Rhd/RGD presenting SLBs, indicated a differential sensing of these two regions by cells. Possibly, for this peptide, domain regions induced more stable (i.e., less dynamic) cell behavior by persistently presenting higher density ligands in a clustered format, as they also remained stable over time, in the presence of cells. Interestingly, on Chol-RGD where domain areas showed extensive remodeling (i.e., they showed diffusion and decrease in size over time), cells could not distinguish the difference between when they were in contact with or outside of the domains. This remodeling of Chol-RGD domains was mainly attributed to a temperature effect (room temperature vs. 37°C), since similar remodeling of domains for this peptide was also observed in the absence of cells (data not shown, given in thesis of Mark Verheijden). On the other hand, cell migration speed on both surfaces remained at similar levels, while on C16:0-RGD, a strikingly higher cell speed was observed. These observations could be linked to adhesion behavior of cells, i.e., cell-surface adhesiveness as marked with focal adhesion formation.^[10] That is, decreased cell speed for C16:0-Rhd/RGD and Chol-RGD is possibly due to stronger adhesion of cells on these surfaces, as revealed by distinct vinculin localization, in contrast to

C16:0-RGD case.^[10] These results once again show the critical influence of the way that ligands are presented to cells, in relation to lipid anchor type conjugated to RGD ligands and peptide clustering on the surfaces.

5.4. Conclusion

In this Chapter, we present a dynamic cell-instructive SLB system that exhibit lipidated peptide clustering (i.e., domain formation) at high ligand densities. Lipidated-peptide-SLB interactions and peptide clustering properties strongly depended on the lipid type that was conjugated to integrin targeting RGD peptide (i.e., C16:0-Rhd/RGD, C16:0-RGD and Chol-RGD). Using a dye conjugated peptide (C16:0-Rhd/RGD) we showed the dynamic presentation and availability of the peptide (at high densities) to cells in SLBs as well as the accumulation of the peptide inside the cell body in real-time, indicative of an active process for peptide take up by the cells. We also show that the lamellipodial dynamics (i.e., the degree of extension and retraction of the lamellipodia) and cell migration speed were dependent on the type of the lipid anchor densities and observations could be related to peptide clustering properties, at high ligand densities. Future work should introduce better insights on the mechanisms of cell-peptide interactions (e.g., in relation to integrin clustering and trafficking in cells) as well as a further understanding on surface properties and peptide clustering in SLBs. Part of the analysis on domain formation and surface characterization is presented in PhD thesis of Mark Verheijden.

5.5. Experimental Section

5.5.1. Peptide synthesis and purification

KGSGRGDSG peptide was synthesized using an automatic solid phase synthesis robot (MultiSyntech, Syro II) following standard Fmoc chemistry on Rink amide resin (Multisyntech Chemicals GmbH). After the synthesis and final Fmoc deprotection step, the resin was washed with dichloromethane (DCM) and methanol and dried under vacuum. In order to conjugate palmitic acid to the N-terminus of the peptide (hence resulting in C16:0-RGD, mono-palmitic acid-RGD), a solution of palmitic acid N-hydroxysuccinimide ester (NHS-Palmitate, Sigma) (5 molar equivalents with respect to the resin) and 1.1 molar equivalents of N,N-diisopropylethylamine (DIPEA) in dimethylformamide (DMF) were added to the reaction vessels containing the peptides on the resin. The reaction was carried out for 4h at room temperature after which the

resin was washed with DMF, DCM and methanol and dried under vacuum. The peptides were cleaved from the resin using a solution of trifluoroacetic acid (TFA):triisopropylsilane (TIS): H₂O (95:2.5:2.5 (v/v%)) using a microwave (40 Watts, CEM Discover Peptide Synthesizer) for 30 min and subsequently precipitated in cold diethyl ether. The crude peptide was purified using RP-HPLC (Reverse Phase High Pressure Liquid Chromatography, C18 Column, Waters, using water/acetonitrile gradient with 0.1% TFA). The purified peptide was analyzed by electrospray ionization mass spectrometry and analytical HPLC (**Figure S1**).

Figure S1. ESI-ToF spectrum of purified C16:o-KGSGRGDSG together with its analytical HPLC trace (calc. $[M] = 1056.63$, found: $[M+2H^+] = 529.15$ m/z, $[M+H^+] = 1057.36$ m/z).

Cholesterol (Chol)-RGD and C16:o-Rhd (Rhodamine)/RGD peptides were synthesized and purified using established methods available in our group (by Mark Verheijden) as explained elsewhere (**Figure S2** and **S3**) (explained in the thesis of Mark Verheijden).

Figure S2. ESI-ToF spectrum of purified Chol-KGSGRGDSG (calc. $[M] = 1287.77$, found: $[M+2H^+] = 644.35$ m/z, $[M+H^+] = 1287.84$ m/z).

5.5.2. Surface characterization of lipidated peptide-SLB interactions

Insertion of lipidated peptides (C16:0-RGD, Chol-RGD and C16:0-Rhd/RGD) into DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine, $T_m = -20^\circ\text{C}$, Avanti Polar Lipids) SLBs was characterized by quartz crystal microbalance with dissipation monitoring (QCM-D) (and spectroscopic ellipsometry for results explained in Figure 5.1B, by Mark Verheijden) as described elsewhere (in the thesis of Mark Verheijden). Furthermore, SLBs (formed on cleaned glass supports, see Section 5.4.3) that present different lipidated peptides (at various concentrations) were characterized with fluorescent microscopy using an Olympus IX71 inverted microscope and a confocal microscope (Nikon A1).

Figure S3. ESI-ToF spectrum of purified C16:0-Rhd/KGSGRGDSG (calc. $[M] = 1539.84$, found: $[M+3H^+] = 513.95$ m/z , $[M+2H^+] = 770.39$ m/z).

Fluorescence recovery after photobleaching (FRAP) was performed to study lipid mobility for peptide presenting and bare SLBs as described in Chapters 3 and 4, with a Nikon A1 confocal microscope. For these experiments TexasRed conjugated 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (TR-DHPE, Molecular Probes, Thermo Fisher Scientific) and TopFluor-phosphoethanolamine (TopFluor-PE, Avanti Polar Lipids) (dye conjugated) lipids were introduced in DOPC vesicles at 0.2 mol% and 0.5 mol%, respectively. In all cases, SLB formation was carried out above the melting temperature of the constituent lipids.

5.5.3. Surface preparation for cell experiments

SLB formation was achieved using the fusion of large unilamellar vesicles (LUVs) on hydrophilic glass supports. DOPC LUVs were sterilized by filtering through 0.2 μm membranes and SLB formation was performed under sterile conditions at room temperature. Glass wells of 96-well plates (Sensoplate, Greiner Bio-one) were used as

glass supports for SLBs. The well plates were filled with 1 M NaOH and left alone for 1h. After removing the solution, the wells were cleaned by pipette rinsing 10X with sterile MQ water with gentle pipetting. Then, 50 μ L of 1X Dulbecco's modified phosphate buffered saline (DPBS, without CaCl_2 and MgCl_2) was added to each well followed by adding 50 μ L of a solution of LUVs (with a final concentration of 0.1 mg/mL). After 1h, excess vesicles were washed off with DPBS by serial dilution (with gentle pipetting, adding and removing 100 μ L DPBS, 10X) in order to avoid damage to the SLBs (leaving 100 μ L DPBS/well).

For the experiments described in section 5.2.1, after SLB formation, but before incubation with lipidated peptides, the well plates with SLBs were further washed 5X, again using serial dilutions, with serum-free basic medium (see below) to add medium components. Then, solutions of Chol-RGD (1.5 and 0.3 μ M, 100 μ L/well in 1X DPBS, stock concentrations) were added to the SLBs and left alone for at least 2h, at room temperature. No further washing was performed and the plates were used as such for cell experiments.

For the experiments described in Sections 5.2.2 and 5.2.3, SLBs were formed on cleaned glass surfaces using DOPC LUVs with or without 0.5 mol% TopFluor-PE (dye conjugated) lipid as explained above. Then, solutions of C16:0-RGD (at 10 μ M), C16:0-Rhd/RGD (10 μ M) and Chol-RGD (5 μ M) peptides were added to the wells (100 μ L/well in DPBS), resuspended gently 2X (each time), followed by removal of 100 μ L solution from the wells. This was repeated 3X where finally 200 μ L solution was left in each well, for at least 2h incubation. Finally, peptide solution was washed 10X with serum-free basic medium (see below) to remove the excess peptide. SLBs prepared from DOPC LUVs containing 0.5 mol% TopFluor-PE were immediately used for live cell imaging experiments (see Section 5.5.5). SLBs prepared from DOPC LUVs without TopFluor-PE lipid were also studied in a side experiment for immunofluorescence analysis.

5.5.4. Cell culture on SLBs

Bone marrow derived human mesenchymal stem cells (hMSCs, Poietics, Lonza) were cultured in basic medium (α MEM (Gibco) containing 10% fetal bovine serum (FBS) (Sigma) and 100 U/mL penicillin and 100 μ g/mL streptomycin (Sigma)) and incubated at 37°C and 5% CO_2 in a humidified environment up to passage 5. Cells were seeded on the surfaces at a density of 5,000 cells/cm² with cell suspension adjusted to have 10% final serum concentration in wells. For these experiments, glass surfaces that were coated

with FBS prior to cell seeding were used as control surfaces to monitor cell adhesion and morphology over time, next to the bare SLB controls.

Cells were cultured on the surfaces for 18h and then fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature. After fixation, cells were washed 3X with PBS and left in PBS with 0.05% sodium azide (Sigma) and kept at 4°C for immunofluorescence analysis.

5.5.5. Live cell imaging experiments

Live cell imaging experiments were performed using a Nikon A1 confocal microscope for bare DOPC, DOPC with C16:0-RGD, DOPC with Chol-RGD and DOPC with C16:0-Rhd/RGD and cells were maintained at normal culture conditions (37°C and 5% CO₂ in a humidified environment) in a climate chamber connected to the microscope. Excitation parameters to monitor TopFluor-PE (488 nm, 0.77% laser power) and C16:0-Rhd/RGD (561 nm, 1.35% laser power) were adjusted to use minimum laser power required for the experiments to achieve cytocompatibility, with corresponding filter settings. Cells were monitored in transmission mode. Images were taken every 5 min (for total 15h) where the first frame corresponds to 20 min after cell seeding. After 15h, cells were cultured on the surfaces for another 3h and fixed (hence at 18h) for immunofluorescence analysis as explained above (**Figure S4**).

Characterization of dynamics of cell behavior and surface properties during cell experiments

Time lapse images were analyzed using ImageJ (NIH). For the analysis of cell migration trajectories and cell speed, at least 10 adherent individual cells were followed (by their nucleus and their center, starting 4h after cell adhesion and for 120 min) while cells that were out of frame and focus (due to focus drift despite autofocus of the camera) or were dividing were excluded. Information on the position of the cell and total path length was obtained with MTrackJ plugin of ImageJ by monitoring each cell in consequent frames. Cell migration speed was calculated simply by dividing the total length of the trajectories (total distance covered, hence not taking the directionality into account) by the time of the trajectory for each cell as one data point.

Bare SLB (DOPC with TopFluor-PE)

10 μ M C16:0-RGD

10 μ M C16:0-Rhd/RGD

5 μ M Chol-RGD

Figure S4. Overview of hMSC adhesion and spreading on DOPC SLBs (with TopFluor-PE lipid). (A) Bare DOPC SLBs (no peptide). DOPC SLBs incubated with (B) 10 μ M C16:0-RGD, (C) 10 μ M C16:0-Rhd/RGD and (D) 5 μ M Chol-RGD. Excess peptide was washed away prior to cell seeding. (Blue: nucleus and red: actin, scale bar: 40 μ m). (Actin staining was performed with a far-red dye to exclude rhodamine signal and false colored as red in this Figure, see experimental section, $t=18$ h).

In order to study the lamellipodial dynamics, cells were first allowed to spread on the surfaces (for C16:0-Rhd/RGD and Chol-RGD, around 6h and 8h after cell seeding, respectively). Then, a vector was drawn from the center of the cell to the defined edge of a lamellipodial extension using ImageJ. The length of the vector was adjusted based on the extension or retraction of lamellipodia in each consecutive frame, measured and reported in terms of displacement every 5 min (in reference to previous frame), corresponding to the time scale of image acquisition, for 30 min.

Furthermore, on functional SLB surfaces, quantification of domain size at the start of the experiment (using the first frame, 20 min after cell seeding, for 10 randomly selected domains) was performed using ImageJ.

5.5.6. Immunofluorescence

PFA fixed cells were permeabilized in 0.5% Triton X-100 in PBS (Sigma) for 10 min at room temperature. After permeabilization, cells were incubated with 0.1% Triton X-100 and 5% (w/v) bovine serum albumin (BSA, Sigma) solution in PBS for at least 20 min to block nonspecific sites. Afterwards, cells were incubated with a solution of AlexaFluor-647 phalloidin or AlexaFluor-568 phalloidin (Molecular Probes, Thermo Fisher Scientific, 1:100) and monoclonal anti-human vinculin-FITC antibody (anti-hVIN-1-FITC, Sigma Aldrich, 1:200) in 0.1% Triton X-100 and 5% (w/v) BSA in PBS for 1h at room temperature in the dark. Far-red actin staining was used in order to be able to multiplex with rhodamine for the case of C16:0-Rhd/RGD presenting SLBs and also used for the other conditions for the experiments described in Sections 5.2.2 and 5.2.3. For vinculin localization, in order to exclude the interference from TopFluor dye, SLBs formed with DOPC LUVs without Topfluor-PE lipid was studied (for Sections 5.2.2 and 5.2.3). For the experiments performed to investigate phosphorylated paxillin (phosphorylated at Y31, pPax) levels, after the blocking step, cells were incubated with rabbit-anti-human paxillin (phospho Y31) antibody (Abcam, ab32115, rabbit monoclonal antibody, 1:500) overnight at 4°C in 0.1% Triton X-100 and 5% (w/v) BSA. Subsequently, cells were gently washed with PBS 3X (10 min each). Then, secondary antibody (AlexaFluor-647 goat-anti-rabbit IgG (H+L), Molecular Probes, Thermo Fisher Scientific, 1:500) was simultaneously incubated with AF-568 phalloidin and anti-hVIN-1-FITC as given above in 0.1% Triton X-100 and 5% (w/v) BSA. After washing 3X with PBS to remove excess molecules, cells were incubated with DAPI nuclear stain in PBS (1:1,000) for 10 min and washed 2X with PBS (10 min each). Imaging was performed with OPERA high content screening system (Perkin Elmer) with corresponding objectives and filter combinations.

For pPax staining, as a background control check, cells were stained with only secondary antibody and without primary antibody; and background subtraction was performed accordingly in the fluorescence images for pPax channel.

Characterization of cell shape factors

Image analysis was performed using ImageJ and Cell Profiler image analysis software (Broad Institute of MIT and Harvard). Quantification of cell shape factors were

performed by using Cell Profiler algorithms. For these analyses, more than 400 cells were taken into account for each condition. For visualization and readability purposes, the brightness of the images in the Figures were adjusted when needed. However, for all the data treatment including quantification, raw images were used following background subtraction.

5.5.7. Statistics

All experiments were performed in triplicates and data are presented as mean $\pm$ standard deviation. Data were analyzed using Kruskal-Wallis one way-ANOVA for statistical significance. Multiple comparison tests were performed with Mann-Whitney U test by adjusting α level downward according to Bonferroni correction comparing all groups of interest (OriginPro v.9, OriginLab).

5.6. Acknowledgments

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5.7. References

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5.8. Videos

Live cell imaging videos of hMSCs cultured on different LCN surfaces which were used for Figures 5.11, 12, 13, 14 and 15 are available:

Video 5.1. Bare DOPC-TF (20 min-15h:20 min)

Video 5.2. C16:0-Rhd/RGD condition, overlay of TopFluor-PE and cells in transmission mode (20 min-15h:20 min).

Video 5.3. C16:0-Rhd/RGD condition, overlay of Rhodamine and cells in transmission mode (20 min-15h:20 min).

Video 5.4. Chol-RGD condition, overlay of TopFluor-PE and cells in transmission mode (20 min-15h:20 min).

Video 5.5. C16:0-RGD condition, overlay of TopFluor-PE and cells in transmission mode (20 min-15h:20 min).

Chapter 6

Exploring a dual peptide presentation on supported lipid bilayers to target integrins and cadherins on endothelial cells

Endothelial cells play a central role in the vascular system, where their function is tightly regulated by both cell-ECM (e.g., via integrins) and cell-cell interactions (e.g., via cadherins). In this chapter, we explored a cell-instructive SLB system to target both integrins and cadherins (i.e., neural (N)-Cadherin) on endothelial cells. Fluid DOPC SLBs were functionalized with integrin targeting lipidated (Cholesterol)-RGD and N-Cadherin targeting Cholesterol (Chol)-HAVDI peptides, where these peptides were presented either individually or at defined molar combinations (1:10, 1:1 and 10:1, Chol-RGD:Chol-HAVDI). First we show that, by studying SLBs with only Chol-RGD conjugation, human umbilical vein endothelial cell (HUVEC) adhesion, spreading and vinculin localization (i.e., focal adhesion formation) in these cells are strongly dependent on ligand density. Second, we demonstrate that the dual presentation of these peptides at a specific molar ratio (1:10) results in a higher extent of cell spreading, where cells exhibited extensive lamellipodia formation. Furthermore, a qualitatively more distinct N-Cadherin localization at the cell periphery was observed for this molar ratio, compared to all other conditions, as indicative of N-Cadherin clustering and mimic of cell-cell contact formation. The antibody blocking (of N-Cadherin on the cell membrane) experiments confirmed the specificity of N-Cadherin-Chol-HAVDI interactions, altogether demonstrating the dual functionality of these SLBs.

6.1. Introduction

Endothelial cells form the linings of the blood vessels, where their interactions with the extracellular matrix (i.e., basal lamina, targeting integrins) as well as with neighboring cells (i.e., cell-cell interactions, targeting cadherins) are critical for blood vessel development and stabilization.^[1] In this process, heterotypic cell-cell (i.e., with another cell type) contacts that are formed with pericytes, across their shared basal lamina, are important for blood vessel stabilization and function, and are known to be mediated by neural (N)-Cadherin.^[1a, 2] Therefore, it is interesting to mimic endothelial cell-pericyte interactions (via targeting N-Cadherin on endothelial cells) in the development of cell-instructive biointerfaces for biomedical applications such as stent endothelialization.

Being cell membrane mimetics, supported lipid bilayers (SLBs), have provided excellent platforms to target cadherins and to study cell-cell junction formation in relation to SLB fluidity.^[3] Furthermore, as explained before (reviewed in Chapter 1, and also presented in Chapters 3-5), SLBs have also been exploited to target integrins, to study receptor-ligand interactions as well as to guide (stem) cell behavior in relation to ligand (i.e., RGD) density and mobility.^[4] However, so far no study reported the dual presentation of integrin and cadherin targeting ligands on SLB biointerfaces, which would represent a better biomimetic approach to reconstitute the endothelial-pericyte interface in which both interactions are intricately related and determine cellular and tissue development (for an extended view please refer to Chapter 1).^[1b, 1c, 5]

Here, by taking the advantage of versatility of SLB-functionalization via lipid insertion, we presented two ligands, in fluid DOPC SLBs, that target integrins and cadherins on the endothelial cell membrane. In order to target integrins, we again used a cholesterol-conjugated RGD (Chol-RGD) peptide (see Chapter 5). In order to target cadherins, we used a peptide that is derived from the extracellular domain 1 (EC1) of N-Cadherin (i.e., HAVDI) and was shown to be bio-active and specifically interacting with N-Cadherin.^[6] We introduced these peptides to SLBs either individually or at different molar ratios (as 1:10, 1:1, 10:1, Chol-RGD:Chol-HAVDI), keeping the peptide concentration in solution constant (**Scheme 6.1**). By investigating the endothelial (HUVEC) cell response on only Chol-RGD functionalized SLBs, we show that also endothelial cell adhesion, spreading and focal adhesion formation in these cells are strongly dependent on ligand density (i.e., Chol-RGD coverage). We then demonstrate that the dual peptide presentation, at a specific molar ratio (1:10), improved cell spreading, compared to each of the individual peptides, suggesting a combined effect of these peptides at this molar ratio.

Furthermore, we show that at this 1:10 molar ratio, cells had qualitatively a more distinct localization of N-Cadherin in the cell-periphery, suggesting N-Cadherin clustering, resembling cell-cell contact formation, where N-Cadherin-HAVDI interactions were shown to be specific. Altogether, these results show the possibility to tune the complexity of cell-surface interactions on SLBs, to guide and investigate tissue type specific cell behavior.

Scheme 6.1. Scheme for the study presented in this Chapter where integrin targeting Chol-RGD and N-Cadherin targeting Chol-HAVDI peptides were introduced in fluid DOPC SLBs, resulting in cell-instructive SLBs with dual functionality, to study endothelial cell adhesion and spreading behavior.

6.2. Results and Discussion

6.2.1. Endothelial cell adhesion and spreading on Chol-RGD functionalized SLBs

As explained above, cell-ECM interactions that are regulated by integrins have important consequences on endothelial cell behavior.^[1b, 1d] Here, we first studied the effect of RGD ligand density on endothelial cell adhesion, spreading and focal adhesion formation, on fluid DOPC SLBs. For this purpose, as also explained in Chapter 5 (Section 5.2.1), DOPC SLBs were functionalized with Chol-RGD peptide at 0.5 μM and 2 μM concentration (leaving the peptide in solution), resulting in surface coverages in the range of 20-30% and 50-60%, respectively (based on spectroscopic ellipsometry (SE) measurements, as discussed in detail in the thesis of Mark Verheijden, in a collaboration with Prof. Ralf Richter). It is important to note that these concentrations of Chol-RGD peptide were chosen based on initial experiments (data not shown) where at 0.1 μM (2-5% coverage)

and $0.2\ \mu\text{M}$ (10-15% coverage), cells had a lower extent of cell adhesion compared to $0.5\ \mu\text{M}$ (20-30% coverage) concentration.

Figure 6.1. Overview of endothelial cell (HUVEC) adhesion on DOPC SLBs incubated with (A) $0.5\ \mu\text{M}$ (20-30% coverage) and (B) $2\ \mu\text{M}$ Chol-RGD (50-60% coverage). HUVEC adhesion on (C) bare DOPC SLBs and (D) fibronectin coated glass surfaces ($t=24\text{h}$, blue: nucleus, red: actin, scale bar: $40\ \mu\text{m}$).

Interestingly, we saw that at 20-30% peptide coverage ($0.5\ \mu\text{M}$), cell adhesion and spreading was improved, compared to 50-60% coverage ($2\ \mu\text{M}$) (**Figure 6.1A-B**). Importantly, on bare (pure) DOPC SLBs, cells remained round and in clusters, while fibronectin coated glass allowed us to monitor cell adhesion and morphology over time (Figure 6.1C-D).

Observations on vinculin localization in cells as a marker of focal adhesion formation,^[7] were in line with the observations on cell adhesion and spreading. A more distinct vinculin localization at the cell periphery could be seen for 20-30% peptide coverage ($0.5\ \mu\text{M}$), compared to 50-60% coverage where rather diffused vinculin was seen also at

lamellipodial extensions (**Figure 6.2A-B**). On bare SLBs, cell adhesion and vinculin localization were impaired, showing that endothelial cell adhesion on these surfaces occurred specifically due to the presence of Chol-RGD peptide (Figure 6.2C).

Figure 6.2. Vinculin localization in HUVECs on DOPC SLBs incubated with (A) 0.5 μM (20-30% coverage) and (B) 2 μM Chol-RGD (50-60% coverage). HUVEC adhesion on (C) bare DOPC SLBs and (D) fibronectin coated glass surfaces ($t=24\text{h}$, blue: nucleus, red: actin and green vinculin, scale bar: 20 μm).

Besides, again in line with the results given in previous Chapters, cells on fibronectin (hence ECM protein) coated glass controls had a more pronounced focal adhesion and stress fiber formation, compared to Chol-RGD functionalized surfaces (Figure 6.2D). These results also show that, similar to what was observed in hMSCs, focal adhesion sensing in endothelial cells on RGD-functionalized SLBs is different than for the cells on fibronectin coated glass.

Figure 6.3. Cell shape factors for HUVECs seeded on Chol-RGD conjugated DOPC SLBs (at two different ligand coverages as indicated) ($t=24\text{h}$, *: $p < 0.05$, $n \geq 250$, each data point represents one cell, mean $\pm$ standard deviation, red line represents the mean value).

Finally, cellular morphological parameters such as cell area, eccentricity (approaches 1 for highly elongated cells), extent (approaches 1 for compact cells with less protrusions) and form factor (approaches 1 for highly circular cells) were quantified in order to further reveal endothelial cell response to ligand density on these SLBs (Figure 6.3 and Table 6.1). Cells on DOPC SLBs at 20-30% coverage (0.5 μM) had a significantly higher cell spreading area (555 ± 309 vs. $418 \pm 297 \mu\text{m}^2$, for 20-30% vs. 50-60% respectively), were more elongated (eccentricity: 0.72 ± 0.16 vs. 0.68 ± 0.16 , respectively), had a higher degree of

protrusions (extent: 0.57 ± 0.13 vs. 0.62 ± 0.13 , respectively) and these cells were less circular (form factor: 0.55 ± 0.19 vs. 0.63 ± 0.19 , respectively) compared to the cells seeded on 50-60% coverage ($2 \mu\text{M}$). These results demonstrate that there may be an optimum ligand density for HUVECs on Chol-RGD functionalized SLBs, to regulate their adhesion, focal adhesion formation and morphological behavior. This is in line with literature, where an optimum surface density (6×10^{10} RGD molecules/ cm^2) of covalently immobilized RGD was detected to for endothelial cell adhesion, cell area and vinculin localization in endothelial cells.^[8] In our case, 20-30% coverage corresponds to an average density of 3×10^{13} molecules/ cm^2 , which shows that on fluid (i.e., laterally dynamic) DOPC SLBs a higher ligand density is required compared to covalent surfaces.^[8-9] These results may also indicate a cell-type specific response on Chol-RGD functionalized SLBs, as for hMSCs, we did not see a decrease in cell spreading when going to higher densities than $0.5 \mu\text{M}$ (i.e., $5 \mu\text{M}$ as given in Chapter 5). However, a direct comparison between two cell types when comparing the same concentrations of the peptides would be necessary to further understand the differences.

Table 6.1. Quantification of cell shape factors for HUVECs seeded on Chol-RGD conjugated DOPC SLBs and fibronectin coated glass ($t=24\text{h}$).

	Cell Area (μm^2)	Eccentricity	Extent	Form Factor
Chol-RGD 20-30% Coverage	555 ± 309	0.72 ± 0.16	0.57 ± 0.13	0.55 ± 0.19
Chol-RGD 50-60% Coverage	418 ± 297	0.68 ± 0.16	0.62 ± 0.13	0.63 ± 0.19
Fibronectin-coated glass	$2,958 \pm 1,846$	0.79 ± 0.14	0.51 ± 0.11	0.41 ± 0.12

Finally, on fibronectin coated glass, cells had a much higher degree of spreading (cell area: $2,958 \pm 1,846 \mu\text{m}^2$), were more elongated (eccentricity: 0.79 ± 0.14), less circular (form factor: 0.41 ± 0.12) with a higher degree of protrusions (i.e., less compact, extent: 0.51 ± 0.11).

6.2.2. Exploring the effects of dual peptide presentation on SLBs to target integrins and cadherins on endothelial cells

Next to cell-ECM interactions, cell-cell communication that is regulated mainly via classical cadherins is critical for vascular homeostasis.^[1c, 2a] As explained above, the contacts between endothelial cells and pericytes, which involve N-Cadherin, is one such important interaction that regulates cell-cell communication in the vascular system.^[1c, 2a, 2c, 2e] Knowing that on our functional SLB biointerfaces we could guide endothelial cell adhesion and spreading in the context of integrin signaling (i.e., mimicking cell-ECM interactions), we next studied the effect of introducing an N-Cadherin targeting peptide in SLBs on endothelial cell (HUVEC) behavior.

Figure 6.4. QCM-D measurements to evaluate the insertion of Chol-HAVDI in DOPC SLBs (already present at the start of the measurements) compared with the insertion of Chol-RGD. Next to individually presented peptides, a 1:1 molar combination of the peptides was introduced to DOPC SLBs to evaluate dual peptide presentation in these experiments. Bare HAVDI is shown as a control peptide to evaluate the specificity of peptide insertion via the cholesterol anchor (F : frequency and D : dissipation).

For this purpose, an N-Cadherin mimetic (and hence N-Cadherin targeting) peptide (i.e., HAVDI)^[6] was synthesized and conjugated with cholesterol, as in the case of RGD peptide, hence allowing SLB functionalization via lipid insertion as well as keeping the anchor type the same for both peptides. Insertion of Chol-HAVDI in SLBs was verified with QCM-D experiments while comparing the extent of the insertion to that of Chol-RGD peptide at the same time (**Figure 6.4**). In addition, in order to investigate the possibility to introduce the dual functionality into DOPC SLBs (i.e., presenting both peptides together) in these experiments, insertion was studied and again verified at a 1:1 molar ratio of both peptides. As a control peptide, interaction of bare HAVDI (without cholesterol conjugation) with SLBs was also studied with QCM-D, where no peptide insertion was observed, showing that the insertion occurred specifically due to the presence of cholesterol anchor on the peptide (**Figure 6.4**).

Showing that we could achieve functional surfaces with Chol-HAVDI (as well as a dual peptide presentation as combined with Chol-RGD), we next studied HUVEC adhesion and spreading on DOPC SLBs where these two peptides were either presented individually or together at defined molar ratios (i.e., 10:1, 1:10 and 1:1 Chol-RGD:Chol-HAVDI). In order to functionalize the surfaces, SLBs were incubated with 0.5 μM of either individual peptides or at given molar ratios (keeping the peptide in solution) as explained before (Section 6.2.1, Chapters 4 and 5). Peptide concentration in each case was kept constant. We chose 0.5 μM total peptide concentration based on the observations on HUVEC adhesion and spreading with only Chol-RGD peptide as explained in the previous section (Section 6.2.1). It is important to note that, for Chol-HAVDI we could not report a peptide coverage (i.e., density) on SLB surfaces, as we could not exclude the effect of bound water in QCM-D measurements on the degree of mass adsorption (i.e., frequency shift)^[10] when this peptide inserted in SLBs. This is in contrast to Chol-RGD case where spectroscopic ellipsometry (SE) measurements allowed us to calculate the dry mass of the peptide, hence could give a more accurate measure of surface coverage (as pointed out in Chapter 4 and explained in more detail in PhD thesis of Mark Verheijden). Therefore, here by keeping the total peptide concentration in solution constant, we aimed at introducing an equal total number of moles of peptides to SLB surfaces, without reporting a coverage in these experiments. Next to functional SLBs, we again used bare SLBs as negative control for cell adhesion. In order to monitor cell adhesion and spreading over time as well as to compare with cell behavior on functional SLBs, in these experiments, we used laminin-coated glass since laminin is one of the important components of endothelial cell ECM (i.e., basal lamina).^[1b]

Figure 6.5. Overview of HUVEC adhesion on DOPC SLBs conjugated with (A) Chol-RGD, (B) Chol-HAVDI, (C) 10:1 (mol:mol) Chol-RGD:Chol-HAVDI, (D) 1:10 (mol:mol) Chol-RGD:Chol-HAVDI and (E) 1:1 (mol:mol) Chol-RGD:Chol-HAVDI peptides. Total peptide concentration incubated with SLBs was kept at $0.5 \mu\text{M}$ (blue: nuclei and red: actin, scale bar: $40 \mu\text{m}$).

In order to understand the effect of individual and dual peptide presentation (at given molar ratios) on cell behavior, we first studied cell adhesion, spreading, cellular morphology as well as cellular organization on functional DOPC SLBs after 24h (**Figure 6.5**). When we compared only Chol-RGD and Chol-HAVDI functionalized SLBs, we observed that on Chol-RGD, cells had a higher degree of spreading and protrusion formation, showing a distinct regulation of cytoskeletal organization in cells in response to these two different peptides (Figure 6.5A-B).^[11]

Figure 6.6. Overview of HUVEC adhesion on bare DOPC and laminin coated glass as control groups (blue: nuclei and red: actin, scale bar: 40 μm).

Interestingly, cells on SLBs that were functionalized at 1:10 molar ratio (Chol-RGD:Chol-HAVDI) of two peptides (dual functionalization), seemed to have the highest degree of spreading among all functional SLBs. Besides, at this molar ratio (1:10), compared to other conditions, an extensive lamellipodia formation in polarized cells was observed, where cells also exhibited thick peripheral actin localization (Figure 6.5D). As lamellipodial extensions are involved in cell migration, these observations may indicate a migratory phenotype for cells at this peptide ratio, which should be further investigated with time-lapse imaging.^[12] Furthermore, at 1:1 molar ratio, a qualitatively higher degree of contact formation (i.e., endothelial cell-cell contacts) between cells was observed compared to other functional SLBs, even though cells remained relatively round (discussed below) (Figure 6.5E). This was not merely a clustering of the cells that we would normally observe on a bare SLB due to impaired cell adhesion, but rather indicated a higher degree of endothelial cell-cell communication when cells adhered and started to spread on these surfaces (1:1 Chol-RGD:Chol-HAVDI) (Figure 6.5E, also shown below). Among all dual-functional SLBs, at 10:1 molar ratio, cells appeared to be smaller

and rounder (discussed below) (Figure 6.5C). Altogether, these results clearly show that cells were sensitive to peptide composition on the surfaces, both when presented individually and at defined molar ratios, which induced differential regulation of cellular morphology as well as cellular organization on functional SLBs. Importantly, again on bare SLBs, cell adhesion was impaired, where on laminin-coated glass, classical morphology of endothelial cells could be observed (**Figure 6.6**).^[1b]

Figure 6.7. N-Cadherin localization in representative HUVECs on DOPC SLBs conjugated with Chol-RGD, Chol-HAVDI and with different molar ratios of these two peptides as well as on bare (pure) DOPC SLBs ($t=24h$, blue: nuclei, red: actin and green: N-Cadherin, scale bars: 20 μm).

Given that endothelial cells (HUVECs) were sensitive to Chol-HAVDI presentation (both individually and in combination with RGD), we next investigated N-Cadherin localization with a closer look up to the cells, in order to understand how cell-functional SLB interactions were mediated via N-Cadherin in cells (**Figure 6.7**, **Figure S3** and **Figure S4**). Intriguingly, among all functional groups, at 1:10 molar ratio, cells had a qualitatively more distinct N-Cadherin localization at the cell periphery, which is indicative of N-

Cadherin clustering and mimics N-Cadherin based cell-cell junction formation between cells and the SLB (Figure 6.7, 1:10 (RGD:HAVDI)).^[3a, 13] In addition, even though we did not quantify here, N-Cadherin localization at the cell periphery (mainly in lamellipodial extensions) was seen to coincide with actin localization at this molar ratio, in line with the role of actin cytoskeleton in cell-cell junction formation and regulation.^[11a] For all other conditions, N-Cadherin was generally rather diffused throughout the cell body (i.e., in cell membrane) even though at 1:1 ratio, we could observe an onset of N-Cadherin localization at cell periphery, yet very slightly (Figure 6.7). Importantly, on bare DOPC SLBs, no functional N-Cadherin localization or cell adhesion was observed, pointing out the specificity of cell adhesion on functional SLBs. Taken together, these results show that next to cellular and cytoskeletal organization on SLBs, N-Cadherin localization was also sensitive to the peptide composition (i.e., ratio of Chol-RGD and Chol-HAVDI peptides) on functional SLBs. Further insight could be gained through the investigation of signaling molecules involved in cadherin signaling, such as β -catenin as well as α -catenin, which is known to link cadherins to actin filaments.^[3a, 14]

Next we quantified cell area and cell shape factors in order to better reveal the morphological consequences of individual and dual peptide presentation at defined molar ratios (**Figure 6.8** and **Table 6.2**). In line with the observations stated above on cellular morphology, cells on Chol-RGD had a significantly higher cell spreading area compared to the cells on Chol-HAVDI (cell area: 376 ± 262 vs. $253 \pm 127 \mu\text{m}^2$, for Chol-RGD vs. Chol-HAVDI, respectively). In fact, among all functional SLBs, Chol-HAVDI induced the smallest cell area, the most circular phenotype (i.e., the highest value for form factor) as well as the lowest degree of protrusions and elongation (the highest value of extent and lowest value for eccentricity, respectively) (Figure 6.8 and Table 6.2). When comparing all functional groups (both individual and dual peptide presentation), at 1:10 molar ratio, cells had the highest spreading area and the lowest degree of circularity (i.e., the lowest value for form factor, Figure 6.8 and Table 6.2) as well as they belonged to the group with higher degree of protrusions (i.e., lower value for extent). Interestingly, among the dual peptide ratios (10:1, 1:1 and 1:10 Chol-RGD:Chol-HAVDI), when compared to Chol-RGD functionalized SLBs, we observed improved cell spreading only at 1:10 molar ratio (cell area: 448 ± 352 vs. $376 \pm 262 \mu\text{m}^2$, for 1:10 ratio vs. Chol-RGD only, respectively) while at other two ratios, cell area was smaller compared to Chol-RGD only case (cell area: 280 ± 180 and $295 \pm 186 \mu\text{m}^2$, for 10:1 and 1:1 ratios, respectively). These results point out the presence of an optimum molar ratio to improve cell spreading, compared to Chol-RGD case.

Figure 6.8. Cell shape factors for HUVECs seeded on DOPC SLBs functionalized with different combinations of molar ratios of Chol-RGD and Chol-HAVDI peptides ($t=24\text{h}$, *: $p < 0.05$, each data point represents one cell, mean $\pm$ standard deviation, red line represents the mean value, $n=190$ for Chol-RGD, $n=65$ for Chol-HAVDI and $n=82, 212$ and 153 , for 10:1, 1:10 and 1:1 molar ratios, respectively).

These observations did not only provide important implications on how cell morphology was regulated by the composition of integrin and cadherin targeting peptides on SLB biointerfaces, but also introduced interesting insights on how cells may be sensing individual vs. dual peptide presentation. For example, even though cells on Chol-RGD had a higher degree of spreading compared to the cells on Chol-HAVDI, when these peptides were presented together, higher Chol-RGD ratio did not induce higher cell spreading. In

contrast, as already mentioned above among the dual peptide ratios, when going from 10:1 to 1:1 ratio (i.e., increasing the relative amount of Chol-HAVDI in SLBs) a slight increase in cell area was observed, where 1:10 ratio (Chol-RGD:Chol-HAVDI) induced the biggest cell area (Figure 6.8 and Table 6.2). Altogether, these results indicate that endothelial cell behavior on ratios or RGD/HAVDI ligands may not be just the sum of the behaviors on two individually presented peptides, as cells may have sensed combinations very differently. It is known that there is a cross-talk between integrin and cadherin mediated adhesions where they share signaling effector molecules, interact with common scaffolding proteins as well as they both connect to actin cytoskeleton.^[5] One of the suggested mechanism of this cross-talk is where the (de)activation of one adhesion type might influence the activity of the other adhesion type.^[5, 15] In this line, it would be interesting to investigate if there is such a cross-talk between integrins and cadherins when we present the peptides in combination in SLBs, in order to be able to reveal mechanisms of observed cell behavior. For this purpose, next to N-Cadherin localization, integrin localization in cells could be investigated. Next to that, studying vinculin (as a scaffolding protein for both type of adhesions) as well as phosphorylated paxillin localization at integrin adhesions and β -catenin localization at cadherin adhesions and co-localization studies could give important insights on cell behavior on these SLBs.^[6d, 11b, 14, 16]

Table 6.2. Quantification of cell shape factors for HUVECs seeded on DOPC SLBs functionalized with Chol-RGD, Chol-HAVDI and different molar ratios of two peptides (t=24h).

	Cell Area (μm^2)	Eccentricity	Extent	Form Factor
Chol-RGD	376 $\pm$ 262	0.70 $\pm$ 0.16	0.60 $\pm$ 0.13	0.61 $\pm$ 0.19
Chol-HAVDI	253 $\pm$ 127	0.68 $\pm$ 0.14	0.64 $\pm$ 0.11	0.67 $\pm$ 0.17
10:1 Chol-RGD:Chol-HAVDI	280 $\pm$ 180	0.70 $\pm$ 0.16	0.62 $\pm$ 0.12	0.65 $\pm$ 0.18
1:10 Chol-RGD:Chol-HAVDI	448 $\pm$ 352	0.69 $\pm$ 0.18	0.61 $\pm$ 0.13	0.60 $\pm$ 0.19
1:1 Chol-RGD:Chol-HAVDI	295 $\pm$ 186	0.70 $\pm$ 0.17	0.61 $\pm$ 0.14	0.64 $\pm$ 0.18

Finally, even though the specific interaction of HAVDI peptide with N-Cadherin was previously demonstrated,^[6c] we aimed at validating the interactions in the context of our experiments. For this purpose, before they were seeded on Chol-HAVDI functionalized SLBs, endothelial cells (HUVECs) were pre-incubated with a specific antibody which targets the half of the extracellular domain of N-Cadherin and is known to block N-Cadherin function.^[6c, 17] Cell adhesion, spreading and N-Cadherin localization in cells when they were pre-incubated with the antibody (i.e., blocking condition, see experimental section) was compared to the case where cells were not treated with the antibody (non-blocking condition) on Chol-HAVDI functionalized SLBs. In addition, as an important control, non-treated cells (without antibody blocking) were seeded on SLBs which were incubated with bare HAVDI peptide, which was shown not to insert into SLBs (Figure 6.4D).

Figure 6.9. HUVEC adhesion on (A) Chol-HAVDI, (B) Chol-HAVDI where cells were pre-incubated with N-Cadherin (extracellular domain) targeting antibody and (C) on bare HAVDI (no cholesterol conjugation) ($t=24h$, blue: nuclei and red: actin, scale bars: 40 μm , in this experiment peptides were dissolved in PBS prior to incubation with SLBs).

It was observed that cells in the blocking condition had a smaller area and were qualitatively rounder when compared to the cells in the non-blocking condition (**Figure 6.9A-B**). Furthermore, for bare HAVDI conditions, cells remained morphologically similar to the blocking condition (**Figure 6.9C**). Besides, for blocking and bare HAVDI conditions, N-Cadherin profiles in cells resembled the profile of the cells on bare DOPC SLBs (**Figure 6.7** and **Figure 6.10B-C**). On the other hand, when cells adhered and started to spread, functional N-Cadherin based adhesions were observed for non-blocking condition, indicating the specificity of the interactions.^[6c] It is important to note that bare DOPC SLB controls are not shown for this experiment due to negligible cell adhesion observed on these surfaces.

Figure 6.10. N-Cadherin localization in HUVECs seeded on (A) Chol-HAVDI-SLB, (B) Chol-HAVDI-SLB where cells were pre-incubated with N-Cadherin (extracellular domain) targeting antibody and (C) on bare HAVDI (no cholesterol conjugation) incubated SLBs ($t=24h$, blue: nuclei, red: actin and green: N-Cadherin, scale bars: 20 μm , in this experiment peptides were dissolved in PBS prior to incubation with SLBs).

Besides, quantification of cell area and cell shape factors showed that cells on blocking conditions had a significantly lower cell area compared to non-blocking condition ($381 \pm 223 \mu\text{m}^2$ vs. $313 \pm 206 \mu\text{m}^2$, for non-blocking vs. blocking, respectively) and were more circular (form factor : 0.69 ± 0.19 vs. 0.70 ± 0.19 , for non-blocking vs. blocking, respectively) (Figure 6.11 and Table 6.3).

Figure 6.11. Cell shape factors on Chol-HAVDI-SLBs, for HUVECs that were pre-incubated with N-Cadherin antibody (blocking) on Chol-HAVDI-SLBs and on bare HAVDI incubated SLBs ($t=24\text{h}$, *: $p<0.05$, each data point represents one cell, mean $\pm$ standard deviation, red line represents the mean value, in this experiment peptides were dissolved in PBS prior to incubation with SLBs).

Furthermore, even though for bare HAVDI-SLBs cell area seemed to be at similar levels to non-blocking (Chol-HAVDI-SLBs) condition, cells had the highest degree of circularity among all the groups (form factor: 0.71 ± 0.19 vs. 0.69 ± 0.19 , for bare HAVDI-SLBs vs. non-blocking (Chol-HAVDI-SLBs), respectively), in line with the observations above for N-Cadherin localization in cells, which did not show a functional adhesion.

Table 6.3. Quantification of cell shape factors on Chol-HAVDI-SLBs, for HUVECs that were pre-incubated with N-Cadherin antibody (blocking) on Chol-HAVDI-SLBs and on bare HAVDI incubated SLBs ($t=24h$).

	Cell Area (μm^2)	Form factor	Eccentricity	Extent
Chol-HAVDI	381 ± 223	0.69 ± 0.19	0.63 ± 0.19	0.66 ± 0.12
Chol-HAVDI (blocking)	313 ± 206	0.70 ± 0.19	0.64 ± 0.17	0.67 ± 0.12
Bare HAVDI	385 ± 229	0.71 ± 0.19	0.61 ± 0.18	0.66 ± 0.12

6.3. Conclusions and outlook

In conclusion, here we show the possibility to fabricate a cell-instructive SLB platform that demonstrates a dual functionality, allowing to simultaneously target integrins and cadherins on endothelial cell membranes. We first showed that endothelial cell (HUVEC) adhesion, spreading and focal adhesion formation was strongly dependent on the density of Chol-RGD (integrin targeting) peptide, as was studied when this peptide was presented alone in DOPC SLBs. Second, by presenting an N-Cadherin targeting peptide (Chol-HAVDI) in SLBs, either individually or in combination with Chol-RGD at defined molar ratios, we showed that cell spreading, morphology and N-Cadherin localization in cells are sensitive to the peptide composition (i.e., the relative amount of Chol-RGD and Chol-HAVDI in SLBs). We also showed that at one defined molar ratio (1:10 Chol-RGD:Chol-HAVDI), endothelial cells had the biggest cell area and exhibited a qualitatively more distinct N-Cadherin localization, compared to all other functional SLBs. Future studies should bring more insight on the molecular mechanisms of cell behavior by investigating both integrin and N-Cadherin signaling in relation to integrin-cadherin

cross-talk.^[5] Furthermore, it would also be interesting to investigate vascular endothelial (VE)-Cadherin localization in these cells and its relation to N-Cadherin localization, on these SLB biointerfaces.^[1c, 2c, 18] Besides, a better understanding of surface (functional SLB) properties and surface characterization would be necessary to determine the surface coverages of Chol-HAVDI peptide, as in the case of Chol-RGD peptide. Therefore, together with the experiments that could reveal the expression levels of N-Cadherin and integrins on the cell membrane, we could have a better understanding on cell-surface interactions in relation to ligand density in SLBs.^[19]

6.4. Experimental Section

6.4.1. Peptide synthesis and purification

Cholesterol (Chol)-RGD (KGSGRGDSG) was synthesized and purified using established methods available in our group (explained in the thesis of Mark Verheijden). Mass spectrometry analysis of purified peptide is given in Chapter 5. KNIDVAHARG (in short HAVDI) peptide was synthesized using an automatic solid phase synthesis robot (MultiSyntech, Syro II) following standard Fmoc chemistry on Rink amide resin (Multisyntech Chemicals GmbH). After the synthesis and final Fmoc deprotection step, the resin was washed with dichloromethane (DCM) and methanol and dried under vacuum. In order to conjugate cholesterol to the N-terminus of the peptide (hence giving Chol-HAVDI) a solution of N-hydroxysuccinimide (NHS)-activated cholesteryl hemisuccinate (5 molar equivalents with respect to the resin) and 1.1 molar equivalents of N,N-di-isopropylethylamine (DIPEA) in dimethylformamide (DMF) were added to the reaction vessels containing the peptides on the resin. The reaction was carried out for 5h at room temperature after which the resin was washed with DMF, DCM and methanol and dried under vacuum. The peptides were cleaved from the resin using a solution of trifluoroacetic acid (TFA):triisopropylsilane (TIS):H₂O (95:2.5:2.5 (v/v/v)) at room temperature for 3h and subsequently precipitated in cold H₂O. The peptide pellet was washed several times in cold H₂O with centrifugation and subsequently all H₂O was removed by centrifugation. Then the peptide was washed in methyl tert-butyl ether (MTBE) several times again using precipitation. Bare HAVDI (no cholesterol conjugation), was directly precipitated in cold diethyl ether after the cleavage. The crude peptides were purified using RP-HPLC (Reverse Phase High Pressure Liquid Chromatography, C18 Column, Waters, using water/acetonitrile gradient with 0.1% TFA). The purified peptides were analyzed by electrospray ionization mass spectrometry (**Figure S1** and **Figure S2**).

Figure S1. ESI-ToF spectrum of purified Chol-KNIDVAHARG (calc. $[M] = 1546.96$, found: $[M+2H^+] = 773.74$ m/z, $[M+H^+] = 1546.31$ m/z).

Figure S2. ESI-ToF spectrum of purified KNIDVAHARG (calc. $[M] = 1078.60$, found: $[M+3H^+] = 360.12$ m/z, $[M+2H^+] = 540.14$ m/z, $[M+H^+] = 1079.37$ m/z).

6.4.2. Surface characterization of cholesterol conjugated peptides-SLB interactions

Insertion of cholesterol conjugated peptides (Chol-RGD and Chol-HAVDI) into DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine, $T_m = -20^\circ\text{C}$, Avanti Polar Lipids) SLBs was characterized by quartz crystal microbalance with dissipation monitoring (QCM-D) system (Q-sense E1, Biolin Scientific), measuring the changes in resonance frequency (ΔF) when a material interacts with the surface. Changes in the resonance frequency of a quartz crystal sensor and the change in dissipation (ΔD) were measured providing information about the mass and the mechanical properties of material adhering to the sensor surface, respectively. SiO_2 coated QCM-D sensors (Q5X303, Biolin Scientific) were used. Before each use, sensors were cleaned with a 2% sodium dodecyl sulfate (SDS) solution, rinsed with MilliQ water and ethanol, dried in under nitrogen flow and finally activated using a UV/ozone chamber (Bioforce, Nanosciences). Measurements were performed under a 100 $\mu\text{L}/\text{min}$ flow and at 22°C . Throughout this work, the fifth overtone was used for the normalized frequency ($\Delta F_5/5$) and dissipation (ΔD_5), unless stated otherwise. As for DOPC SLB formation, the experiment was performed using 1X PBS (Gibco, pH=7.5) solution in order to give a higher ionic strength for vesicle rupture. After bilayer formation, the baseline was stabilized with MilliQ water in which the peptides were dissolved for QCM analysis. All peptides were handled with the same buffer and a titration experiment with different concentrations of the peptides was performed to study insertion. Next to Chol-RGD and Chol-HAVDI, bare HAVDI was used as a negative control for insertion, in order to show that insertion occurs specifically via the cholesterol linker. Furthermore, a mixture of Chol-RGD and Chol-HAVDI peptide solution at 1:1 molar ratio was used in order to study the dual insertion which is the case in cell experiments.

6.4.3. Surface preparation for cell experiments

For cell experiments, LUVs (large unilamellar vesicles) of pure DOPC lipids were prepared as described before (in Chapters 3, 4 and 5) and were diluted to a concentration of 0.4 mg/mL in sterile MilliQ water (MQ, Millipore, $18.2\text{ m}\Omega$). Vesicles were sterilized by filtering through 0.2 μm polyethersulfone (PES) membranes and SLB formation was performed at room temperature. The glass wells of 96-well plates (Sensoplate, Greiner Bio-one) were treated for 1h with sterile filtered 1 M NaOH to clean and activate the surface, followed by the removal of solution and washing the surfaces 10X with sterile MilliQ water. After the surface was cleaned, a vesicle suspension with a final concentration of

0.2 mg/mL prepared in 0.5X Dulbecco's modified phosphate buffered saline (DPBS, without CaCl_2 and MgCl_2) was added to each well for 1h incubation.

Pre-formed SLBs were washed with DPBS following serial dilutions, in order to avoid damage to the SLBs (leaving 100 μL DPBS/well, 10X, as explained in previous chapters). Before incubation with peptides, SLBs were further washed 5X, again using serial dilutions, with serum-free basic medium (see below), in order to add medium components in this step. Glass controls (i.e., glass wells) were incubated at a concentration of 10 $\mu\text{g/mL}$ either with fibronectin (Sigma) or laminin (Sigma), to monitor cell adhesion and spreading over time.

For the experiments that are described in Section 6.2.1, DOPC SLBs were functionalized with Chol-RGD peptide by incubating the SLBs with Chol-RGD solution (1.5 μM and 6 μM , 100 μL /well in 1X DPBS, stock concentrations) for at least 2h, at room temperature. For the experiments that are described in Section 6.2.2., Chol-RGD and Chol-HAVDI peptides were first dissolved in MilliQ water at 15 μM concentration and then diluted to 1.5 μM in 0.9XPBS (as stock concentration to add to SLBs). Then, peptide solutions (100 μL /well) of 1.5 μM were added either individually or at defined molar ratios of Chol-RGD:Chol-HAVDI (i.e., 1:10, 1:1 and 10:1) peptides (keeping the total peptide concentration the same), incubated for at least 2h. In both cases, no further washing was performed and the plates were used as such for cell experiments.

Besides functionalized SLBs, bare SLBs were used as negative controls for cell adhesion.

6.4.4. Cell culture and experiments on SLBs

Human umbilical vein endothelial cells (HUVECs, pooled donors, Lonza) were cultured in Endothelial growth medium (EGM) kit (Lonza or Promega, without exogenous VEGF to exclude associated effects of this growth factor) with 2% FBS (Sigma) and incubated at 37°C and 5% CO_2 in a humidified environment up to passage 4 and sub-cultured every 3 days or used for cell experiments. For cell adhesion and spreading experiments, cells at passage 4 were seeded at pre-studied density of 8,000 cells/ cm^2 with cell suspension adjusted to have 2% final serum concentration. Cells were incubated on surfaces for 24h and then they were fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature. After fixation, cells were washed 3X with PBS and left in PBS with 0.05% sodium azide (Sigma) and kept at 4°C for immunofluorescence analysis.

For characterization of cell-cell contact protein expression, cells were seeded at passage 4, at a seeding density of 7,500 cells/cm² on fibronectin coated glass surfaces (10 µg/mL, Sigma), and cultured for 4 days until they become a monolayer (**Figure S3**). Then cells were fixed as described above and used for immunofluorescence analysis (see section 6.4.6).

Figure S3. Characterization of (A) N-cadherin, (B) β -catenin and (C) VE-cadherin basal expression levels in HUVECs at p4 as used for this study (blue: nuclei and green: N-cadherin, β -catenin and VE-Cadherin, respectively, scale bar: 20 µm) (cells were seeded on fibronectin coated glass).

Figure S4. (A) Vinculin localization in HUVECs on laminin coated glass (blue: nuclei, green: vinculin and red: actin). (B) N-Cadherin expression and localization in HUVECs on laminin coated glass (blue: nuclei, green: N-cadherin and red: actin)(t=24h, scale bars: 20 μm).

6.4.5. Studying the specificity of cell-peptide interactions

For antibody blocking experiments, HUVECs were incubated with anti-GC-4 (50 $\mu\text{g/mL}$, Sigma), which binds to the N-terminal half of the extracellular domain of human N-cadherin. Cells were trypsinized (0.25% trypsin, 2 min) and incubated with antibody in 2% FBS in PBS buffer, for 45 min on ice as described in ref.^[6c] Cells used for seeding on non-blocking conditions (i.e., SLBs that were functionalized with Chol-HAVDI, bare SLBs, SLBs and protein coated glass substrates) were also incubated on ice and kept in buffer solution to treat the cells in the same way. Afterwards, cells were washed twice in buffer centrifuged at 1,000 rpm for 5 min and resuspended in growth media with adjusted serum concentration for cell experiments. The expression of N-Cadherin was determined via immunostaining of the HUVECs incubated on surfaces for 24h. Bare SLBs were used as additional negative controls for cell adhesion as described in previous sections.

6.4.6. Immunofluorescence

For characterization of cell adhesion (i.e., focal adhesion formation) and spreading on SLBs, actin cytoskeleton and vinculin stainings were performed. PFA fixed cells were permeabilized in 0.5% Triton X-100 in PBS (Sigma) for 10 min at room temperature. After permeabilization, cells were incubated with 0.1% Triton X-100 and 5% (w/v) bovine serum albumin (BSA, Sigma) solution in PBS for 1h to block nonspecific sites. Afterwards, cells

were incubated with a solution of AlexaFluor-568 phalloidin (Molecular Probes, Thermo Fisher Scientific, 1:100) and monoclonal anti-human vinculin-FITC antibody (anti-hVIN-1, Sigma Aldrich, 1:200) in 0.1 % Triton X-100 and 5% (w/v) BSA in PBS for 1h at room temperature, in the dark. Cells were gently washed with PBS 3X, for 10 min each. For nuclear staining in each experiment, after washing 3X with PBS to remove excess molecules, cells were incubated with DAPI in PBS (1:1,000) for 10 min and washed 2X for 10 min with 1X PBS.

For characterization of cell-cell contact protein expression, actin cytoskeleton, N-cadherin, β -catenin and Vascular endothelial (VE)-cadherin stainings were performed. PFA fixed cells were permeabilized in 0.5% Triton X-100 in PBS (Sigma) for 10 min at room temperature. After permeabilization, cells were incubated with 0.1% Triton X-100 and 5% (w/v) BSA (Sigma) solution in PBS for 1h to block nonspecific sites. Afterwards, cells were incubated with monoclonal mouse anti-human N-cadherin antibody (BD Transduction Laboratories, 1:500), mouse anti-human- β -catenin antibody (BD Transduction Laboratories, 1:500) and rabbit anti-human VE-cadherin antibody (Cell Signaling Technology, 1:500), overnight at 4°C in 0.1 % Triton X-100 and 5% (w/v) BSA. Subsequently, cells were gently washed with PBS 3X (10 min each). Then, secondary antibody (AlexaFluor-488 goat-anti-rabbit IgG or goat-anti-mouse IgG (H+L), Molecular Probes, Thermo Fisher Scientific, 1:1000) was simultaneously incubated with AF-568 phalloidin as given above in 0.1 % Triton X-100 and 5% (w/v) BSA. After washing 3X with PBS to remove excess molecules, cells were incubated with DAPI nuclear stain in PBS (1:1,000) for 10 min and washed 2X with PBS (10 min each).

Validation of the anti-GC-4 antibody using immunofluorescence (**Figure S5**), was performed by incubation of PFA fixed cells in 0.1% Triton X-100 and 5% (w/v) BSA in PBS (Gibco), for 1h, to block nonspecific sites. Then, cells were incubated with monoclonal mouse anti-human N-cadherin antibody (clone GC-4) (Sigma, 1:40) in 1% (w/v) BSA in PBS and incubated overnight. Cells were gently washed 3X with 1% (w/v) BSA in PBS, 10 min each. Afterwards, cells were incubated with secondary antibody (Molecular Probes, Thermo Fisher Scientific, 1:200) in 1% (w/v) BSA in PBS. Nuclear staining was performed as explained before.

Figure S5. HUVECs stained for the extracellular domain (EC) of N-Cadherin (with Anti-GC4 antibody used in blocking experiments) on fibronectin coated glass (scale bar: 20 μm , blue: nuclei and green: N-Cadherin (at EC)).

In order to reveal the effects of Chol-HAVDI presentation in SLBs on N-cadherin expression, actin cytoskeleton and N-cadherin stainings were performed as described above for cells that were cultured on functionalized SLBs at different conditions. Imaging was performed using OPERA high content screening system (Perkin Elmer) with corresponding objectives and filter combinations.

6.4.7. Data Analysis

All experiments were performed in triplicates. In section 6.2.1., Data were analyzed by using Mann-Whitney U test. In section 6.2.2, one-way ANOVA followed by Bonferroni post-hoc test was performed comparing all groups of interests, to test statistical significance (OriginPro v.9, OriginLab). Image analysis was performed using Image J (NIH) and Cell Profiler image analysis software (Broad Institute of MIT and Harvard). Quantification of cell number and cell shape factors were performed by using Cell Profiler algorithms. For these analyses, more than 250 cells were taken into account for each condition, unless stated otherwise.

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6.6. References

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Chapter 7

Epilogue

7.1. Introduction

Supported lipid bilayers (SLBs) introduce many opportunities to fabricate cell-instructive biointerfaces with tunable physical and chemical properties as well as enable multiple functionalization routes to study specific cell-surface interactions in a highly biomimetic manner.^[1]

In previous chapters, we have shown how to exploit SLBs as cell-instructive biointerfaces to guide hMSC behavior in relation to ligand (i.e., integrin targeting RGD ligands) mobility, density, functionalization route and peptide-SLB interactions. We also showed how the complexity of SLB biointerfaces can be tuned in terms of peptide composition on the SLBs (to target different receptors on the cell membrane) in the context of endothelial cell behavior, hence guiding a tissue type specific cell response.

With these results in hand, yet realizing that the previous work was performed on glass supports as in vitro platforms, we aimed at translating these SLB biointerfaces on a widely known biomaterial, i.e., polycaprolactone (PCL),^[2] in an attempt to develop a biomaterial coating to specifically control surface chemistry while preserving bulk (e.g., mechanical and degradation) material properties.^[2a] For this purpose, we used a method that was previously developed in our group by Dr. Jasper van Weerd, where PCL surfaces were conjugated with a cholesterol linker to achieve biomaterial supported lipid bilayers (BSLBs) (**Scheme 7.1**). Importantly, at a given cholesterol linker incubation time (i.e., cholesterol linker density on the surface), air-stable BSLBs were achieved, which introduces many advantages as a biomaterial coating and overcomes inherent instability of traditional SLBs, while preserving their physicochemical properties.^[2a, 3]

7.2. Supported lipid bilayers as biomaterial coatings

The steps that were taken for the preparation of BSLBs are given in Scheme 7.1, where the PCL surface first goes through an oxygen plasma treatment (10s, 40 mA, 200 mTorr), previously optimized for the generation of aldehyde groups on these surfaces.^[2a, 4] In fact, purpald staining verified the formation of aldehyde groups on oxygen plasma treated PCL surface, while blank OP treated CHCl_3 (i.e., solvent for PCL) control and native PCL (no OP treatment) did not show any staining (data not shown). Furthermore, when an OP treated sample was incubated with NaBH_4 for reduction of aldehydes, no staining was observed, showing the specificity of the staining to detect aldehydes (data not shown).^[2a]

Scheme 7.1. Scheme for the polymer modification with cholesterol conjugation and bilayer formation on cholesterol conjugated polymeric support. Reproduced from ref. ^[2a]

After the OP treatment step and hence generation of aldehyde groups on the surfaces, they were immediately incubated with an amine-terminated cholesterol linker solution (1 mM in ethanol) at defined incubation times, which would result in different surface densities of cholesterol linker on the PCL surface (resulting in PCL-Chol surface).^[2a, 3] As the amine group on the cholesterol linker readily reacts with aldehydes (yielding an imine bond), this method introduces an easy way to covalently modify the surface without additional steps.^[2a] Subsequently, the imine groups were converted to hydrolytically stable secondary amines by reduction using NaBH₄.^[2a, 5] The covalent attachment of the cholesterol linker on PCL surfaces was monitored with water contact angle measurements. As expected, OP treatment of PCL resulted in a decrease in contact angle values.^[2a] Subsequent addition of the cholesterol linker to surfaces resulted in an increase in contact angle values (as measured after removing possibly non-specifically adsorbed molecules), which was above the contact angle values that were observed for native surfaces (no treatment) (Figure 7.1). These results altogether confirms the surface modification occurred through the covalent attachment of cholesterol linker (Figure 7.1A).^[2a] In addition, when we measured contact angle values for different cholesterol linker incubation times (i.e., 10, 30, 60, 120 and 180 min), we did not observe any correlation between these two parameters. Therefore, in order to have a better understanding of surface properties, surface sensitive techniques such as X-Ray photoelectron spectroscopy (XPS) could be used to reveal the molecular density of cholesterol linker at different incubation times.^[3]

Figure 7.1. Contact angle measurements for PCL films that are treated with oxygen plasma (OP, 40 mA, 10s) and then functionalized with a cholesterol linker at Day 0, Day 1 and Day 2 of cholesterol linker functionalization of the surfaces. Background surfaces correspond to PCL surfaces that went through OP treatment, ethanol incubation (as in the case of cholesterol addition) and NaBH_4 reduction, as a reference surface to cholesterol linked surfaces with identical treatment but without cholesterol linker (Chol: cholesterol). PCL-Chol and background surfaces were kept under argon protection, while OP treated surfaces were kept in water.

Furthermore, we monitored the contact angle values over time (Day 0, Day 1 and Day 2 of cholesterol linker attachment), in order to study the stability of surface modification (Figure 7.1B-C). In time, a slight decrease in contact angle values for PCL-Chol surfaces could be seen. However, contact angle values always persisted above the values for native (i.e., starting) PCL surfaces, which again confirms the presence of cholesterol linker on the surface (PCL-Chol) even 2 days after the addition (Figure 7.1C).

Figure 7.2. Characterization of lipid bilayer formation on cholesterol conjugated PCL surfaces. (A) Fluorescence images to monitor homogeneity of DOPC based coating (i.e., bilayer) on PCL surfaces that were incubated with cholesterol linker (i.e., PCL-Chol) for 5 min, 20 min and 60 min for conjugation (Red originates from TexasRed-DHPE lipid that was introduced to DOPC vesicles at 0.2 mol%, scale bar: 100 μm). (B) A fluorescence recovery after photobleaching (FRAP) experiment on PCL-Chol surfaces with 60 min cholesterol incubation time, giving the bleach spot at the bleach moment and 10 min after bleaching (Region of interest was a 15 μm square bleach region, yellow square depicts the region that was used to plot the profiles given in C, scale bar: 25 μm). (C) Profile plots of the regions given in B, showing the extent of bleaching and recovery after 10 min. X axis (distance) is identical for both plots.

Next we evaluated SLB formation on PCL-Chol surfaces, with cholesterol linker incubation times of 5 min, 20 min and 60 min (**Figure 7.2**). For this purpose, a modified vesicle fusion method was used where DOPC vesicles that contain 0.2 mol% TexasRed-DHPE (as dye conjugated lipid) were first deposited on the surfaces (in 0.5X PBS, 0.5 mg/mL vesicle concentration) and went through a freeze-thaw cycle (freezing to -80°C for 1h and then thawing at room temperature), to further promote and ensure vesicle rupture on the surfaces for SLB formation.^[2a, 6] Confocal imaging showed a more homogeneous fluorescence (indicating a homogeneous lipid coating, as explained in Chapters 3-5), for 20 and 60 min cholesterol linker incubation times compared to 5 min, where at 60 min a slight improvement was observed compared to 20 min (Figure 7.2A). When a fluorescence recovery after photobleaching (FRAP, as explained in Chapters 3-5) experiment was performed (Figure 7.2B), lipid mobility was observed on surfaces that were incubated with cholesterol linker for 60 min, indicating SLB formation albeit with low mobility. As such, profile plotting showed an increase in average normalized intensity in a bleached area from 0.61 to 0.74, from the bleach moment and 10 min after bleach, respectively (Figure 7.2C). As also shown in Figure 7.2B, recovery either stayed at very low levels or stopped after 10 min. It is known that high cholesterol linker densities on the surfaces may result in low to no mobility in the lower leaflet of the SLB, which could explain these observations.^[2a] However, again knowing the molecular density of cholesterol linker on the surfaces could give a better insight on the observed effects.

Based on these results, in order to investigate hMSC behavior on BSLBs, we chose 60 min as the incubation time to fabricate PCL-Chol surfaces. BSLBs on these PCL-Chol surfaces were formed as explained above using DOPC ($T_m=-20^{\circ}\text{C}$, mobile SLBs) and DPPC ($T_m=41^{\circ}\text{C}$, immobile SLBs) based vesicles, which were either functionalized with 10 mol% bipalmitic acid conjugated RGD peptide (Bipal-RGD, RGD-SLBs) or without functionalization (hence to form bare DOPC and DPPC SLBs). We first studied hMSC adhesion and spreading as well as vinculin localization in cells on functional (RGD-SLBs) or non-functional (bare SLBs) as well as on only PCL-Chol (no SLB) surfaces, 18h after cell seeding. On all surfaces, cell adhesion and spreading was present after 18h (**Figure 7.3**). When we compared bare and RGD presenting SLBs, individually for each lipid composition (i.e., DOPC and DPPC), on RGD-SLBs a qualitative improvement (slight to moderate) in stress fiber formation and vinculin localization could be observed (Figure 7.3B and D and Figure 7.3C and E, for DOPC and DPPC SLBs, respectively).

Figure 7.3. Vinculin localization in hMSCs on different PCL-Chol surfaces. (A) PCL-Chol only. PCL-Chol surfaces coated with (B) bare DOPC, (C) bare DPPC, (D) DOPC SLBs presenting 10 mol% bipalmitic acid-RGD and (E) DPPC SLBs presenting 10 mol% bipalmitic acid-RGD ($t=18h$, blue: nucleus, red: actin and green: vinculin, scale bar: 20 μm).

We then investigated how the hMSC differentiation capacity was affected on different PCL-Chol surfaces (with and without defined SLBs). For this purpose, cells were seeded

on the surfaces and then allowed to adhere for 6h in basic medium (as given in Chapters 3-5). Then, the medium was changed to a double induction medium, which contained a 1:1 ratio of osteogenic (as given in Chapter 3) and adipogenic differentiation (as explained in refs.^[2a, 7]) media.

Figure 7.4. Alkaline phosphatase (ALP) expression in cells on different PCL-Chol surfaces (with and without defined SLBs), as a marker of their osteogenic differentiation capacity. For PCL-Chol surfaces background correction was performed and contrast was adjusted for visibility where sites of ALP activity appear as dark blue. All images in this case were treated identically. Cells on polystyrene surfaces that were treated with osteogenic differentiation medium were used as control (polystyrene osteogenic, sites for alkaline phosphatase activity appears as purple). Cells on polystyrene surfaces in basic medium were used as negative control for differentiation and did not show any staining ($t=7$ days, scale bar: 20 μm , bipal: bipalmitic acid).

When we stained cells for alkaline phosphatase (ALP) activity after 7 days, we observed that on only PCL-Chol surface (no SLBs), a qualitatively higher number of cells showing

ALP activity was present compared to other conditions (both RGD-SLBs and bare SLBs), while on functional RGD-SLBs only a slight increase compared to bare SLBs was observed. These results indicate that when basic medium was changed to differentiation medium, at 6h, cells seeded on BSLBs may have had a difference in adhesion and spreading behavior compared to cells on only PCL-Chol surfaces, suggesting an effect of SLB coating on the PCL-Chol surfaces.^[2a, 7-8] Therefore, for future studies, cell adhesion could be studied for shorter time points such as 6h as in this case to reveal the differences at earlier stages, next to 18h that was used in this Chapter.

Figure 7.5. Oil deposition by cells on different PCL-Chol surfaces (with and without defined SLBs), as a marker of their adipogenic differentiation capacity. Cells on polystyrene surfaces that were treated with adipogenic differentiation medium were used as control (polystyrene adipogenic, sites for oil deposition appears as red, via Oil-Red O staining). Cells on polystyrene surfaces in basic medium were used as negative control for differentiation and did not show any staining ($t=7$ days, scale bar: 20 μm , bipal: bipalmitic acid).

Furthermore, we observed that, on all surfaces, a qualitatively higher number of cells showed ALP activity (i.e., ALP positive), compared to the cells showing oil deposition (via oil red O staining), which was used as a marker of adipogenic differentiation. This may indicate that, as cells could spread on the surfaces, when they were given the double induction medium (i.e., the choice between going through either osteogenic or adipogenic differentiation) they preferably differentiated into osteogenic lineage as was previously shown in literature.^[7-8]

7.3. Conclusion and outlook

In conclusion, in this Chapter we showed the steps of translating cell-instructive SLB biointerfaces as biomaterial coatings, which was demonstrated on PCL surfaces. PCL surfaces were modified with a cholesterol linker, which was previously shown to result in air-stable SLBs at given incubation time points (i.e., surface densities on PCL-Chol surfaces).^[2a, 3] We showed that coating homogeneity (i.e., SLB homogeneity as revealed by fluorescence signal of dye-conjugated lipid in SLBs) increased with increasing cholesterol incubation times. We further showed that at a defined incubation time (60 min) a rather homogeneous coating (SLB) was observed that show lipid mobility, albeit at low levels. Furthermore, cell experiments showed the potential of applicability of BSLBs for guiding hMSC behavior. Future work should bring more insights on surface properties, such as cholesterol linker density per incubation time point, as well as surface topological properties (that can be inspected by atomic force microscopy, AFM) before and after OP treatment to reveal any effects on BSLB properties and subsequent cell response. Besides, next to PCL, other plasma-treated polyesters (as was also described previously in our group^[2]) could be utilized to achieve stable BSLBs through identical modification via the cholesterol linker and could be investigated for their potential as cell-instructive biointerfaces. In addition, investigating cell adhesion at shorter time points can give insights on early stages of cell response on BSLBs (on PCL-Chol in this case).^[2a] Finally, cell experiments on PCL-Chol surfaces (with and without SLBs) in basic medium (i.e., without differentiation components) that investigate differentiation capacity at gene expression level can introduce more insights on cell-surface interactions without the effect of soluble factors.

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7.5. References

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Summary

The extracellular environment is a highly dynamic milieu where instructive cues (i.e., signals) that originate from the surrounding matrix and neighboring cells are tightly controlled in a spatiotemporally regulated manner. Incorporation of bioinspired dynamic cues, while designing new cell-instructive biointerfaces, has recently been an important focus in biomaterials development for regenerative medicine applications. In this line, progressive developments in materials chemistry as well as introduction of non-covalent chemistries and stimuli-responsive elements in biomaterials design has opened up tremendous opportunities to achieve such highly biomimetic cell-instructive biointerfaces with dynamic regulation of physical, chemical and mechanical cues, to guide desired cell and tissue response. In this thesis, chemical approaches to fabricate dynamic cell-instructive biointerfaces using light-responsive liquid crystal polymer networks (LCNs) and supported lipid bilayers (SLBs) are presented and discussed with an emphasis put on investigating (stem) cell behavior. In the first part of the thesis, temporal control over cell migration upon light-induced in situ changes in nanotopographical cues is achieved on azobenzene conjugated LCNs. This work introduces these materials as new biointerfaces. In the second part of the thesis, central focus is on how to exploit SLBs as dynamic cell-instructive platforms providing control over ligand density, mobility, functionalization route, composition (i.e., type of the peptide ligand) as well as peptide-SLB interactions. On SLBs, functionalized via biotin-neutravidin interactions, ligand (i.e., integrin targeting RGD) density and mobility are shown as important parameters to guide hMSC behavior. Next, using lipid insertion (i.e., using alkyl tail or cholesterol modified (lipidated) RGD ligands) as functionalization route, both lateral (in-plane) and out-of-plane dynamicity of the ligands are controlled. The length of the alkyl tail (i.e., interaction strength of lipidated peptide and SLB) that is conjugated to RGD peptide is shown to be critical to control hMSC adhesion, spreading and focal adhesion formation in these cells on laterally mobile ligands (i.e., in fluid SLBs). Using a dye-conjugated lipid-modified RGD, the dynamic presentation of the peptide is further demonstrated together with the propensity of hMSCs to take up the peptide as well as the dependency of cell migration on the lipid anchor type conjugated on RGD. In the final part, the complexity of the surfaces is tuned by controlling lipid-modified peptide compositions for mimicking cell-matrix and cell-cell junctions in fluid SLBs, to guide endothelial cell behavior. In the epilogue, first steps are explored and discussed that are taken to employ SLBs as cell-instructive interfaces on biomaterials.

Samenvatting

De extracellulaire matrix is een zeer dynamische omgeving waarin stimuli aan cellen uit de nabijgelegen matrix strak zijn gereguleerd, zowel in de ruimte als door de tijd. In het onderzoek naar biomaterialen voor regeneratieve geneeskunde wordt steeds meer focus gelegd op het inbouwen van dynamische prikkels bij het ontwerpen van cel-regulerende bio-oppervlakken. De snelle ontwikkelingen op het gebied van de materiaalkunde, met name gericht op het introduceren van niet-covalente chemie en stimulus-gevoelige elementen in het ontwerp van biomaterialen, opent een wereld aan ontwerpmogelijkheden voor het maken van cel-regulerende bio-oppervlakken die de natuur nabootsen. Fysische, chemische, en mechanische prikkels van zulke oppervlakken worden dynamisch gereguleerd om zo de reactie van cellen en weefsel te sturen. In dit proefschrift worden chemische methoden beschreven om dynamische cel-regulerende bio-oppervlakken te fabriceren, waarbij licht-gevoelige polymeernetwerken (LGPs) en ondersteunde lipide dubbellagen (OLDs) worden gebruikt, met de nadruk op onderzoek naar het gedrag van (stam)cellen. In het eerste deel van het proefschrift wordt beschreven hoe de migratie van cellen over de tijd kan worden gecontroleerd door gebruik te maken van nanotopografische veranderingen, die *in situ* met licht kunnen worden opgewekt, in LGPs die gemodificeerd zijn met azobenzeen. Dit materiaal wordt hier voor het eerst gebruikt als bio-oppervlak. In het tweede deel ligt de nadruk op het gebruik van OLDs als dynamisch cel-regulerend substraat waarbij de ligand dichtheid en mobiliteit, de manier van functionalisatie, de samenstelling (bijvoorbeeld het type peptide ligand), en de peptide-OLD interacties kunnen worden gecontroleerd. We laten zien dat het gedrag van hMSC op OLDs, die door middel van biotine-neutravidine interacties zijn gefunctionaliseerd, kan worden beïnvloed door de ligand (in dit geval een RGD ligand dat integrine bindt) dichtheid en mobiliteit. Vervolgens beheersen we zowel laterale (in-het-vlak) als uit-het-vlak bewegelijkheid van liganden, door gebruik te maken van alkyl- of cholesterol-gemodificeerde RGD liganden als manier van OLD-functionaliseren. We laten zien dat de lengte van de alkyl staart die aan het RGD peptide vast zit, die de sterkte van de interactie tussen het peptide en de OLD bepaald, cruciaal is voor het beheersen van adhesie, spreiding, en vorming van focale adhesie van humane stamcellen op lateraal mobiele liganden in fluïdische OLDs. Door RGD te modificeren met een lipide staart en een kleurstof, waren we in staat de dynamiek van het peptide verder inzichtelijk te maken en werd duidelijk dat de cellen deze liganden kunnen opnemen. Verder blijkt cel migratie afhankelijk te zijn van de lipide staart van het RGD. In het laatste deel worden de OLD-oppervlakken aangepast met verschillende typen van liganden die cel-matrix en cel-cel verbindingen na kunnen bootsen op OLDs.

In de epiloog zijn de eerste stappen naar het gebruik van OLDs als cel-regulerenden biomaterialen beschreven en besproken.

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Gülistan

26-03-18, Enschede

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Gülistan Koçer was born in Silvan, Turkey on March 10th, 1988. She received her BSc degree in Chemical Engineering from the Middle East Technical University (METU), Ankara, Turkey in 2011. As a part of her undergraduate studies, in the fall semester of 2009, she attended the Technical University of Denmark within the scope of an ERASMUS exchange programme. In August 2013, she received her MSc degree in Biomedical Engineering (*cum laude*) at the Department of Tissue Regeneration, University of Twente (UT) where she investigated the genome wide regulation of gene expression in osteogenic cells in response to surface structural properties of calcium phosphate ceramics. The work was carried out in the group of Prof. Dr. Jan de Boer and co-supervised by Dr. Roman Truckenmüller and Prof. Dr. Pamela Habibovic, now at Maastricht University. From October to December 2013, she worked at the same department under the supervision of Prof. Dr. P. Habibovic, on a project involving topographical modification of polymer substrates for cell studies.

In December 2013, she joined the group of Prof. Dr. Ir. Pascal Jonkheijm, at the Department of Molecular Nanofabrication, University of Twente, as a PhD candidate. The results of her work are described in this thesis.

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2. G. Koçer, J. ter Schiphorst, M. Hendrikx, H. G. Kassa, P. Leclère, A. P. H. J. Schenning and P. Jonkheijm, *Light-responsive hierarchically structured liquid crystal polymer networks for harnessing cell adhesion and migration*, Adv. Mater. **2017**, 29, 1606407.
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2. M. L. Verheijden, G. Koçer, R. P. Richter and P. Jonkheijm, *Controlling cell adhesion and spreading by tuning the binding of lipid-modified peptides in supported lipid bilayers.*
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The ability to think differently today from yesterday, distinguishes the wise from the stubborn.

-John Steinbeck