Supramolecular Binding of Vesicles, Viruses and Cells to Biomimetic Lipid Bilayers

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2018

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Mark Lloyd Verheijden

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DISSERTATION

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on the authority of the rector magnificus
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on account of the decision of the graduation committee,
to be publicly defended
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Chapter 1

The lipid bilayer as dynamic platform for receptor-ligand interactions

The design of biointerfaces that can dynamically interact with their environment is a key step in the development of systems that can mimic or steer biological function. Lipid bilayers are highly suitable to be used in such designs because they can be rendered dynamic and biomimetic. In this chapter, we briefly highlight those properties of lipid bilayers that are relevant when applying them as dynamic receptor platforms, such as lateral mobility, nonfouling behavior and their self-assembled nature. We then review some key examples of artificial systems that illustrate dynamic interactions at lipid bilayers and discuss the general concepts and considerations that play a role when using lipid bilayers as receptor-ligand platforms. The main focus concerns amphiphilic receptors or ligands that directly insert into lipid bilayers and the various factors playing a role in this interaction.

1.1. Introduction

Dynamic receptor-ligand interaction platforms are interfaces on which receptors reside that bind specific ligands in a non-covalent fashion. The dynamic nature means that the binding between the molecules and receptors is intrinsically reversible. Dynamic binding can also be modulated by applying a stimulus to induce conformational and/or electronic changes. Various stimuli can be applied, such as light, temperature, electrochemical, magnetic, and enzymatic stimuli. Dynamic receptor-ligand interaction platforms are unique due to their self-assembly characteristics, large tunability, and biomimetic nature. These properties make them particularly suitable for applications such as drug delivery, tissue engineering, and studying cell-material and cell-cell interactions. Indeed, a wide variety of such dynamic platforms have been developed and recently reviewed.^{1, 2}

A particular example of dynamic platforms are lipid bilayers. Lipid bilayers are self-assembled structures in which amphiphilic molecules are arranged in two leaflets, where the hydrophilic parts of the amphiphiles are facing the aqueous environment and the hydrophobic segments are facing inwards to minimize their interaction with the aqueous phase. Lipid bilayers are better known as the key constituent of cell membranes. With the above mentioned biological applications in mind, lipid bilayers have the unique advantage that they are intrinsic mimics of the cell membrane.³ In addition, the self-assembly character of the lipid bilayers makes it relatively easy to introduce functional molecules such as (artificial) receptors or ligands. Together with their nonfouling characteristics,⁴ lipid bilayers are highly suitable for the fabrication of dynamic receptor-ligand interaction platforms.

Fabrication, characterization, and modification of supported lipid bilayers (SLBs) as well as their use as cell analysis platform has recently been reviewed in detail by our group.⁴ Therefore, general properties of lipid bilayers will be discussed in section 1.2 only briefly and when relevant for the work described in this thesis. Section 1.3 gives a more detailed discussion on the various ways of using lipid bilayers as platforms that facilitate dynamic and reversible interactions. The use of the hydrophobic interior of the lipid bilayer as a sort of hydrophobic binding pocket as well as the parameters that influence direct interaction with this interior by receptors or ligands bearing hydrophobic segments will be the particular focus of section 1.3.

1.2. General properties of lipid bilayers

1.2.1. Self-assembled structures of amphiphiles

Lipid bilayers are self-assembled structures that exist in aqueous environments and consist of two layers of amphiphilic molecules, generally referred to as lipids. A vast amount of lipids have been identified,⁵ typically glycerophospholipids, sphingolipids, and sterols. The shape and structure of the lipids and the ratio in which the lipids are present determine a number of properties of lipid bilayers, such as phase behavior and curvature.

The relative size of the hydrophilic head group compared to that of the hydrophobic tail segment –the “packing parameter” introduced by Israelachvili *et al.*⁶– will largely determine the final shape of the self-assembled lipid bilayer structure. This is generalized in Figure 1.1.^{7, 8} For natural phospholipids, the packing parameter is usually close to unity and this results in a flat, bilayer structure analogous to the cellular membrane. Small deviations in the packing parameter will result in membrane curvature and mixtures of lipids might lead to a local curvature when specific lipids phase separate in a certain region. Generally observed structures in solution are uni- and multilamellar vesicles ranging from tens of nanometres to over a micrometre.⁹ Alternatively, lipid bilayers can be formed on a surface to achieve supported lipid bilayers. Commonly used methods for making SLBs are the Langmuir Blodgett/Langmuir Schäfer method and the vesicle fusion method.⁴

1.2.2. Phase behavior and mobility of lipid bilayers

One of the most intriguing properties of lipid bilayers is the mobility of lipids in the bilayer and the related phase behavior.¹⁰ Although lipid mobility and phase behavior have been reviewed exhaustively,¹¹⁻¹⁴ a brief discussion on this topic is given because of its relevance when studying interactions with lipid bilayers. The possible movements of lipids in bilayers are shown in Figure 1.2A. Lateral diffusion of lipids illustrates the ability of lipids to move laterally, *i.e.* within the individual leaflets of the bilayer. The degree of lateral mobility mainly depends on the structure of the lipid tail and the temperature (Figure 1.2B). Above the phase transition temperature (T_m), the lipid bilayer will be in the liquid-disordered (L_d or fluid) phase and the individual lipids show a high lateral mobility, typically in the order of $\mu\text{m}^2/\text{s}$. Below the T_m , the lipid bilayer will be in the solid-ordered (S_o or gel) phase and the individual lipids diffuse very slowly, typically below $10^{-3} \mu\text{m}^2/\text{s}$.

Figure 1.1 | Self-assembly behavior of amphiphilic molecules. Schematic amphiphile structures with various shapes and the effect thereof on self-assembled structures.⁷ Adapted with permission, copyright 2015, Hindawi.

Generally, shorter lipid tails or the presence of unsaturation in the tails lowers the T_m .⁴ Mixtures of gel and liquid state lipids can lead to phase separation. A special case that has been widely investigated is the effect of the addition of cholesterol to either gel or liquid state lipids. Addition of a certain density of cholesterol to either of these phases, results in a so called liquid ordered (L_o) phase (Figure 1.2B). This phase is quite densely packed but still demonstrates a high lateral mobility of the lipids. Next to lateral mobility of lipids, another possible movement of lipids that can occur is

between the two leaflets of the bilayer. This so-called flip-flop rate has been observed to occur on the timescale of minutes as was measured for 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) SLBs above their T_m .¹⁵ However, rates in the order of days were found using 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) vesicles above their T_m .¹⁶ This difference might be ascribed to lipids moving from one to the other leaflets through defects in SLBs (Figure 1.2C). This *apparent* flip-flop does not occur in vesicles, which are defect-free.¹⁶ This suggests that conventional flip-flop of regular phospholipids is a relatively slow process. At the same time, flip-flop is highly dependent on the temperature and the head and tail groups. Flip-flop in the order of seconds has been observed in the case of dye-functionalized fatty acids (single lipid anchor).¹⁷ Another, and often neglected, movement of lipids is the exchange of lipids with the solution. Indeed, this process is generally slow, which can be correlated to the very low solubility (often nM or below) of individual lipids. Again, this process is strongly dependent on temperature and the structure of the head and tail group of the lipids.¹⁷

Figure 1.2 | Mobility in phospholipid bilayers. (A) The various types of lipid mobility in lipid bilayers.¹¹ (B) Main phases in lipid bilayers and the effect of temperature and presence of cholesterol.¹² (C) Defect mediated *apparent* flip-flop process.¹⁶ Adapted with permission, copyright: (A) 2003, WILEY-VCH, (B) 2009, BASE, (C) 2017, American Chemical Society.

1.2.3. Nonfouling properties of lipid bilayers

Bio-sensing in complex fluids such as blood, urine, or saliva requires elimination of nonspecific adsorption at sensor surfaces by any undesired solute. Typically, an increase in surface hydration stability using polar chemistry improves the nonfouling

character of surfaces. Stable surface hydration minimizes interaction energies with other hydrated solutes. Some polar, uncharged hydrophilic surfaces such as polyethylene glycol (PEG) are hydrated and therefore this polymer has often been used in the design of nonfouling (background) layers. Interestingly, balanced anionic and cationic groups on surfaces also cluster water around each charged group based on ion-dipole forces as is the case for lipid head groups such as the ubiquitously used zwitterionic phosphatidylcholine (PC) head group.^{18, 19} The PC head group for example holds up to 15 water molecules in its primary hydration shell, compared to approximately one water molecule per monomer for PEG.¹⁸ Fouling of surfaces has also been found to be affected by the presence of defects in the surface (coating). The fluidic nature of lipid bilayers however minimizes surface defects during its fabrication and to some extent also afterwards.²⁰ Indeed, clear repression of nonspecific adhesion of for example 3T3 cells²¹ as well as proteins such as fibrinogen and fetal bovine serum (FBS) have been reported for SLBs.²⁰ In addition to being nonfouling, SBLs are readily functionalized. Due to the combination of these properties, SLBs are starting to be used for functionalization of specialist devices²² and multifunctional targeting particles.²³

1.2.4. Introducing receptors and ligands at lipid bilayers

Biological interactions are defined by receptors, ligands and their mutual arrangements on cell membranes. In this chapter we will not cover descriptions of classes of natural receptors and ligands, but we will focus on descriptions of model systems designed to artificially study specific properties of receptor-ligand interactions on lipid bilayers. Many interactions with cellular receptors are transient, *i.e.* ligands can bind, possibly resulting in a signaling cascade, and unbind again. Or, ligands can bind and assemble in larger structures that can later disassemble again. Lipid bilayer platforms constitute an interesting model system as it can capture such dynamics and structural arrangements. These artificial systems can teach us about the general concepts that govern complex biological processes where multiple types of interactions often play a role simultaneously.

The first step is to introduce receptors and/or ligands on lipid bilayers. Strategies to achieve these functionalized (supported) lipid bilayers have been discussed in literature.^{4, 24} In short, lipid-functionalized receptors/ligands or lipid-functionalized reactive groups can be mixed-in during the fabrication of lipid bilayers. When using reactive lipids, the receptor or ligand of choice can be reacted to the lipid bilayer after bilayer formation. Alternatively, a lipid-functionalized receptor or ligand can be inserted

from solution after bilayer formation. A specific case concerns the situation where this interaction of the receptor or ligand to the lipid bilayer is reversible, which will be discussed in detail in section 1.3. After functionalization of the lipid bilayer, a variety of interaction types can be used to achieve dynamic interactions with the receptor or ligand that is bound to the lipid bilayer. The main interaction types are briefly highlighted here.

Metal-ligand interactions at lipid bilayers have been described in many systems.^{8, 9} Generally, hydrophobic anchors based on cholesterol, aliphatic hydrocarbons and/or pyrene have been used to anchor receptors such as terpyridine or iminodiacetate to the lipid bilayer where these receptors can bind metal ions like Cu^{2+} , Ni^{2+} , Ca^{2+} and Zn^{2+} .^{8, 9} Often, these surfaces are then used to bind a second ligand, such as proteins functionalized with multiple histidines.^{25, 26} A recent and remarkable example consists of a sterol-functionalized molecule with on one side a Cu^{2+} binding moiety and on the other side a Zn^{2+} binding moiety (Figure 1.3A).²⁷ This molecule is, by design, slightly too short to span the entire lipid bilayer. Addition of EDTA to the outside of the vesicles scavenges the Cu^{2+} from the Cu^{2+} binding moiety, lowering its polarity. This change allows the entire molecule to translocate to face the inside of the vesicle, where in turn Zn^{2+} is bound by the Zn^{2+} binding moiety.²⁷ In addition, the Zn^{2+} receptor acts as a ester hydrolysis catalyst, making the entire system a synthetic transmembrane signal transduction systems.²⁷

Hydrogen bonding interactions have also been used at lipid bilayers, however less frequently than metal-ligand interactions.^{9, 28} These interactions are generally weak in aqueous media but are enhanced at the bilayer interface where the polarity is reduced compared to aqueous solutions.²⁹ Hydrogen bonding interactions at lipid bilayers are generally demonstrated for vesicle-vesicle binding, but have also been shown for vesicles-SLB binding. The latter has been achieved using lipids that were functionalized with either cyanuric acid or melamine³⁰ (Figure 1.3B). Formation of melamine-cyanuric acid complexes led to binding of vesicles and subsequently fusion of vesicles was observed. Interestingly, fusion was accelerated when an antimicrobial peptide, rather than a lipid was used as membrane anchor.³⁰ Another type of interaction exploited at lipid bilayers represents DNA base pairing, which has emerged as a field on its own and this has recently been reviewed.³¹ In these DNA-base pairing systems, the aim has generally been to achieve stable rather than dynamic interactions. Figure 1.3C shows a schematic that depicts the binding of a DNA origami structure to SLBs. The binding was a consequence of single stranded DNA (ssDNA), which was anchored *via*

its cholesterol to the SLB, hybridized to complementary ssDNA sticking out of the DNA origami. The entire DNA origami structure was laterally mobile on the fluid SLB.³²

Another interaction motif relates to the interaction between lectins and the glycocalyx, which have inspired many to develop (semi)synthetic systems on lipid bilayers.³³⁻³⁸ Since the monovalent interaction between a lectin and the corresponding sugar receptor is typically weak, these interactions occur in a multivalent fashion. Two examples are presented in section 1.2.5.

Figure 1.3 | Examples of supramolecular interactions at membrane bound receptors or ligands. (A) Synthetic transmembrane signal transduction systems based on metal-ligand interactions. The orange triangle represents EDTA that upon addition binds Cu^{2+} and triggers translocation.²⁷ (B) Vesicle fusion as a result of multiple hydrogen bonding interactions either between two vesicles or between vesicles and an SLB.³⁰ (C) Cholesterol-modified ssDNA presented at SLB binds DNA origami with sticky, complementary ssDNA through DNA base pairing.³² (D) Anchored by alkyl tails, cyclodextrins (host) on lipid bilayers can bind adamantane or azobenzene guests as an example of host-guest interactions.³⁹ Adapted with permission, copyright: A) 2017, American Chemical Society, B) 2009, American Chemical Society, C) 2014, American Chemical Society, D) 2013 American Chemical Society.

Host-guest interactions are a last category employed at lipid bilayers. There have only been a few systems where either the host or the guest molecule was anchored to the lipid bilayer.³⁹⁻⁴⁴ For example, lipid-modified cyclodextrins were embedded in the lipid bilayer and shown to bind adamantane or azobenzene (Figure 1.3D). Azobenzene affords stimulus responsiveness to the systems due to its reversible, light induced cis-trans isomerization. Only the trans isomer effectively binds to cyclodextrin and this behavior was also demonstrated for the interaction between azobenzene and vesicle-bound cyclodextrin.³⁹

1.2.5. General considerations for receptors and ligands bound to lipid bilayers

When using the lipid bilayer as a receptor platform, the first consideration concerns the hydrophobic core of the lipid bilayer in an otherwise aqueous environment. Receptors that are linked to a lipid through a hydrophilic spacer but that are themselves hydrophobic, can fold back into the hydrophobic core of the lipid bilayer (Figure 1.4A). This backfolding was observed in the case of *e.g.* dinitrophenyl (DNP) which, when presented at the membrane rather than free in solution, binds orders of magnitude weaker to the anti-dinitrophenyl antibody. The degree to which this backfolding occurs and affects the binding affinity depends on the exact hydrophobicity of the receptor. The decrease in affinity was for instance much stronger for DNP compared to the more hydrophilic biotin (Figure 1.4A-B). The use of a hydrophilic spacer between the receptor and the lipid anchor can partially overcome this backfolding.⁴⁵ Inversely, if the binding site of the free ligand is hydrophobic and the membrane bound receptor has a hydrophobic binding pocket, a certain degree of competition occurs between binding of the ligand to the receptor and to the hydrophobic core of the lipid bilayer. That means that the ligand directly interacts with the lipid bilayer and not only with the receptor (Figure 1.4C). To which degree this plays a role will depend on the affinity of the hydrophobic ligand towards the receptor on the one hand and the affinity for the lipid bilayer on the other hand. Adamantane carboxylate for instance binds to β -CD ($K_a = 0.14 \text{ mM}$)⁹ but has also been used as membrane anchor, though without quantifying the affinity.⁴⁶

Figure 1.4 | Important considerations when using lipid bilayers as receptor platform.

(A) Binding of dinitrophenyl (DNP) with anti-DNP in solution (top) compared to when DNP is presented on a lipid bilayer (middle) and the effect of using a PEG spacer between the lipid and DNP (bottom). **(B)** Binding of biotin with anti-biotin.⁴⁷ **(C)** Competition of guest binding between its membrane anchored host and the hydrophobic interior of a lipid bilayer. A-B) Adapted with permission, copyright 2008, Elsevier.

Another two considerations need to be taken into account when introducing the receptor or ligand into a pre-formed lipid bilayer through insertion. First, the dissolved amphiphilic receptors or ligands might aggregate, generally into micelles, at their respective critical micelle concentration (CMC). The propensity of the amphiphile to insert into the lipid bilayer will be lost or at least significantly reduced when it is part of a micelle.⁴⁸ In particular, when the CMC of a certain amphiphilic receptor or ligand is low, functionalization of lipid bilayers through insertion from solution might become impossible. In general, this will be the case for amphiphiles with a relatively large hydrophobic segment. There is an obvious trade-off between stable insertion on the one hand and a high CMC to allow insertion on the other hand. This might explain the general use of cholesterol as membrane anchor, since cholesterol is suggested to have a relatively low tendency to self-aggregate in solution, while it is relatively hydrophobic at the same time.⁴⁹ Second, high concentrations of amphiphilic molecules in solution can destabilize and dissolve the lipid bilayer. This detergent effect is stronger when there is a larger hydrophobic mismatch between the amphiphile and the lipid bilayer and seems to occur mainly above the CMC of the amphiphile.^{50, 51} The detergent effect should not only be taken into account for inserting the amphiphilic receptor but also

for binding the complementary ligand, in the case that this ligand is amphiphilic. Fortunately, this effect is mostly negligible as it occurs generally in the high micromolar range, well above the usual concentration range to insert receptors or bind ligands.⁵¹

Two other considerations are important for the specific case of lipid bilayers that are supported on a solid surface. The first consideration in this respect is the fact that supported lipid bilayers need to be hydrated at all times. When removing the water, the self-assembled bilayer structure will delaminate. Some strategies have been developed to overcome this problem, for instance the stabilization of the SLB using cholesteryl functionalized substrates,⁵² using a small fraction of PEGylated lipids,⁵² or using the disaccharide trehalose as stabilizer.⁵³ The second consideration is that electrostatic interactions between the surface and the membrane bound receptors or ligands could repel or attract them. As a result, they might be distributed asymmetrically over the leaflets and not be presented in the density that is expected based on the mixing ratio, particularly considering the fast defect mediated exchange between leaflets that has been described in section 1.2.2 (Figure 1.2C). This asymmetrical distribution is generally known and accepted for a certain type of (charged) lipids,⁵⁴ though not very well described and often not taken into account for lipid bound receptors or ligands. Probably the extent to which this asymmetrical distribution occurs will depend on the structure and charge of the receptor or ligand as well as on the support material. If an asymmetrical distribution occurs, quantification of interactions at SLBs will become challenging.

Finally, we recognize the specific case that ligand binding to receptors displayed at fluid SLBs occurs in a multivalent fashion. In this case, the lateral mobility of the lipid bilayer can have important consequences for the receptor-ligand binding characteristics, mainly in two ways. Firstly, the lateral mobility of receptors in the SLB can facilitate the alignment of multiple receptors upon interaction with their multivalent ligands. For a static receptor display, as is the case on regular surfaces where receptors are covalently attached, you would often have either insufficient receptors and/or the receptors would not be perfectly aligned with ligands. As a result, the effective valency would be lower and/or the binding imposes a stress. For example, a static and homogeneous display of biotin at a certain, low density would only allow one biotin to interact to streptavidin, its tetravalent binding partner. For the same density of biotin displayed on a fluid SLB, each streptavidin would still be able to bind to multiple (generally two) biotin moieties due to their lateral mobility. In fact, it has been found experimentally that even at high streptavidin concentration and low biotin density, no monovalent binding takes place on fluid SLBs. This makes sense since the lateral

dynamics are much faster than the binding process.⁵⁵ Secondly, receptors on SLBs can in some cases reorganize themselves into dense clusters. Clustering of receptors generally increases the overall affinity for multivalent binding due to the possibility of reaching a higher valency. This was observed for His₆-tagged proteins binding to clustered Cu²⁺-chelating lipids with at least an order of magnitude increase in affinity compared to freely distributed Cu²⁺-chelating lipids (Figure 1.5A).²⁵ However, receptor clustering does not always have much effect or can even result in a decrease on the binding affinity, due to steric crowding. This was observed, respectively, for concanavalin A (ConA) binding to clustered mannosylated lipids³³ (Figure 1.5B) and for cholera toxin binding to an SLBs presenting clustered glycosylated GM₁ lipids⁵⁶ (Figure 1.5C).

Figure 1.5 | Effect of receptor clustering upon binding of multivalent ligands. (A) Clustering of chelator lipids induced by Cu²⁺ binding promotes the binding of His₆-tagged proteins. Cu²⁺ can be removed again by complexation with EDTA.²⁵ **(B)** Clustering of mannosylated lipids has no significant effect on binding of concanavalin A.³³ **(C)** Binding of pentameric cholera toxin B subunits (CTB) is hindered by clustering of the complementary glycolipid (GM₁) at a lipid bilayer.⁵⁶ Adapted with permission, copyright: A) 2009, American Chemical Society, B) 2009, Royal society of Chemistry, C) 2007, American Chemical Society.

1.3. Reversible anchoring of receptors and ligands at lipid bilayers

In the previous sections, examples of supramolecular interactions at lipid bilayers were discussed, as well as routes to anchor receptors and ligands to lipid bilayers. One of these routes is the insertion of lipidated receptors or ligands from solution to a pre-formed lipid bilayer. The interaction between the hydrophobic interior of the membrane and the hydrophobic segment of the amphiphile is by itself a supramolecular interaction and can be tuned by *e.g.* selecting a specific hydrophobic molecular structure. Various examples have been reported in literature that make use of this type of interactions to achieve reversible interactions of receptors or ligands directly with the lipid bilayer. Parameters that have been found to play a role in these interactions, such as the structure of the lipid anchor and the composition and curvature of the membrane, will be discussed in more detail in the following sections. In nature, this type of interactions are also regularly used: various enzymes modify the membrane-affinity of proteins by adding one or more lipid chains, such as palmitoyl, myristoyl, farnesyl, or geranylgeranyl, to the proteins. The interaction of the resulting lipidated proteins is often stabilized by electrostatic interactions between the protein and charged lipids.⁵⁷ The contribution of electrostatic interactions in membrane binding will also be discussed, in section 1.3.7.

1.3.1. CMC and lipid backfolding

The propensity of amphiphiles to aggregate in aqueous solutions and the relevance thereof has been discussed in section 1.2.4. Additionally, it is interesting to note here that longer lipid tails generally correspond to a higher propensity to form aggregates (*i.e.* a lower CMC). Contrarily, shorter lipid tails will generally be selected to achieve dynamic rather than stable interactions of amphiphiles with lipid bilayers. This means that a larger range of concentrations of freely dissolved amphiphiles is available to achieve dynamic insertions compared to stable insertion.

In addition to *intermolecular* aggregation of lipidated ligands or receptors into micelles, *intramolecular* interactions can also cause inefficient membrane insertion. In the latter case, the hydrophobic lipid anchor folds back and interacts with another part of the amphiphile, as shown schematically in Figure 1.6 (R_b). This will result in a decreased affinity for the lipid bilayer. Such a process has been reported for the recoverin protein where the C14:0 (a linear lipid with 14 carbons and 0 unsaturated bonds) lipid anchor folds back onto the protein in a Ca²⁺-dependent manner.⁵⁸ Such

backfolding of (parts of) lipid tails to modulate binding to lipid bilayers has not been employed in designing systems to anchor lipidated receptors and ligands on lipid bilayers.

Figure 1.6 | Behavior of amphiphilic receptors or ligands at lipid bilayers. Schematic representation of the binding of a lipid anchored receptor or ligand at a lipid bilayer and possibilities for subsequent binding or sorting as well as processes that can obstruct membrane binding of the amphiphile.

1.3.2. General energy and entropy considerations for inserting lipidated ligands and receptors in lipid bilayers

Lipid insertion is mainly driven by hydrophobic interactions. Hydrophobic interactions are most easily understood by imagining that a hydrophobic molecule (or a molecule that contains a hydrophobic section) is introduced into water. Placing a hydrophobic molecule in water results in re-orientation of water molecules to ensure that a maximum amount of hydrogen bonds between the water molecules is retained. However, retaining these hydrogen bonds around the hydrophobic molecule limits the freedom of the water molecules and is thus entropically unfavorable. This entropy effect occurs for both small and large hydrophobic molecules, however, it plays a predominant role only in the case of small hydrophobic molecules (radius below approximately 0.5 nm). These small molecules 'fit' in between water molecules without

breaking hydrogen bonds whereas larger hydrophobic molecules placed in water result in breaking of hydrogen bonds between water molecules, in addition to the re-orientation of water molecules. This results in an additional enthalpic penalty. Now, imagine that the hydrophobic or amphiphilic molecule is already dissolved in water, it will be both entropically and enthalpically favorable to move the hydrophobic (part of the) molecule from water to a hydrophobic environment.

It is now clear that the hydrophobic part of an amphiphile will partition into the hydrophobic core of the lipid bilayer due to hydrophobic interactions. Conversely, this insertion will restrict the conformational freedom of the amphiphile as the hydrophobic section is now restricted to the core of the bilayer and the hydrophilic section is limited to the surface of the lipid bilayer. This means an entropic penalty has to be paid.⁵⁹ Taken together, the exact affinity of the amphiphile for the lipid bilayer will depend on the exact conditions and structure of both head and tail group as will be discussed in more detail in the following sections.

Hydrophobic interactions will generally increase when increasing the temperature, up to a certain point.⁵⁹ This was also shown experimentally for the binding of lipidated proteins to 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) vesicles, between 10 °C and 40 °C. This increase in temperature resulted in observing an additional -5.4 kJ/mol⁶⁰ associated with this interaction.^{59, 61}

It seems intuitive that, when partitioning of the amphiphile into the lipid bilayer is favorable, that aggregation of the amphiphiles in aqueous solution should also be favorable. However, this is not necessarily the case as aggregation requires the thermodynamically costly creation of a (solvated) interface. This interface is already in place for insertion of amphiphiles into a lipid bilayer.⁵⁹ Also, if repulsive forces are present between the head groups of the amphiphile, they will hinder aggregation while it would not necessarily hinder (initial) insertion into a lipid bilayer.

1.3.3. Chain length and structure of lipidated ligands and receptors

Hydrophobic interactions provide access to design and tune the interaction strength between an amphiphile and the lipid bilayer. Increasing the exposed area of the hydrophobic section should increase the free energy of insertion into a lipid bilayer. This relationship was shown almost four decades ago by Tanford, who studied the partitioning of a range of saturated linear fatty acids (C8:0-C22:0).⁶² A clear linear trend was observed between the lipid chain length and the free energy of partitioning of the fatty acids between water and n-heptane. This trend was expressed by equation 1.1:⁶²

$$\Delta G_p = 17.8 - 3.45 \times n_c \quad (\text{Eq. 1.1})$$

1 With G_p the free energy of partitioning into the organic phase (in kJ/mol) and n_c the number of carbons in the fatty acid. Note that here the partitioning of the molecule to the bulk of the organic phase is described, not to the interface as is the case for insertion in lipid bilayers. Thus, the entropy penalty mentioned in section 1.3.2 is not included in this equation. Instead, a penalty for the partitioning of the hydrophilic carboxylic acid into the organic phase is additionally present. However, when this value (17.8 kJ/mol) is replaced by the entropy penalty for lipid bilayer insertion, the equation provides a powerful tool to estimate the free energy for the insertion of amphiphiles into lipid bilayers. Indeed, the value of 3.45 kJ/mol for each additional CH_2 in the lipid tail was also observed for lipidated peptides that bind to vesicles of various compositions (Figure 1.7A).⁶³ The entropy penalty is graphically reflected in Figure 1.7A as a positive free energy of binding in absence of a lipid tail. Interestingly, this relation was found to be valid also for quite long lipid chains up to C22:0. This suggests that even these long chains do not coil up in aqueous solution, as that would reduce the free energy gain for additional CH_2 groups.^{59, 62} Analogously, the dissociation *rate* constant of fatty acids from vesicles was found to decrease by 10-fold for each additional two CH_2 groups, as was measured for the range C14:0 to C24:0.¹⁷

The Tanford relation holds for linear and saturated lipid anchors. However, depending on the application or biological system, the structure of the lipid anchor might be different. For those cases, predicting the affinity is more difficult and some examples will be highlighted in this section. One commonly used anchor is cholesterol, which is often assumed to provide stable, irreversible anchoring of molecules to the membrane. To study the interaction of cholesteryl-modified ssDNA (59 nucleotides) with SLBs, QCM-D was used.⁴⁸ Figure 1.7B shows a decrease in the frequency (negative Δf) indicating that mass adsorbs to the pre-formed SLB. Contrary to expectations, complete desorption of the cholesteryl-modified ssDNA was observed upon washing with buffer. The interaction was instead irreversible when two saturated C18 alkyl tails $(\text{C18:0})_2$ were used to anchor the same ssDNA to a lipid bilayer. The interaction was also found irreversible when cholesteryl-modified ssDNA was hybridized with a complementary ssDNA that also carried a cholesteryl anchor, which effectively gave two cholesteryl anchors for dsDNA yielding a much more stably inserted ligand.⁴⁸ Stably inserted dsDNA carrying two cholesterol groups, one on each strand, was also observed by Höök *et al.*⁶⁴

Figure 1.7 | Effect of the chain length and structure of the lipid anchor on membrane binding. (A) Effect of chain length on the free energy of membrane insertion for linear, saturated lipid anchors. The grey area indicates an uncertainty in the entropic penalty.⁶⁵ (B) ssDNA modified with three different anchors interact with SLBs followed by QCM-D.⁴⁸ (C) Coiled-coil forming peptides inserted in vesicles induce fusion depending on the choice of the anchor.⁴⁶ Adapted with permission, copyright: A) 2011, Elsevier, B) 2014, American Chemical Society, C) 2013, American Chemical Society.

The influence of unsaturated bonds in lipid alkyl chains on the free energy for binding to lipid vesicles has been studied using isothermal titration calorimetry.⁶⁶ The fatty acids C18:1, C18:4 and C22:6 resulted in a binding free energy of -31, -24 and -29 kJ/mol, respectively.⁶⁶ Clearly, the introduction of each additional unsaturated bond in the chain leads to a decrease in affinity of the chain to the lipid bilayer. In line with these observations, the dissociation *rate* of a series of fatty acids from the lipid bilayer was evaluated and found to increase 5-fold when going from C18:0 to C18:1 and 10-fold from C18:0 to C18:2.¹⁷

Coiled-coil forming peptides have been functionalized with hydrophobic anchors such as adamantane, DOPE and cholesterol.⁴⁶ The affinities of these lipidated peptides to the lipid bilayers were not determined, but the differences in the structure of the hydrophobic chains had effect on their affinity to the lipid bilayer as was studied in content mixing assays.⁴⁶ In these assays, vesicles that contain a self-quenching concentration of a fluorescent dye were incubated with one of the lipidated peptides and subsequently mixed with empty vesicles incubated with the complementary

lipidated peptides. An increase in fluorescence emission signified vesicle fusion. A correlation was found between the hydrophobicity of the anchor and the ability of the corresponding peptide to induce vesicles fusion (Figure 1.7C). In this study, high pressure liquid chromatography (HPLC) was used to compare the hydrophobicity of the various lipid anchored peptides while the hydrophilic segment remained the same.⁴⁶

1.3.4. Influence of the hydrophilic segment of lipidated receptors and ligands

The role of the hydrophilic segment of the amphiphile in the binding process of the amphiphile to the lipid bilayer is limited, as it remains in the aqueous environment.⁵⁹ Provided that there is no interaction of the hydrophilic segment with the head groups of the lipid bilayer (electrostatic interactions will be discussed in section 1.3.7), the contribution of the hydrophilic segment on the free energy of binding is limited to a decrease in conformational freedom. Nevertheless, amphiphiles with the same anchor can show significantly different binding behavior. It has been shown recently that when a stably anchored biotin-functionalized lipid binds a monovalent streptavidin variant, the lipid is pulled out of the lipid bilayer as was traced using QCM-D.⁵⁵ It intuitively makes sense that a large hydrophilic segment draws the equilibrium towards the dissolved, unbound state. Indeed, the entropy penalty for immobilizing a protein of 0.1, 1 and 100 kDa was calculated to be approximately 33, 42 and 63 kJ/mol respectively. This calculation overestimates the entropic costs, yet it nicely shows the effect of the size of the hydrophilic section on the binding efficiency to the lipid bilayer. In addition, the flexibility of the structure will influence the entropic penalty for the binding of the entire receptor-ligand complex to the lipid bilayer.⁶³

1.3.5. The role of the membrane

In addition to the partitioning of the amphiphile into the lipid bilayer, it is often found that amphiphiles sort into a part of the bilayer with a specific curvature or into a specific phase of a heterogeneous bilayer. This has been observed in various studies where fluorescently labelled amphiphiles or atomic force microscopy was used.⁶⁷⁻⁷¹ Although there are no clear rules, some general trends can be observed: saturated lipid tails and sterols often associate with the L_o phase, while unsaturated lipid tails containing at least one cis double bond often associate with the L_d phase.^{70, 71} For example, a double cholesteryl-modified ssDNA partitioned into the L_o phase on heterogeneous giant unilamellar vesicles (GUVs) as visualized using confocal microscopy (Figure 1.8A). In contrast, peptides modified with two C16:0 tails, partitioned into the L_d phase

as was visualized using AFM.⁶⁷ Recently, the insertion of peptides modified with C14:0 to lipid bilayers was evaluated. Using fluorescently labelled lipids and fluorescently labelled lipidated peptides, a FRET assay showed small differences in affinity depending on the curvature, for vesicles in the range of 30 to 400 nm in diameter.⁶⁸ In addition, small differences in affinity of lipidated peptides for L_o , L_d and S_o phases could be determined by using heterogeneous GUVs.⁶⁸ These results are summed up in Figure 1.8B. Similar subtle differences in phase preference were found in nearest neighbour recognition measurements and Monte Carlo simulations using peptides anchored with combinations of myristoyl, palmitoyl, cholesterol and a myristoyl derivative with a cis-cyclopropyl moiety, which has a permanent cis configuration in the lipid chain.⁷²

Figure 1.8 | Lipid sorting towards specific curvatures or phases. (A) Left and right: same GUV composed of 1:1:1 DOPC:DPPC:Chol with rhodamine-PPPE (red) as lipid dye partitioning into the L_d phase and (Chol)₂-ssDNA partitioning into the L_o phase as shown by fluorescently labelled (green) complementary DNA.⁷³ **(B)** Preference of lipidated peptides to partition into a certain phase (top) or a certain vesicle size and with that curvature (bottom).⁶⁸ **(C)** AFM image of a lipid bilayer consisting of DOPC, DPPC and cholesterol (respectively 1:2:1) exposed to N-Ras protein equipped with two farnesyl chains. Left: 2 hours after exposure and right: 25 hours after exposure.⁶⁷ Adapted with permission, copyright: A) 2009, American Chemical Society, B) 2017, WILEY-VCH, C) 2009, American Chemical Society.

One factor that can play a role in whether inserted lipid chains end up in a certain lipid phase or membrane curvatures is the hydrophobic mismatch. This mismatch indicates a difference in size of a lipid anchor or the hydrophobic part of a

transmembrane protein compared to the thickness of the hydrophobic part of the membrane. As a result, either the membrane can locally adapt its thickness, or the lipid anchor can adapt its length. Either adaptation gives an unfavorable contribution to the binding of the amphiphile to the lipid bilayer. Adaptation of the membrane results in perturbations where phospholipids in the membrane have to adapt their extended length. This is observed for transmembrane proteins as these proteins themselves cannot easily adapt their thickness.⁷⁴ Adaptation of the anchor of the receptor or ligand is also not commonly encountered, but has been observed for the N-Ras protein equipped with a C16:0 anchor.⁷⁰ Using ^2H NMR, the C16:0 chain was found to reduce its length by introducing so-called *gauche* defects. These *gauche* defects are C-C bonds that are transiently in a less favorable *gauche* configuration. Depending on the thickness of the membrane, the C16:0 anchor was found to be between 8.7Å and 15.5Å, corresponding to an average of eight and two *gauche* configurations at any time, respectively.⁷⁰ Surprisingly, in a similar system with lipidated α -helix peptides with various alkyl chain lengths, no significant adaptation of the chain length in the host membrane was observed.⁶⁵ Hence, it is unclear how generally applicable this process is for inserted lipids. In more general terms, an amphiphile will partition to the location in the bilayer where both the membrane and the amphiphile have to adapt as little as possible. This might result in partitioning of the amphiphile into a certain phase, thickness or curvature. It has even been observed, by AFM, that N-Ras proteins that were equipped with two farnesyl chains partition to the interface of L_o and L_d domains, thus alleviating the line tension between the phases (Figure 1.8C).^{67, 74} The various sorting effects and secondary binding modes are schematically depicted in Figure 1.6.

1.3.6. Amino acids as hydrophobic anchor

Considering their omnipresent appearance in cellular lipid membranes, the use of amino acid sequences as a chain to anchor a larger entity to the membrane deserves special attention. That amino acids can be used as anchor in the lipid bilayer is perfectly illustrated by (trans)membrane proteins where hydrophobic amino acid residues in a certain segments of the protein, often in a α -helix or β -barrel, provide affinity to the membrane.

Cell penetrating peptides^{75, 76} and antimicrobial peptides⁷⁷⁻⁷⁹ are interesting classes of molecules that use amino acid residues to provide dynamic interaction with the membrane. Cell penetrating peptides generally contain both positively charged and hydrophobic amino acid residues. This allows dynamic interactions with membrane-

bound glycosaminoglycans, resulting in endocytosis, or with the membrane, resulting in direct translocation through the membrane.⁷⁵ Antimicrobial peptides are small (<10 kDa) peptides that can be found in *e.g.* macrophages and can kill pathogens such as bacteria, enveloped viruses, and fungi. Due to their amphiphilic character and specific structure, these peptides often have a disruptive effect on lipid membranes, which in turn is related to their function.⁷⁷ Their small size makes that these peptides can be readily synthesized and adapted if desired. Binding studies of such peptides to lipid bilayers give some interesting insights. It has been found that the LAH4 peptide (with Leu-Ala-His repeat unit) binds fluid lipid vesicles with a 0.24 μM affinity.⁷⁸ Interestingly, it has a random coil conformation in solution, but adopts an α -helix structure upon association with the membrane.⁷⁸ Another example is the GALA peptide (with Glu-Ala-Leu-Ala repeat unit) that adopts an α -helix only at $\text{pH} < 6$ at which point it associates to the membrane. Above this pH , deprotonation of the glutamic acid residues results in the destabilization of the helical structure. Together with the increased polarity, this results in detachment from the membrane when the peptide is in this random coil state (Figure 1.9A).⁸¹ It should be noted that in both examples above, the peptide and the lipid bilayer are oppositely charged and, therefore, this peptide-bilayer interaction is not just based on hydrophobic interactions but also on electrostatic interactions.

Figure 1.9 | Amino acid based membrane interactions. (A) Antimicrobial α -helix peptide interacting with lipid monolayer in a pH dependent manner.⁸¹ (B) Contributions of individual amino acid residues to the binding to lipid bilayers based on molecular dynamics simulations (y-axis) and experimental data from equilibrium dialysis combined with HPLC (x-axis).⁸⁰ Adapted with permission, copyright: A) 2015, American Chemical Society, B) 2016, American Chemical Society.

From these examples it is clear that even for relatively short amino acid sequences, predicting the affinity of proteins or peptides for lipid bilayers is not straightforward, as the environmental conditions and secondary structure can have large influences. Still, attempts have been made to determine the contribution of various amino acids to membrane binding.⁸⁰ For short peptides without secondary structure, individual amino acid contributions were found to be largely additive and their contributions are

presented in Figure 1.9B. Note that the values relate to bringing the amino acid residue into contact with the lipid bilayer and not necessarily indicate full incorporation of amino acid residues into the hydrophobic core of the bilayer. Therefore, the hydrophobic amino acids give a positive value while the hydrophilic amino acids do not give (large) negative values.⁸⁰

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1.3.7. Electrostatic interactions at lipid bilayers

Electrostatic interactions can contribute or even fully determine the interaction of a molecule or larger structure with lipid bilayers. A few examples are selected in this section to show the role and potential use of electrostatic interactions at lipid bilayers. Note that these systems require the use of charged lipids, which conflicts with achieving nonfouling lipid bilayers that are made best using overall neutral membranes consisting of zwitterionic lipids.

The potential of using electrostatic interactions on lipid bilayers was shown by the assembly of negatively charged RNA structures at partially cationic lipid bilayers (Figure 1.10A). These RNA structures were attached as a whole or could be build up step-by-step on the SLB. The charge repulsion between RNA structures was reduced by the interactions with the oppositely charged lipids, allowing efficient packing of the RNA structures.⁸² The binding of positively or negatively charged ferritin protein cages occurred only to oppositely charged lipid bilayers, however the charge density at the lipid bilayer determined the extent of binding, up to full coverage of the bilayer with ferritin cages.⁸³ The important role that pH can have on these electrostatic interactions was also demonstrated by monitoring the binding of ferritin to SLBs as function of the pH (Figure 1.10B). In this case, the charge of the ferritin cage can be inverted by changing the pH to be above or below the isoelectric point (pI).⁸³ For proteins in general, an approximate 16-25 kJ/mol is estimated per interaction between a charged amino acid and an oppositely charge lipid.⁸⁴ Particularly insightful and comprehensive in this respect, is the work from Geiger *et al.*⁸⁵ The binding of negatively charged allylamine hydrochloride (AH) and its polymeric equivalent (PAH) were studied using second harmonic spectroscopy, vibrational sum frequency generation spectroscopy, and QCM-D, as well as molecular dynamics calculations. AH was observed to bind to lipid bilayers containing 10% of anionic lipids with a binding constant of 0.16 M and a corresponding ΔG of -14.6 kJ/mol. For binding PAH with 190 repeat units to the lipid bilayer, a binding constant of 3.7×10^{-8} M was obtained with a corresponding ΔG of -52.7 kJ/mol.⁸⁵ Although a clear increase in affinity of almost seven orders of magnitude was observed when comparing binding of AH and PAH to the lipid bilayer, it also shows

the non-additive nature of the binding free energy. This was ascribed to polymers that retain their conformation when bound to membranes, yielding only partial contact with the lipid bilayer, as schematically shown in Figure 1.10C. Additionally, it was shown that an increase in ionic strength decreases the binding affinity both for the monomeric and the polymers case as a consequence of charge screening.⁸⁵ Altogether, the capacity of such polymeric systems for designing reversible interactions at lipid bilayers is demonstrated as well as the main parameters that should be taken into account.

Figure 1.10 | Example systems using electrostatic interaction at lipid bilayers. (A) Assembly of RNA (negatively charged) polyhedron at DOTAP (positively charged) doped lipid bilayer followed using QCM-D.⁸² (B) Binding of ferritin is highly dependent on lipid bilayer charge in combination with buffer pH, which determined the charge of ferritin.⁸³ (C) Positively charged allylamine hydrochloride (AH) and the polymeric equivalent (PAH) binding to lipid bilayers doped with anionic lipids.⁸⁵ Adapted with permission, copyright: A) 2014, Royal Society of Chemistry, B) 2012, Royal Society of Chemistry, C) 2017, American Chemical Society.

1.4. Interplay of interactions: biological function

The focus in this chapter is on fabricating (semi)synthetic systems that show dynamic interaction with model lipid membranes with a specific focus on the direct interaction with the hydrophobic core of lipid bilayers using amphiphilic molecules. In most of the presented examples the aim has been to mimic biological systems, either to better understand them or to recreate them or their function. We will conclude with

one example from biology that elegantly demonstrates how various effects described in this chapter come together to generate biological function.

1 The bin-amphiphysin-rvs (BAR) domain characterizes the BAR-protein family which is involved in reshaping the cell membrane, for instance for shaping membranes into tubes and for assisting in endocytosis. The BAR family consists of proteins that dimerize to give rigid, 'banana' shaped structures with various degrees of curvature (Figure 1.11A).⁸⁶ Initial binding to the membrane is driven by electrostatic interactions.⁸⁷ The N-BAR dimer presents positively charged residues at the concave surface of the dimer, which interacts with negatively charged lipids. This results not just in binding but also induces membrane curvature, simply by functioning as scaffold (Figure 1.11B). In addition, some of the negatively charged lipids that are attracted in the protein-membrane interface can also induce membrane curvature. This is the case for the negatively charged phosphorylated phosphatidylinositol (PI) lipids. These lipids have an inverted conical shape that induce membrane curvature (Figure 1.11C).⁸⁸ Furthermore, both monomers of the N-BAR dimer present two amphiphilic helices, which stabilize the interaction with the membrane by inserting in the core of the lipid bilayer. In fact, the localization of these helices in the membrane was found to be optimal for inducing membrane curvature by functioning as molecular 'wedge'. This wedging mechanism is schematically shown in Figure 1.11B.⁸⁷ Intriguingly, the overall effect works both ways: N-BAR proteins do not just induce curvature, but they also preferably partition into curved membrane regions. This can be partially dedicated to an increased concentration of packing defects in curved membranes that can accommodate amphiphilic helices.⁸⁹ In addition, the curved shape of the BAR dimer will also result in preferred partitioning to membranes with (similar) curvature, to optimize electrostatic interaction without having to deform the membrane. Together, this will result in a self-reinforcing concentration of BAR dimers in a curved membrane region.⁸⁹ This was also shown experimentally by pulling a tubular lipid bilayer structure out of a GUV using optical tweezers and a streptavidin coated bead (Figure 1.11D). In this experiment, I-BAR proteins were shown to partition into the highly curved tube region. Curvature sorting was only observed when I-BAR proteins were inside the GUV and not when they were outside. This is expected as I-BAR proteins have their positive charge on the convex side and therefor associate with negatively curved membranes.⁹⁰

Figure 1.11 | Structure and function of BAR-domain proteins are highly coupled. (A) Shape of various BAR proteins. The grey line indicates the curvature and the side of the protein dimer that is positively charged.⁸⁶ **(B)** The N-BAR protein presents amphiphilic helices at the concave side (left) which together with the curved structure results in membrane curvature (right).⁹¹ **(C)** Electrostatically induced accumulation of negatively charged PI lipids at the interface between the membrane and the BAR-protein induces membrane bending.⁹² **(D)** Schematic (top) and fluorescence microscope (bottom) picture of a lipid tube being pulled out of a GUV using a streptavidin coated bead. Green colour shows the partitioning of the I-BAR protein dimer in the highly curved tube.⁹⁰ Adapted with permission, copyright: A) 2011, WILEY-VCH Royal Society of Chemistry, B) 2012, ELL & Excerpta Medica, C) 2014, The American Physiological Society, D) 2015, Nature Publishing Group.

In the context of this work but also in a biological context, reversibility of membrane association is very important. If the curvature inducing proteins would not detach, they might result in undesired effects further on, or at least be lost for repeated use. For the BAR proteins, scission of the endosome from the original membrane results in a decrease in the curvature which will result in detachment of the protein. This nicely demonstrates how the whole circle of attachment, function and detachment is 'programmed' into the design of the protein. It should be noted that the BAR protein

family was highlighted here as example, but that it is just one of many proteins involved in membrane reshaping processes and their effect is generally a collective effect.

1.4.1. Scope and outline of the thesis

In many biological processes, dynamic interactions and multivalency play a crucial role. This is particularly true for interactions at the cell membrane, which is by itself a highly dynamic, multivalent ligand platform. Many biological interaction are not (fully) understood. For example: how many interactions are needed for a virus to be endocytosed? How do cells sense the dynamics of their environment and what is the effect on cell behavior? What is the role of receptor density and mobility on the binding of multivalent ligands? Answering this kind of questions is not just fundamentally relevant. It will also aid in the design of biosensors, provide more control in tissue engineering and help the development of multivalent constructs for cell targeting and pathogen inhibition.

In this thesis, a variety of interactions platform are designed, characterized and applied that help to understand and use these biologically relevant interactions. To this end, we used the many advantages of lipid bilayers described in this chapter, such as nonfouling behavior, ease of (dynamic) functionalization, lateral mobility and biomimicry. While supported lipid bilayers are at the base of the interaction platforms in all chapters of the thesis, the proposed applications range from cell adhesion studies to drug delivery and virus detection.

In chapter 2, SLBs are functionalized with methyl viologen and cell surfaces are modified with naphthol *via* metabolic labelling and subsequent click-chemistry. This facilitated the programmed assembly of cells at SLBs in the presence of cucurbit[8]uril (CB[8]) that binds methyl viologen and naphthol together in a heteroternary supramolecular complex.

In chapter 3, integrin-binding Arg-Gly-Asp (RGD) ligands with various lipid tails are synthesized and the interaction of these peptides with SLBs are characterized. The range of binding affinities of these ligands to the membrane is related to hMSC cell adhesion and spreading.

In chapter 4, the lipidated RGD peptides discussed in chapter 3 are shown to spontaneously assemble into domains at SLBs at sufficiently high peptide densities. In addition, the behavior of lipidated peptides in terms of domain formation, lateral mobility and interactions with cells are investigated by using a lipidated peptide labelled with a fluorescent dye.

In chapter 5, specific and selective binding of nickel(II)-complexing nitrilotriacetic acid (NTA(Ni)) presenting vesicles is achieved on SLBs that are functionalized with histidine bearing peptides. This interaction platform allowed for demonstration of the possibility to target mobile, membrane bound receptors based on receptor density, *i.e.* superselective targeting. In addition, recruitment of histidine receptors in the fluid SLB is also demonstrated and is hypothesized to have a reinforcing effect on superselective targeting behavior.

In chapter 6, the SLB is used as virus detection platform. Biotinylated SLBs are fabricated to first bind streptavidin and then bind a polyacrylic acid polymer functionalized with biotin and trisaccharides to mimic either the avian or human receptor. The binding of hemagglutinin (HA) clusters, used as virus model, is characterized in detail using QCM-D and shows high selectivity for the receptor type.

Chapter 7 discusses various studies on how to expand and use the toolbox of interactions with lipid membranes. Specifically, we describe how to supramolecularly attach fluorescent proteins to SLBs, how to generate SLBs on generally applicable polyelectrolytes, and how to functionalize various cell types with (desthio)biotin handles *via* vesicle fusion by using cholesteryl or DOPE anchored biotin and DOPE anchored desthiobiotin.

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Chapter 2

Functionalizing the glycocalyx of living cells with supramolecular guest ligands for cucurbit[8]uril-mediated assembly

Multiple naphthol ligands were installed on the glycocalyx of white blood cells via metabolic labeling and subsequent strain promoted azide-alkyne cycloaddition. Only when cucurbit[8]uril was present to drive the formation of ternary complexes, cells specifically assembled on a methylviologen functionalized supported lipid bilayer through multivalent interactions.

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2.1. Introduction

The development of strategies to engineer cell-surface interactions is essential to achieve control over cell-cell and cell-material interactions. These interactions are mediated by *e.g.* cell receptors and their ligands and are dynamic and vital for correct cell behaviour.^{1, 2} Supramolecular chemistry provides dynamism, reversibility and responsiveness to the design of cell-surface interactions.³⁻⁶ Installing non-natural supramolecular receptors and ligands on the exterior of cell membranes provides access to programmable cell behaviour.⁷⁻⁹ In addition, cell adhesion can be controlled in space and time when the supramolecular interaction motifs are sensitive to remote stimuli.¹⁰⁻¹⁵

Several methods have been described to install non-native motifs on cell membranes without interfering with natural cell receptors and signalling, *e.g.* by engineering the cell genome,¹⁶ by performing covalent chemistry on membrane proteins,¹⁷ by inserting artificial lipids into the cellular lipid bilayer,^{2, 18-25} or by metabolic labeling of the glycocalyx.^{26, 27} For example, non-natural sugars containing a bio-orthogonal azido group²⁸ have been metabolically incorporated into the glycocalyx structure on the cell membrane, as depicted in Figure 2.1. These azido-modified cell membranes were reacted with DNA strands via either strain-promoted azide-alkyne cycloaddition (SPAAC), copper(I)-catalyzed Huisgen azide-alkyne cycloaddition (CuAAC) or Staudinger ligation. This elegant metabolic approach has enabled the site-specific assembly of living cells labeled with single DNA strands on surfaces displaying complementary DNA sequences,^{29, 30} but also multiple cell types have been used to form microtissues with controlled stoichiometry.⁷

Although DNA-immobilization strategies hold great potential, the use of synthetic supramolecular host molecules provides access to cellular assemblies responsive to remote stimuli and avoids susceptibility to nucleases. Among the supramolecular host molecules that are suitable to apply under physiological conditions are the pumpkin-shaped macrocyclic cucurbit[n]uril (CB[n]) host molecules.^{6, 31-33} For example, we previously used CB[8] ($n=8$) as the host to anchor guest Trp-Gly-Gly-Arg-Gly-Asp-Ser (WGGRGDS) peptides via their N-terminal tryptophan to surface-bound guest methylviologens as ternary complexes.¹² Detachment of the cells that adhered to these supramolecular RGD surfaces occurred under electrochemical control. Clearly, this cellular self-assembly strategy relies on the ability to bind RGD ligands to their natural occurring integrin receptors on the cell membrane. This strategy is integrin-dependent

Figure 2.1 | Experimental outline. At the top, schematic representation of a Jurkat cell modified via metabolic labeling and SPAAC to display naphthol (Nph) moieties on the cell membrane. In the middle, the naphthol (guest) functionalized Jurkat cell assembles on a methylviologen (MV) (guest) bearing supported lipid bilayer (SLB) in the presence of CB[8] (host) by the formation of multiple ternary host-guest complexes. At the bottom, structures of the molecules used in this chapter.

and therefore lacks in flexibility when dealing with different cell types with different adhesiveness characteristics. Therefore strategies are required that install non-native supramolecular motifs on cell membranes that enable programmable assembly protocols orthogonal to natural cell receptor-ligand interactions. Very recently we have incorporated CB[8]-binding motifs at the bacterial surface by genetically modifying a transmembrane protein to form multiple intercellular ternary complexes leading to the assembly of bacteria.¹⁵

In this chapter we report the installation of supramolecular CB[8]-binding ligands on the cellular membrane using metabolic labeling and the subsequent CB[8]-mediated assembly on a supported lipid bilayer (SLB). To achieve specific CB[8]-mediated cell adhesion, this receptor-independent strategy was combined with the use of a suspension cell line, such as blood cells. Supramolecular naphthol (Nph) guest moieties were introduced onto the membrane of white blood cells by using metabolic oligosaccharide engineering.³⁰ Administration of peracetylated *N*-azidoacetyl-*D*-mannosamine (MAN) to living Jurkat cells incorporates azido groups into the glycocalyx on the cell surface (Figure 2.1). These azido-functionalized cells were then reacted with bicyclononyne-functionalized naphthols (NphBCN, for synthetic details see experimental section) in a SPAAC reaction²⁷ to install the naphthol groups on the cell surface. Finally we show that specific cellular supramolecular interactions are established by heteroternary complex formation between CB[8] and two different guests, *i.e.* SLB-bound methylviologen (MV) and cell surface-bound naphthol causing cellular assembly at surfaces.

2.2. Results and discussion

The metabolic introduction of azido groups and the progress of the subsequent reaction with bicyclononyne derivatives was validated using flow cytometry. Jurkat cells were incubated for 3 days with 50 μM MAN in cell culture medium and subsequently, after washing, a PBS solution of 48 μM bicyclononyne functionalized fluorescein (fluoBCN) was added for 1 h at 37°C (see experimental section 2.5.3 for details) for the SPAAC reaction. Subsequently, the cells were washed and analysed by flow cytometry (Figure 2.2A-B). As controls, cells were reacted either with only MAN (condition +MAN-fluoBCN), or with only fluoBCN (condition -MAN+fluoBCN), or with neither of these two (condition -MAN-fluoBCN). All controls consistently showed low fluorescence intensities with respect to the condition when both MAN and fluoBCN (condition +MAN+fluoBCN) were present indicating that the reaction occurred

specifically. Moreover, inspection of the cells that were treated with both MAN and fluoBCN using confocal fluorescence microscopy revealed green emission that was localized on the cellular membrane (Figure 2.2B, inset).

Figure 2.2 | Cell surface functionalization. Analysis of Jurkat cell surface functionalization after 3 day incubation with 50 μM MAN and subsequent SPAAC with fluoBCN. **(A)** Distributions of the green fluorescence intensity of cells labeled with 48 μM fluoBCN and controls as assessed by flow cytometry. **(B)** Average fluorescence intensities over three independent experiments is shown for all conditions: for each individual measurement the mean fluorescence intensity was normalized against a set of calibration beads (error bars = standard deviation). The inset shows a representative confocal image of fluoBCN labeled Jurkat cells. **(C)** Turnover study of azide (reactive group of MAN) and of fluoBCN groups at the cell surface by flow cytometry. Turnover of azide (+MAN+1day+fluoBCN, n=1) was tested by introducing 1 day of cell incubation in medium between the metabolic labeling and the click reaction. Turnover of fluoBCN (+MAN+fluoBCN+1day, n=2) was tested by introducing 1 day of cell incubation in medium after the click reaction. For both turnover experiments the corresponding negative controls are presented as well. **(D)** FluoBCN concentration dependence of the fluorescence intensity of labeled Jurkat cells as measured by flow cytometry.

These results confirm earlier findings that metabolic labeling procedures yield azido groups at the cell surface that can react with fluorescent molecules carrying complementary reactive groups for the SPAAC reaction. To evaluate the stability of

functional groups after metabolic labeling and subsequent SPAAC, the change in mean fluorescent intensity in the flow cytometry data was followed as function of storage time. After incubation of the cells with azido-functionalized mannose (MAN), cells were washed, kept in culture medium for one more day without MAN prior to SPAAC coupling with fluoBCN. Similarly, the azido-functionalized mannose was administered to cells and directly reacted with fluoBCN, and then kept in culture medium for an additional day without any reactant. In both cases, most of the fluorescence intensity was lost, yet remained higher than the intensity of their corresponding negative controls (Figure 2.2C).

Next, we correlated the concentration of fluoBCN to the number of fluorophores on the cell. Flow cytometry (Figure 2.2D) shows a linear trend of increasing fluorescence intensity of cells treated with increasing concentrations of fluoBCN in the range of 10 to 240 μM . This result indicates that the number of supramolecular CB[8]-binding ligands on the cell membrane can be modulated. In fact, the number of molecules at the cell surface can be determined quantitatively (see experimental section 2.5.5). When using for example 50 μM of MAN and 48 μM of fluoBCN, the number of fluorescein groups per cell was determined to be $1.1 \cdot 10^6$, in agreement to values found in literature.⁷ As a result, this approach seems promising to understand the thermodynamic and kinetic parameters that control cell-cell and cell-surface interactions via defining the density of the supramolecular guest molecules on the cell surface.

We envisioned that such modified cells with artificial supramolecular naphthol guest moieties would interact in a multivalent fashion with a surface displaying methylviologen as complementary guest and CB[8] as the host that binds both Nph and MV groups in a ternary complex. We introduced the complementary MV guest moieties on a supported lipid bilayer to benefit from its excellent nonfouling characteristics as we have demonstrated previously in forming heteroternary complexes between MV and azobenzene-modified sugars at the SLB as an example of cell-material interactions based on a naturally occurring receptor-ligand combination.³⁴

SLBs were produced on glass from vesicles that adsorb, fuse and rupture, forming a supported lipid bilayer covering the surface. Vesicles were made of DOPC, a maleimide headgroup modified lipid (MCC, 0.5 mol%) and a lipid labeled with Texas Red (TR, 0.2 mol%) mixed in chloroform. After drying and rehydration in PBS buffer the vesicles were extruded through a polycarbonate membrane with 100 nm pore size (see experimental section 2.5.6 for details). To incorporate MV into the vesicles, 100 μM of thiol-functionalized methylviologen (MV-SH, for synthetic details see

experimental section) was added to a suspension of the maleimide-functionalized vesicles during the rehydration step. The pH of the rehydration buffer was set to 7.4, which is suitable for the Michael addition reaction to occur.³⁵ After reacting for 2 h at room temperature, the lipid suspension was extruded and the resulting vesicles were used for the formation of SLBs bearing MV groups. The presence of MV on the surface of these vesicles (Figure 2.3A) was verified using zeta potential measurements (Figure 2.3B). Vesicles incorporating the doubly positively charged MV moieties showed a positive potential. A negative potential originating from the negatively charged MCC and TR lipids was retained in a control experiment where paraquat, which bears no thiol, was used during the reaction. A decreased positive potential was detected after adding CB[8] to the MV-bearing vesicles indicative of complexation of CB[8] and MV, which would result in shielded positive charges. Together, these results confirmed that vesicles were decorated with MV and that complexation with CB[8] can occur. The vesicle size was not significantly influenced by the MV labeling or CB[8] complexation, as measured with DLS (Figure 2.3C).

Figure 2.3 | Vesicle functionalization and characterization. Characterization of vesicles of DOPC : MCC : TR (99.3 : 0.5 : 0.2 mol%). **(A)** Vesicle bearing MV before and after complexation by CB[8] is depicted schematically. **(B)** Zeta potential results are shown for vesicles reacted with MV-SH (vesicles+MV) and upon addition of CB[8] (vesicles+MV+CB[8]). As control, vesicles that were not reacted with MV but that were still containing negatively charged MCC and TR were measured in the presence of paraquat (vesicles+paraquat). Error bars are standard deviations based on 3 measurements. **(C)** Diameter size distribution as measured using DLS of the same conditions show stable and uniformly sized vesicles for each condition.

To test whether the formation of multiple ternary complexes between MV:CB[8]:Nph would drive the selective adhesion of cells on the SLB, a flow channel setup was used (see experimental section 2.5.7). Vesicles (0.1 mg/ml) bearing MV functionalities were prepared as described above and flown over the activated glass bottom of the PDMS flow channel. After extensively washing with PBS, a uniform SLB was formed and imaged by fluorescence microscopy (see experimental section, Figure 2.10B).

Jurkat cells were functionalized with Nph ligands using the same procedure that was used to equip the Jurkat cells with fluorescein but now using NphBCN instead of fluoBCN. Prior to flowing the naphthol-functionalized cells, a solution of 20 μM CB[8] was injected in the flow channel and allowed to interact with the MV on the SLB via host-guest interactions.

The formed CB[8]-methylviologen complex constituted the artificial receptor for the naphthol-functionalized Jurkat cells. These cells were then loaded in the flow channel in the presence of 20 μM CB[8]. In a period of 10 min without flow, the Jurkat cells settled on the SLB (Figure 2.4). Subsequently, a low flow rate was applied and then stepwise increased while monitoring the adhesion of the cells in the chip using bright field microscopy (Figure 2.4).

Figure 2.4 | Supramolecular cell immobilization on SLBs. Immobilization of Jurkat cells on a MV-modified SLB in a flow channel setup. Cells were metabolically labeled with MAN and reacted with NphBCN. Cells were loaded (10^6 cells in PBS, 5 pulses of 2 s at 100 $\mu\text{l/min}$) in the flow channel and after 10 min the flow was started again and its rate was increased stepwise. Experiments were performed in the presence of 20 μM CB[8] (+MAN+NphBCN+CB[8]) or 20 μM CB[7] as a control (+MAN+NphBCN+CB[7]). As additional controls, cells reacted with MAN but not with NphBCN in presence of CB[8] (+MAN-NphBCN+CB[8]) and unmodified cells (-MAN-NphBCN-CB[8]) were tested. **(A)** Representative bright field images at increasing flow rates for the conditions (+MAN+NphBCN+CB[8]) and (+MAN+NphBCN+CB[7]). Inset, higher magnification. Scale bars 200 μm , and 40 μm for the inset. **(B)** Dependence of the number of immobilized cells on the applied flow rate for the conditions (+MAN+NphBCN+CB[8]), (+MAN+NphBCN+CB[7]), (+MAN-NphBCN+CB[8]) and (-MAN-NphBCN-CB[8]). Between 300 and 700 cells were counted per condition using ImageJ software in triplicate experiments.

As negative controls, the same procedure was applied but altered either by replacing the CB[8] with CB[7], or to have unmodified cells, or to have cells with MAN but for which the naphthol moieties were missing. Cells in all negative controls already started to disappear from the SLB at flow rates as low as 0.25 $\mu\text{l}/\text{min}$ and beyond a flow rate of 1 $\mu\text{l}/\text{min}$ hardly any cells remained visible at the SLB (Figure 2.4). On the contrary, in the presence of CB[8], cells displaying Nph ligands selectively adhered on the MV functionalities on the lipid bilayer and cells started to flow away from the SLB when flow rates were applied higher than 10 $\mu\text{l}/\text{min}$. Only beyond 100 $\mu\text{l}/\text{min}$ all of the initially adhered Jurkat cells could be flushed away. The results demonstrate that the display of CB[8]-binding moieties that we introduced via metabolic labeling and strain promoted azide-alkyne cycloaddition seem sufficiently stable to provide supramolecular ligands at the cell surface within the time scale of the surface assembly, and that these ligands interact in a specific manner.

2.3. Conclusions

We presented a system that can simplify the investigation of interactions between cells and material surfaces, through the use of synthetic ligands and receptors. Such a system provides a bioorthogonal modification of the cell surface independently from natural cell ligands and with numbers of supramolecular handles that can in principle be modulated. We have demonstrated the successful use of host-guest chemistry to introduce non-natural ligand-receptor interactions while it also enables the specific control over cell adhesion, possibly even on a spatial and temporal scale. This artificial, yet biomimetic approach paves the way for quantifying the relation between the number of receptors and the forces involved in the supramolecular cell adhesion.

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2.5. Experimental section

2.5.1. General methods

Chemicals were purchased from Sigma Aldrich or from Acros Organics unless differently specified and used without further purification. The purity of each batch of cucurbit[8]uril (Sigma Aldrich) was assessed by UV-Vis titration with cobaltocenium according to a literature procedure.³⁶ Dulbecco's Phosphate Buffered Saline (PBS, Sigma Aldrich) with pH 7.4 at 25 °C containing 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride was used. TR was purchased from Invitrogen and all other lipids as well as polycarbonate membranes were purchased from Avanti Polar Lipids. Cell staining reagents were purchased from Invitrogen. FluoBCN was purchased from Synaffix. Reactions were monitored using thin-layer chromatography (TLC), which was performed on 0.2 mm Merck precoated silica gel 60 F254 aluminium sheets. Spots were visualized using a basic KMnO₄ solution unless otherwise specified. Column chromatography was carried out on silica gel 60 (0.063-0.2 mm, Merck). NMR spectra were recorded on a Bruker spectrometer (Ascend 400). Chemical shifts are given in units of parts per million (ppm) and expressed relative to the signals of deuterated solvents. Coupling constants (J) are reported in Hertz (Hz). High-resolution mass spectrometry (HRMS) was performed using a Waters ESI(+)-ToF spectrometer (Micromass LCT) calibrated on the molecular ion of reserpine. The elemental analysis was performed on an Interscience analyser (Flash 2000 CHN). Zeta potentials of lipid vesicles (0.5 mg/ml in 0.5x PBS) were measured in folded capillary cells at 25 °C on a Malvern Instrument system (Zetasizer NanoZS) using a laser with a wavelength of 633 nm and backscatter detection at an angle of 173°. Fluorescence microscopy was performed using an Olympus (1X71) microscope. For confocal microscopy, a Nikon confocal (A1) microscope was used equipped with a 488 nm laser and a 500-550 nm emission filter. Flow cytometry was carried out on BD bioscience flow cytometer (FACS Aria II) equipped with a 488 nm laser. For each measurement, the flow cytometer was calibrated using a Molecular Probe (L14821, 2.5 µm) LinearFlow™ Green Flow Cytometry Intensity Calibration Kit. Fluorescence spectroscopy was performed using a Perkin Elmer fluorimeter (LS 55).

2.5.2. Synthesis procedures

Synthesis of NphBCN (5). NphBCN (5) was synthesized as shown in Figure 2.5. The individual steps are described in more detail below.

Figure 2.5 | Synthesis of NphBCN (5). Synthesis route of NphBCN (5) starting from 2-naphthol.

Compounds **1**³⁷, **2**³⁸ and **6**²⁷ were synthesized according to known literature procedures.

For the synthesis of compound **3**, compound **1** (98 mg, 0.25 mmol, 1eq.) was dissolved in dry CH₂Cl₂ (20 ml) followed by the addition under argon of EDC (0.2 ml, 1.3 mmol, 3.1 eq.), HOBt (61 mg, 0.25 mmol, 1 eq.) and a catalytic amount of DMAP (20 mg). The solution was stirred for 30 minutes followed by the addition of compound **2** (100 mg, 0.4 mmol, 1eq.) and DIPEA (0.1 ml). Stirring was continued for another 18 h at room temperature. The solution was then diluted with 50 mL of CH₂Cl₂ and extracted three times with water (20 ml). The organic layer was dried over MgSO₄ and the solvent was evaporated *in vacuo*. The residue was subjected to silica column chromatography (CH₂Cl₂/MeOH: 9/1). The product was obtained as brownish oil. Yield: 68 mg (58 %); ¹H-NMR (400 MHz, CDCl₃): δ = 7.76-7.70 (m, 3H, 3CH); 7.42 (t, J = 7.9 Hz, 1H, CH); 7.32 (t, J = 7.9 Hz, 1H, CH); 7.14-7.12 (m, 2H, 2CH); 6.16 (s, 1H, NH); 5.00 (s, 1H, NH); 4.10 (m, 2H, CH₂); 3.60-3.47 (m, 10H, 5 CH₂); 3.31 (bs, 2H, CH₂); 2.30 (t, J = 6.8 Hz, 2H, CH₂); 1.89 (m, 4H, 2CH₂); 1.44 (s, 9H, 3CH₃). ¹³C-NMR (101 MHz, CDCl₃): δ = 157.10, 134.72, 129.48, 129.10, 129.02, 127.76, 126.85, 126.47, 123.64, 119.07, 109.43, 106.70, 100.98, 77.36, 70.43, 70.36, 70.31, 70.05, 67.67, 38.87, 36.07, 28.88, 28.55, 22.60.

For the synthesis of compound **5**, compound **3** (100 mg, 0.19 mmol, 1 eq.) was dissolved in 5 ml of dry MeOH. The solution was cooled to 0 °C followed by the dropwise addition under argon of acetylchloride (0.05 ml). Stirring was continued at room

temperature for another 3 h. Completion of the reaction was monitored using TLC. After that time all solvent was evaporated and the product was obtained as brown oil. The product (**4**) was used without further purification in the next step. Compound **4** (50 mg, 0.13 mmol, 1.3 eq.) was dissolved in 5 ml of DMF followed by the addition of TEA (0.1 mL). After stirring for 5 minutes, **6** (30 mg, 0.10 mmol, 1 eq.) dissolved in 2 mL of DMF was added dropwise. The solution was stirred at room temperature for 24 h followed by evaporation of all solvent *in vacuo*. The residue was purified by silica column chromatography (CH₂Cl₂/MeOH: 9/1). The desired product was obtained as pale yellow oil. Yield: 33 mg, (60%); ¹H-NMR (400 MHz, CDCl₃): δ = 7.70-7.76 (m, 3H, 3CH); 7.42 (t, J = 7.8 Hz, 1H, CH); 7.32 (t, J = 7.8 Hz, 1H, CH); 7.11-7.14 (m, 2H, 2CH); 6.12 (s, 1H, NH); 5.18 (s, 1H, NH); 4.14 (d, J = 7.9 Hz, 2H, CH₂); 4.09 (bs, 2H, CH₂); 3.48-3.59 (m, 10H, 5 CH₂); 3.36 (bs, 2H, CH₂); 2.21-2.30 (m, 8H, 4CH₂); 1.88 (s, 4H, CH₂); 1.52-1.58 (m, 2H, CH₂); 1.33 (q, J = 8.5 Hz, 1H, CH); 0.87-0.83 (m, 2H, CH₂). ¹³C-NMR (101 MHz, CDCl₃): δ = 172.87; 156.99; 156.92; 134.68; 129.47; 129.02; 127.73; 126.82; 126.46; 123.67; 119.01; 106.67; 98.91; 70.39; 69.67; 67.66; 67.39; 65.97; 63.72; 62.97; 61.78; 40.87; 40.13; 39.35; 38.80; 38.27; 37.15; 36.36; 30.37; 29.14; 28.86; 26.77; 22.59; 21.52; 20.22; 17.86; 15.39. Traces of ethyl acetate are present in the ¹H-NMR and ¹³C-NMR. HRMS [M+Na]⁺ = 573.2936 (calc. 573.2941), [M+K]⁺ = 589.2888 (calc. 589.2680), [M+2ACN+H]⁺ = 655.2214 (calc. 655.3471).

Synthesis of MAN. The procedure from Laughlin and Bertozzi³⁰ for the synthesis of peracetylated N-azidoacetylmannosamine was used. For TLC, spots were visualized by treatment with 0.05 M solution of triphenylphosphine in toluene and subsequent staining with a 0.08 M ninhydrin solution in 1-butanol containing 0.5 M acetic acid. Yield: 91 mg (6.6%), white wax. ¹H-NMR: (400MHz, CDCl₃) δ = 2.00 (s, 3H), 2.06 (s, 3H), 2.11 (s, 3H), 2.12 (s, 3H), 3.83-3.87 (ddd, 1H, J_{5,6a} = 2.4, J_{5,6b} = 4.5, J_{5,4} = 9.4), 4.12-4.16 (dd, 1H, J_{6a,5} = 2.4, J_{6a,6b} = 12.0), 4.16 (s, 2H), 4.23-4.27 (dd, 1H, J_{6b,5} = 4.5, J_{6b,6a} = 12.0), 4.72-4.75 (ddd, 1H, J_{2,3} = 1.4, J_{2,NH} = 3.6, J_{2,1} = 9.0), 5.14-5.19 (app t, 1H, J_{4,5} = 9.4), 5.91 (d, 1H, J_{3,2} = 1.4), 6.85-6.88 (d, 1H, J_{1,2} = 9.0). ¹³C-NMR: (101MHz, CDCl₃): δ = 20.6, 20.6, 20.6, 20.7, 50.1, 52.3, 61.7, 64.9, 71.4, 73.3, 90.1, 168.4, 168.7, 169.8, 170.3, 170.8. Minor peaks in the ¹H-NMR and ¹³C-NMR are attributed to the anomeric isomer. HRMS [M+Na]⁺ = 453.1248 (calc. 453.1234), [M+K]⁺ = 469.0982 (calc. 469.0973), [2M+Na]⁺ = 883.2366 (calc. 883.2570), [2M+K]⁺ = 899.2091 (calc. 899.2309). IR: 2100 cm⁻¹ v_{as} N₃ (strong). Anal. Calcd. for C₁₆H₂₂N₄O₁₀: C 44.65, H 5.15, N 13.02; found: C 43.34, H 4.88, N 12.33.

Figure 2.6 | Structure of MAN. Structure of MAN with numbered carbon atoms.

Synthesis of MV-SH (10). MV-SH (**10**) was synthesized as shown in Figure 2.7. The individual steps are described in more detail below.

Figure 2.7 | Synthesis of MV-SH (10). Synthesis route of MV-SH (**10**) starting from 4,4'-bipyridine

Syntheses of compounds **7**, **8** and **9** were adapted from Rauwald, et al.³⁹ and González-Campo, et al.³⁷

For synthesis of compound **7**, 4,4'-bipyridine (5.0 g, 32 mmol) was dissolved in DCM (75 ml). Methyl iodide (2.5 ml, 40.5 mmol) was dissolved in DCM (25 ml) and this solution was added dropwise to the refluxing solution while stirring. Upon addition, the colorless solution turned into an orange suspension. The suspension was refluxed for 1 hour and allowed to cool down overnight while stirring. The product was filtered and washed with DCM. The residue was recrystallized from methanol. The product was filtered and washed with diethyl ether, yielding a yellow, crystalline solid. Compound **7** was used for the following synthetic step without further characterization.

For synthesis of compound **8**, 1,11-Dibromoundecane (2.07 g, 6.6 mmol) was dissolved in acetonitrile (15 ml) and DMF (3 ml). Compound **7** (500 mg, 1.68 mmol) was dissolved separately in a warm mixture of acetonitrile (10 ml) and DMF (2 ml) and

added dropwise to the refluxing solution while stirring. Stirring and refluxing conditions were continued for 48 h. The lukewarm solution was filtrated and the residue was washed with acetonitrile. Ether was slowly added to the clear filtrate while stirring. The product was collected by centrifugation (10 minutes at 7000 rpm) and decantation. Recrystallization from methanol and acetonitrile and drying *in vacuo* yielded an orange crystalline solid. Compound **8** was used for the following synthetic step without further characterization.

For synthesis of compound **9**, compound **8** (226 mg, 0.4 mmol) was dissolved in a mixture of Milli-Q water (12.5 ml) and ethanol (5 ml). Potassium thioacetate (114 mg, 1.0 mmol) was dissolved in Milli-Q water (12.5 ml) and ethanol (5 ml) and added to the refluxing solution while stirring. The reaction mixture was kept under N₂ and refluxed for 48 h resulting in a light green-blue solution. NH₄PF₆ (0.26 g, 1.6 mmol) was added to the reaction mixture. The crème-white precipitate was filtered under vacuum and redissolved in acetonitrile. Any brown solids were removed by filtration. The product was again precipitated by adding tetrabutylammonium chloride (0.44 g, 1.6 mmol) resulting in a white precipitate. The product was filtered under vacuum and further dried *in vacuo*. The product was obtained as a pale yellow powder. Compound **9** was used for the following synthetic step without further characterization.

For synthesis of compound **10**, compound **9** (44 mg, 72 µmol) was dissolved in a 1.25 M HCl solution in methanol (0.58 ml) while stirring. The mixture was refluxed overnight. The product was dried *in vacuo* and then dissolved in Milli-Q water (25 ml). The product was precipitated by adding NH₄PF₆ (0.53 g, 3.25 mmol). After vacuum filtration, the residue was dissolved in acetonitrile (10 ml) and again precipitated by adding tetrabutylammonium bromide (1.08 g, 3.25 mmol). Vacuum filtration and further drying *in vacuo* yielded the final product as a yellow-green solid. Yield: 19 mg (51%). ¹H-NMR (400 MHz, D₂O): δ = 9.12-9.05 (dd, 4H, CH); 8.56-8.52 (m, 4H, CH); 4.72 (t, 2H, CH₂), 4.50 (s, 3H, CH₃); 2.71 (t, 2H, CH₂), 2.08 (m, 2H, CH₂), 1.63 (m, 2H, CH₂), 1.36-1.26 (m, 14H, CH₂). ¹³C-NMR: (150 MHz, D₂O): δ = 23.74-38.45, 48.34, 62.21, 126.60, 126.93, 145.41, 146.31, 149.93. HRMS [M²⁺-H]⁺ = 357.2354 (calc. 357.2354), [M²⁺-C₁₁H₂₂SH]⁺ = 171.0896 (calc. 171.0917).

2.5.3. Cell culture

Jurkat cells were cultured in RPMI medium with 10% fetal bovine serum at a density of 10⁵ - 10⁶ cells/ml at standard cell culture conditions (37 °C with 5% CO₂). Cells between passages 11 and 25 were used. For metabolic labeling of cells, 2.5 · 10⁶ cells in 10 ml of the same culture medium were incubated with MAN in the incubator for 3

days and then washed with PBS three times. After that 10^6 cells per condition were allowed to react for 1 h in the incubator with NphBCN (5% DMSO in PBS) or fluoBCN (PBS) depending on the experiments. Finally cells were washed with PBS three times. Cells for negative controls were treated according to the same procedures but replacing the addition of only solvents as described in the main text.

The viability of cells was tested for a range of concentrations of DMSO and CB[8]. The concentrations of 5% DMSO and 20 μ M CB[8] were found to be optimal. The staining experiments were conducted according to standard procedures of the LIVE/DEAD Cell Viability assay from Invitrogen using fluorescence microscopy. Green colour ($\lambda_{\text{ex}} = 460\text{-}490$ nm and $\lambda_{\text{em}} = 525$ nm) corresponds to live cells and red colour ($\lambda_{\text{ex}} = 510\text{-}550$ nm and $\lambda_{\text{em}} = 590\text{-LP}$) corresponds dead cells.

Figure 2.8 | Live-dead assay of Jurkat cells. Cells were exposed for 1 h in the incubator to percentages of DMSO between 0 to 10% (top panel) and of CB[8] between 0 and 50 μ M in PBS (bottom panel).

2.5.4. Flow cytometry

Samples of cells (10^6 cells/ml, 1 ml) were loaded in the flow cytometer after brief vortexing and each measurement was set to count a total of 10^5 cells. For all experiments the fluorescence intensity was calibrated with commercial beads. The flow cytometer was operated with a 488 nm laser and emission was detected at 530 nm.

2.5.5. Determining cell coverage

Cells were labeled with fluoBCN. Their mean fluorescence was measured by flow cytometry. For each measurement, standard beads with various coverages of fluorescein were also measured. The average number of fluorescein molecules per cell could be calculated by quantifying the amount of fluorescein molecules per bead *via* a

calibration obtained by fluorescence spectroscopy. Here, an example calculation is given for cells labeled with 50 μM of MAN and 48 μM of fluoBCN.

First, a calibration curve of concentrations of fluorescein ($C_{\text{fluorescein}}$) between 0 and 0.1 μM in Milli-Q water was made using a fluorescence spectrophotometer. Linear fitting of this calibration curve results in:

$$C_{\text{fluorescein}}(M) = 1.38 \times 10^{-10} \times I_{\text{max,fluorescence}} \quad (\text{Eq. 2.1})$$

With I_{max} the maximum fluorescence emission. Similarly, fluorescence spectra were measured for a series of commercial beads with a range of coverages of fluorescein. The intensity values obtained from the bead series were converted to fluorescein concentration using equation 2.1. This resulted in a known concentration of fluorescein in each sample of beads. The possible influences of scattering of the beads or fluorescence quenching due to proximity of dye molecules to the beads surface were assumed to be negligible.

The same set of beads was measured by flow cytometry as well. This resulted in Figure 2.9, giving a mean intensity for each type of beads. The values of mean intensities from flow cytometry were correlated to the corresponding intensity measured by fluorescence spectroscopy as follows. Each stock solution of beads had a given density of 6×10^7 beads/ml. For fluorescence spectroscopy, samples were prepared by adding one droplet of the sonicated bead solution in 2 ml of PBS. The volume of the droplet was determined to be $38 \pm 1 \mu\text{l}$ (standard deviation, $n=3$) resulting in a final concentration of 1.1×10^6 beads/ml. With the concentration of fluorescein as well as the density of beads known, the coverages (number of fluorescein molecules/beads) of the different beads were determined.

Combining these calculated coverages of the beads, with their mean intensities from representative flow cytometry experiments, resulted in Figure 2.9B. Linear fitting (red line) of these data points results in:

$$\theta (10^6 \text{ molecules/object}) = 3.59 \times 10^{-3} \times I_{\text{FlowCytometry}} \quad (\text{Eq. 2.2})$$

Equation 2.2 provides the average fluorescein coverage (θ) of the cells. For a representative cell experiment with conditions comparable to the ones used for naphthol labeling of cells, the +MAN+fluoBCN sample had a mean intensity of 317.5, giving a coverage of 1.1×10^6 (fluorescein molecules/cell). This is in the same order of magnitude as values obtained in similar studies using metabolic labeling.⁷

Figure 2.9 | Determining fluorescein coverage. **(A)** Fluorescence intensity obtained with flow cytometry for a commercially available set of fluorescein labeled beads. **(B)** Fluorescein coverage of fluorescent beads A-E as a function of their flow cytometry mean intensity. Coverages were calculated as described in the text.

2.5.6. Vesicle preparation

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) was stored as a 10 mg/mL stock solution in chloroform at -20°C and used as the main constituent in SLBs. 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidomethyl)-cyclohexane-carboxamide] (MCC) was stored as a 2 mg/ml stock in chloroform at -20°C . The lipid-dye conjugate, Texas Red-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (TR) was stored as a 1 mg/ml stock solution in methanol at -20°C . The dissolved lipids were mixed in a molar ratio DOPC/MCC/TR of 99.3/0.5/0.2 and dried under a flow of nitrogen in a glass vial in order to create a film of lipid material at the glass wall. This film was further dried under vacuum for at least 1 h and subsequently hydrated by vortexing in Milli-Q water to form multilamellar vesicles (MLVs) at 1 mg/ml. In the case of methylviologen functionalized vesicles, 100 μM of thiol-functionalized methylviologen in PBS was used for the hydration step and the reaction was carried out for 2 h. The lipid suspension was extruded 11 times through a polycarbonate membrane with 100 nm pore size resulting in large unilamellar vesicles (LUVs) as confirmed by DLS measurements (Figure 2.3C). The resulting LUVs were kept at 4°C and used within two weeks or within one day in case of the methylviologen functionalized vesicles.

2.5.7. Supported lipid bilayer (SLB) fabrication

SLBs were fabricated in a flow channel setup which was built as previously described.³⁴ In short, the flow channel consisted of a standard glass microscope slide on which a polydimethylsiloxane (PDMS) flow channel was attached with a width of 1.5 mm and a height of 50 μm . A bright field microscopy overview is presented in Figure 2.10A. For repeated use of flow channels, the channels were rinsed with 1 wt% sodium dodecyl sulfate in Milli-Q water and the glass was activated for 1 h with a 1 M solution of sodium hydroxide in Milli-Q water. The channels were subsequently thoroughly rinsed with Milli-Q water. Supported lipid bilayer formation was achieved by dilution of the vesicle suspension to 0.1 mg/ml in PBS. Prior to vesicle incubation, the channels were flushed briefly with PBS. The channels were then incubated with the vesicle suspension for at least 30 min to allow for vesicle adsorption and rupture to occur. Subsequently, the channels were washed with an excess of Milli-Q water. From this point forth, care was taken to ensure no air bubbles entered the device.

The quality of the SLBs was checked always before and after each cell experiment via fluorescence microscopy by having a uniform red intensity from the TR present in the composition of the bilayer (Figure 2.10B). Moreover a negative control of untreated Jurkat cells (-MAN-fluoBCN-CB[8] in the main text) was flown on the SLB prior to using the same SLB for further experiments to further verify the consistent performance of different individual SLBs.

Figure 2.10 | Visualizing cells and SLB. (A) Stack view of the flow channel. Inlet and outlet are visible at the corners. (B) Fluorescence images of the same SLB before and after an experiment with cells. Red colour from 0.2% TR in the SLB composition.

2.6. References

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Chapter 3

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

Fluid supported lipid bilayers (SLBs) were reversibly (i.e. with out-of-plane dynamicity) functionalized with integrin targeting lipid-modified Arg-Gly-Asp (RGD) ligands via lipid insertion. By varying the length and structure of the lipid chain, both the affinity and the density of the lipid-modified RGD to the SLB was tuned. Analysis by quartz crystal microbalance with dissipation monitoring (QCM-D) combined with spectroscopic ellipsometry (SE) showed that increasing the length of the alkyl chain resulted in higher binding affinities, providing a window of affinities ranging from 10^3 M^{-1} to over 10^5 M^{-1} and ligand coverages that could reach over 30% (with respect to the density of lipids in the top leaflet). We further show that, on C14:0-RGD presenting SLBs and in the context of human mesenchymal stem cell (hMSC) behavior, focal adhesion formation (vinculin localization) and cell spreading are dependent on ligand coverage. Moreover, going from low (C12:0-RGD) to moderate (C14:0-RGD) affinity resulted in 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, inducing less cell spreading, but a similar cellular morphology. In a broader perspective, we have demonstrated that surface affinity can be used as cue to guide cell behavior at material surfaces and that SLBs functionalized with lipid-modified RGD ligands can be used as biointerface to achieve the required tunable dynamicity.

3.1. Introduction

Functionalization of surfaces with ligands that interact with cell receptors represents a synthetic cell-instructive substitute for ligands that reside in the extracellular matrix (ECM) and interact with cells. For example, cell-adhesive Arg-Gly-Asp (RGD) peptide ligands, which are derived from fibronectin and recognized by transmembrane integrin receptors, have been used to fabricate cell-instructive coatings on biomaterials. When anchoring these RGD ligands to material surfaces, control over molecular flexibility, binding affinity, ligand density, and ligand mobility is critical to most effectively guide cells to the desired responses. Such synthetic cell-instructive 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 Enormous progress has been documented over the last decades in terms of integration of instructive properties of the ECM in biomimetic interfaces. Notwithstanding this progress, 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 cell-instructive interfaces.¹ This dynamicity can be achieved in an “on-off” fashion by introducing moieties in the ligand that are responsive towards *e.g.* light, electrical potential, pH or enzyme activity. For example, Del Campo and co-workers immobilized an inactive RGD peptide that became bioactive upon uncaging using light of a suitable wavelength.²⁻⁴ Another sophisticated example from Mrksich and co-workers demonstrates electrochemical control over ligand availability to cells. In their case an RGD ligand was first electrochemically removed from the surface by oxidation of the O-silyl hydroquinone ether bond to give surface-bound benzoquinone moieties to which, subsequently, a diene-functionalized RGD ligand was electrochemically attached by a Diels-Alder reaction.⁵ Stupp *et al.* 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 $1.6 \times 10^3 \text{ M}^{-1}$ and $5.0 \times 10^4 \text{ M}^{-1}$, for naphthyl and adamantyl guests, respectively) on cell response.⁶ Another intrinsically dynamic system was designed in our group, in which ternary complexes of cucurbit[8]uril (CB[8]), tryptophan-modified RGD peptides, and surface-bound methyl viologen were used. Release of the RGD ligands was electrochemically

achieved by reduction of methyl viologen that causes dissociation of the ternary complex.⁷ These and other synthetic responsive strategies have provided important knowledge in engineering cell-instructive interfaces to more closely mimic the natural cellular environment.^{1, 8}

Dynamic behavior can also be achieved by making use of supported lipid bilayers (SLBs), which are often used as artificial mimics of cell membranes.⁹ SLBs can be formed using naturally occurring lipids (*e.g.* the zwitterionic 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, DOPC) introducing a highly biomimetic and biocompatible way to engineer cell-instructive interfaces with intrinsic lateral dynamics and nonfouling properties.⁹⁻¹¹ SLBs have been widely used as platforms to study membrane properties and biophysical parameters as well as to generate surface gradients;^{12, 13} as interfaces to study receptor-ligand interactions in relation to ligand mobility, which was seen to support receptor clustering and signaling;¹⁴⁻¹⁷ as cell surface models to study cadherin mediated cell-cell interactions¹⁸⁻²⁰ in which also a certain extent of mobility was shown to be critical for proper junction formation;¹⁸ to pattern surfaces in order to introduce different domains of ligands,^{11, 21, 22} and to form (responsive) surfaces for reversible cell binding.^{23, 24} Next to these studies, the use of SLBs has been reported for investigations of understanding cell adhesion,²⁵⁻²⁹ cell spreading, and focal adhesion signaling in response to ligand density and mobility. Recently, a few studies appeared regarding stem cell behavior and fate on SLBs, pointing out the importance of ligand density and lateral mobility.³⁰⁻³²

Although the achievements of employing SLBs as cell-instructive interfaces are intriguing and vast, the ligands were incorporated into SLBs stably over time in all of the cases mentioned above, for instance by using streptavidin-biotin interaction, which was verified by quartz crystal microbalance with dissipation monitoring (QCM-D) measurements in some cases.^{30, 32} In some other cases, lipid-modified RGD peptides that contained two alkyl chains, in analogy to phospholipids (*e.g.* DOPC), were incorporated in SLBs which is also expected to provide stable anchoring of the peptide over time.^{25, 26, 28}

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.³³ Various other examples of reversible interactions at lipid bilayers have been described in chapter 1 of this thesis. In the current chapter, cell-instructive interfaces in which cell-adhesive ligands reside reversibly were employed to investigate effects of the affinity of ligands for the substrate surface on

cell behavior, at the SLB interface. Such relation between ligand affinity in cell-instructive interfaces and cell adhesion and spreading has to the best of our knowledge not been described previously.

In this chapter, we rationally designed and synthesized 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 (Figure 3.1). We characterized the binding affinities and coverages of the ligands at the SLB with QCM-D combined *in-situ* with 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,^{34, 35} we show that on fluid DOPC SLBs, lipid-modified ligand affinity and density critically influenced stem cell behavior.

Figure 3.1 | Experimental outline. Schematic representation of the equilibrium between dissolved and inserted lipid-modified peptides. Structures are given for the lipid used for SLBs and for lipid-modified RGD peptides, where "R" represents lipid chains as indicated at the bottom.

3.2. Results and discussion

3.2.1. Characterization of binding of lipid-modified peptides to SLBs

A nine amino acid peptide (KGSGRGDSG) containing an Arg-Gly-Asp (RGD) ligand for specific recognition by integrin receptors for cell adhesion^{36, 37} was synthesized and modified with six different lipid chains that allow insertion into SLBs (Figure 3.1).

To characterize the binding of the lipid-modified ligands to DOPC-based SLBs, QCM-D was used. High-quality, fluidic DOPC (Figure 3.1) SLBs were formed following literature procedures and this was confirmed using QCM-D (Figure 3.23) and fluorescence recovery after photo bleaching (FRAP, Figure 3.24).^{38, 39}

Important to note, is that no changes in frequency and dissipation were detected by QCM-D (Figure 3.2A) and also no changes in coverage were detected by SE (Figure 3.2B) during the incubation of the SLB with peptide ligands that were not modified with lipids, indicative of the absence of nonspecific interactions between the peptide chain and the SLB. Additionally, no micelle formation was observed for any of the lipid-modified peptides in the range of the concentrations used for QCM-D and SE analysis and cell studies (Figure 3.25).⁴⁰

Figure 3.2 | Control for nonspecific interaction of the RGD peptide with the SLB. Combined (A) QCM-D and (B) SE measurement (after DOPC SLB formation, not shown) in open chamber, operated under continuous stirring, of the incubation of RGD ligands without lipid modification to the SLB. The small change in frequency observed in QCM-D resulted from the slight change in temperature upon addition of the sample to the open chamber.

On the contrary, a shift in frequency was detected by QCM-D when DOPC SLBs were exposed to lipid-modified RGD ligands, shown in Figure 3.3A for C14:0-RGD. The rate of association as well as dissociation was found to be dependent on the flow rate (Figure 3.3A-B), suggesting transport limited binding.

Figure 3.3 | Flow rate dependent insertion and desorption. QCM-D measurement (flow chamber) at DOPC SLBs, made prior to the measurement, of the **(A)** insertion of 100 μ M C14:0-RGD and the **(B)** desorption of C14:0-RGD at various flow rates as indicated in the figure. Areas that are not highlighted in grey correspond to 0.5x PBS buffer (pH 7.5).

For sufficiently rigid layers, the Sauerbrey equation (Eq. 3.1)⁴¹ relates the observed changes in frequency (ΔF) detected by QCM-D to changes in adsorbed mass (Δm):

$$\Delta m = -\frac{C \times \Delta F_n}{n} \quad (\text{Eq. 3.1})$$

With C the mass sensitivity constant (17.7 ng/cm² for the 5 MHz sensors used in this chapter) and n is the overtone number. Note that all the frequency shifts given in this chapter are already divided by n . Even for cases where the Sauerbrey equation is valid, hydrodynamically bound water associated with hydrophilic ligands accounts for a considerable part of the observed changes.⁴²

This unknown fraction of hydrated water prevents accurately determining surface ligand coverages based on QCM-D results. To more reliably estimate the ligand density at the surface, the dry mass of the surface bound lipid-modified RGD was determined using spectroscopic ellipsometry (SE), which is based on the change in refractive index with respect to the buffer and can be converted in a change in thickness or areal mass and surface coverage.^{40, 42} The surface coverages of the lipid-modified peptides were determined from SE data by considering layers as Cauchy layers and using de Feijter's optical model, for details see section 3.5.3.

In the case of C14:0-RGD the observed changes in dissipation (ΔD) were small ($\Delta D/\Delta F \ll 4 \times 10^{-7} \text{ Hz}^{-1}$)⁴³ with respect to the observed changes in frequency (Figure 3.5A) during stepwise increase in concentrations of C14:0-RGD flown over the SLB (Figure 3.4). Therefore, the Sauerbrey model can be applied and differences in coverages derived from SE and QCM-D as plotted in Figure 3.4 for C14:0-RGD can be related to hydrodynamically bound water.

Figure 3.4 | Ligand coverage based on QCM-D and SE. Coverages corresponding to titration plateau values from combined QCM-D/SE measurements (open chamber) of the insertion of C14:0-RGD to DOPC SLBs. Coverages from QCM-D, detecting the hydrated mass, are obtained using the Sauerbrey model. Open and closed symbols represent results of measurements *in duplo*.

Titration series of lipid-modified ligands C12:0-RGD, C14:0-RGD and C16:0-RGD were performed in a combined QCM-D/SE setup. In Figure 3.5A-B the combined QCM-D/SE traces for C14:0-RGD are given as example. Association constants (K_a) were derived based on the shifts in frequency (QCM-D) or change in equilibrium coverage (SE) (Figure 3.5C). When comparing the K_a values derived from the stand-alone QCM-D in flow conditions with those values derived in static format in the combined QCM-D/SE set-up, these values were found to be similar (Table 3.1) and plotted as averaged QCM-D derived K_a values in Figure 3.6. Here, K_a values are plotted as a function of the hydrophobicity of the lipid-modified ligands for which we used the reverse phase HPLC retention time of the lipid-modified peptides.⁴⁴ Important to note is that only in the case of C18:0-RGD, we observed a significant depletion effect in the open, combined QCM-D/SE set-up and those results are therefore not used. This was avoided under flow conditions in the stand-alone QCM-D set-up.

Furthermore, Table 3.1 and Figure 3.6 demonstrate that the introduction of an unsaturation (C18:1-RGD instead of C18:0-RGD) in the lipid chain resulted in a reduction of the affinity of the lipid-modified RGD ligand to the SLB. An even more pronounced reduction in affinity was observed when changing from one long lipid chain to two short chains (2xC8-RGD instead of C16-RGD).

The averaged K_a values derived from QCM-D are consistently higher than the K_a values derived from SE. Indeed, the binding curves of the QCM-D measurements, either measured stand-alone or in the combined set-up, saturated beyond a certain concentration that was consistently reached earlier than in the binding curves of the SE measurements (Figure 3.5C and Figure 3.21 in section 3.5.3). Lower saturation

Figure 3.5 | Interaction of C14:0-RGD with SLBs. Titrations of C14:0-RGD (concentrations of the peptide are shown in the graph) to DOPC SLBs, made prior to the measurement, using **(A)** QCM-D (flow chamber, 100 $\mu\text{l}/\text{min}$) and **(B)** SE (open chamber, operated under continuous stirring). **(C)** QCM-D and SE titration plateau values (data points) for the binding of C14:0-RGD to a DOPC SLB, both derived from the same *in-situ* combined (open chamber) measurement and fit (solid lines) using the Langmuir model.

values yield higher K_a values. The underlying cause is related to the QCM-D detecting both the adsorbing molecule and the hydrodynamically bound water, while SE detects only the former. This means that, for QCM-D, the binding of lipid-modified peptides to the SLB contributes more during the early stages of the binding, while in the later stages of the binding, when the surface becomes more crowded, the hydrodynamic volumes of the peptides start to overlap and each additional peptide contributes relatively to a lesser extent to the frequency shift. This observation of hydration shell overlap is in line with literature in the case of the adsorption of proteins and other large biomolecules.^{43, 45} Here, we additionally note that this can yield slightly overestimated K_a values.

Taking together, the binding analysis of the lipid-modified peptides shows that the length of the lipid chain is a suitable design variable to tune the affinity of cell-adhesive RGD peptides to the SLB by over two orders of magnitude, roughly ranging from 10^3 M^{-1} to over 10^5 M^{-1} .

Table 3.1 | Binding constants (K_a) obtained by QCM-D (flow or open chamber) and SE (open chamber).

Lipid-modified peptide	K_a (M^{-1})		
	QCM-D (flow chamber)	QCM-D (open chamber)	SE (open chamber)
C12:0-RGD	1.1×10^3	1.5×10^3	9.3×10^2
C14:0-RGD	1.6×10^4	2.7×10^4	1.1×10^4
duplo		1.8×10^4	0.5×10^4
C16:0-RGD	1.2×10^5	4.6×10^5	1.2×10^5
duplo		1.7×10^5	1.1×10^5
C18:0-RGD	5.5×10^5	-	-
C18:1-RGD	1.8×10^5	-	-
2xC8-RGD	4.0×10^3	-	-

Figure 3.6 | **Affinity and hydrophobicity of lipid-modified peptides.** Binding constants (K_a) of lipid-modified peptides obtained from QCM-D (average of both flow chamber and open chamber) as well as from SE measurements (open chamber). K_a is plotted as a function of the reverse phase HPLC retention time (using the same solvent gradient, flow rate and column) on each of the lipid-modified peptides as a measure for their hydrophobicity.⁴⁴

The lipid-modified RGD coverages derived from SE for C12:0-RGD, C14:0-RGD and C16:0-RGD are converted to relative coverages, with 100% being defined as one lipid-modified peptides per phospholipid in the distal leaflet of the SLB (a surface area of 0.725 nm^2 was used for DOPC).⁴⁶ From the relative coverages it becomes even more apparent that remarkably high peptide coverages at the SLB were achieved (Figure 3.7). Coverages exceeding 30% illustrate the ability of this system to serve as a highly versatile surface coating strategy. In this line, it is also important to note that the affinity of lipid-modified peptides to the surface determines the concentration that is required to obtain a certain coverage, which can hold important implications for cell studies (section 3.2.2 and further).

Figure 3.7 | Peptide coverages. SE measurements (open chamber) of the titration of C12:0, C14:0, and C16:0 lipid-modified peptides to SLBs, expressed as relative coverage with respect to the lipid coverage in the top leaflet of the SLB. Measurements were performed *in duplo* as indicated by the closed and open symbols.

Correlating the changes in frequency and dissipation, can give additional insight into the structure of the formed adlayer.⁴⁰ For the binding of increasing concentrations of the various lipid-modified peptides, the dissipation leveled off while the frequency continued to increase. An example is shown for the insertion of C14:0-RGD in Figure 3.8A-B and an overview using plateau values from the titrations of the different lipid-modified peptides (*in duplo*) is shown in Figure 3.8C. This behavior indicates that initially, a soft and hydrated layer was formed, but that later in the binding process softening of the adlayer came to an end, although additional mass continued to adhere to the surface. This inflection point (at a ΔF value of around 4-5 Hz) indicated the start of the formation of a more densely packed peptide layer, resulting in a relatively less hydrated and more rigid layer.²⁹ Observations that might be related to this behavior are further discussed in chapter 4.

Similarly, the frequency shift from QCM-D (ΔF) was plotted against the coverage from SE (Γ) for various lipid-modified peptides (Figure 3.8D). Fitting of all the data point with a 2nd degree polynomial shows a decreasing slope for increasing coverage. Considering that hydrated mass is only detected by QCM-D and not by SE, this further consolidates the hypothesis that for higher coverages, the peptide layer becomes so dense that the hydration shells of the peptides start to overlap.

Figure 3.8 | Peptide layer properties (A) QCM-D measurement (flow chamber, 20 $\mu\text{l}/\text{min}$) of the insertion of C14:0-RGD in an SLB, made prior to the measurement, and subsequent desorption. Areas not highlighted in grey represent washing with 0.5 x PBS buffer (pH 7.5). (B) Corresponding ΔD_5 vs $-\Delta F_5$ plot of the C14:0-RGD insertion step between 40 and 70 min (the striped area in graph (A)). (C) Overlay of ΔD_5 vs $-\Delta F_5$ plots from various QCM-D (open chamber) titration measurements performed with different lipid-modified peptides. Data points correspond to the plateau values of ΔF_5 and ΔD_5 of each of the titrations. (D) Frequency shift (ΔF_5) from QCM-D as function of coverage (Γ) from SE from combined QCM-D/SE measurements. Overlay of results (*in duplo*) from C12:0-RGD, C14:0-RGD and C16:0-RGD.

3.2.2. 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 in affinity influence cell behavior, to explore these platforms as dynamic biomimetic stem cell microenvironments. This was done by studying human mesenchymal stem cell (hMSC) adhesion and spreading as critical regulators of (stem) cell fate.^{34, 35}

3.2.3. 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 are marked with the localization of key regulator proteins such as vinculin, form and mature under appropriate conditions.^{36, 47, 48} 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, 36, 48, 49} Therefore, to study surface coverage dependent cell response, DOPC SLBs were functionalized with C14:0-RGD, which showed a moderate affinity of $K_a = 8.0 \times 10^3 \text{ M}^{-1}$ (based on SE results) to the SLB. A number of concentrations were defined that were related to certain surface coverages (18-20, 10-12, 6-8, 3-5 and 0.2-1%) based on SE results (Figure 3.7 and Table 3.2 in section 3.5.6 for corresponding C14:0-RGD concentrations). Once equilibrium conditions were achieved for the peptide insertion process (after 2 h 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. hMSC adhesion was studied by investigating vinculin localization at focal adhesions after 18 h.⁵⁰

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 3.9). Furthermore, on bare SLBs a lower extent of cell adhesion and an impaired cell shape compared to functional SLBs were seen (data of nonfouling properties of the bare SLB are shown and discussed in more detail in the thesis of Gülistan Koçer). In addition, the ability of bare SLBs to minimize hMSC cell adhesion was further demonstrated by live-cell imaging shown in chapter 4.

As mentioned above, it is important to note that in our system, lipid-modified peptides were incubated at defined concentrations to achieve certain coverages and kept in culture medium in presence of cells because of the dynamic nature of interactions between lipid-modified 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.⁵¹ However, the competing RGD in those systems has generally no interaction with the surface (therefore freely available in solution at all times),^{51, 52} which is in contrast to our system where peptides spontaneously insert to

Figure 3.9 | 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 various coverages indicated in the figure, after 18 h. (Scale bar: 20 μm , blue: nucleus, red: actin and green: vinculin, yellow line represents the one dimensional region of interest for profile plotting in Image J).

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 , 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 3210 and 3295 cells/cm², respectively and higher than bare SLBs (1976 cells/cm²). Therefore, in this system, we are able to compare the effects of lipid-modified peptide surface coverage on cell behavior.

3 Interestingly, even though at all coverages in the case of 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 filaments⁵³ was seen only at 10-12% coverage, which was supported with one dimensional profile plots of representative focal adhesions (Figure 3.9 and Figure 3.10). 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 3.9). 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 cells³⁶ (Figure 3.9 and Figure 3.10). 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 3.9 and Figure 3.10), pointing out an optimum coverage for C14:0-RGD to control focal adhesion maturation.⁵³

Further analysis towards the effect of C14:0-RGD density on hMSC cell behavior can be found in the thesis of Gülistan Koçer, altogether suggesting the existence of an optimum surface density for C14:0-RGD to induce effective cell adhesion and spreading, which is in agreement with previous observations on covalent RGD presenting surfaces.⁵⁴ 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 require a larger number of ligands to elicit the same cell binding and spreading response.^{26, 50, 55}

Figure 3.10 | Vinculin profiles. Cumulative vinculin profile plots for four representative focal adhesions in hMSCs on DOPC SLBs functionalized with C14:0-RGD at different coverages after 18 h (FA: focal adhesion).

3.2.4. Effect of lipid-modified peptide affinity on 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 lipid-modified peptide affinity to SLBs on cell behavior by introducing RGD ligands having different lipid anchors. For this purpose, next to C14:0-RGD ($K_a=8.0 \times 10^3 \text{ M}^{-1}$), 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 high affinity binding C16:0-RGD ($K_a=1.2 \times 10^5 \text{ M}^{-1}$) to study focal adhesion signaling as well as cell spreading.

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, ligand affinity can influence how cells perceive their physical environment at the level of single integrin-RGD bond, 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.^{36, 37, 56, 57} 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.^{53, 56}

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 3.9 and Figure 3.10). 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.⁵³ At higher affinity (*i.e.* going from C14:0-RGD to C16:0-RGD at 10-12%) cells generally still had distinct, but also qualitatively longer focal adhesions (vinculin localization Figure 3.11 and these data are shown in more detail in the thesis of Gülistan Koçer). 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.

Interestingly, going to a lower affinity at the same coverage (*i.e.* C12:0-RGD vs. C14:0-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 3.11).

However, as the lipid-modified peptide affinity to the surface determines the effective concentrations to achieve certain coverages (Figure 3.7), for C12:0-RGD a relatively high concentration (210 μM) of the peptide was needed to achieve 10-12% coverage (Table 3.2). This free RGD concentration may result in prevention of cell binding and therefore focal adhesion formation as discussed above.^{6, 51, 52} 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 of C12:0-RGD, which is in the concentration range that does not inhibit cell binding and spreading)^{51, 52} 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 3.11 and Figure 3.9, 0.2-1% coverage for C12:0-RGD and C14:0-RGD). Thus, the effect of RGD concentration in solution is not the cause for the change in 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.^{36, 53, 56}

A very low level of cell spreading for low affinity C12:0-RGD functionalized SLBs, at low coverages (0.2-1%) was observed, which was significantly lower than the cell area

Figure 3.11 | Lipid-modified peptide affinity dependent focal adhesion formation in hMSCs. Vinculin localization and corresponding profile plots for given focal adhesions (yellow line) in hMSCs on DOPC SLBs functionalized with C12:0-RGD at 0.2-1% (low) coverage and C12:0-RGD, C14:0-RGD and C16:0-RGD at 10-12% (high) coverage ($t=18$ h, scale bar: 20 μm , blue: nucleus, red: actin and green: vinculin, yellow line represents the one dimensional region of interest for profile plotting in Image J).

that was seen on C14:0-RGD functionalized surfaces at the same coverages (Figure 3.12 and Figure 3.13A). 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 3.12, Figure 3.13A). 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 3.12 and E and Figure 3.13A). Most intriguingly, on C14:0-RGD at the 10-12% coverage, cells had a significantly higher cell area compared to all other conditions, indicating again an optimum condition in terms of peptide affinity to control cell spreading (Figure 3.12, Figure 3.13A).

Figure 3.12 | Lipid-modified peptide affinity dependent cell morphology. Overview of cell morphology on DOPC SLBs functionalized with C12:0-RGD at 0.2-1% and at 10-12% coverage, with C14:0-RGD at 0.2-1% and 10-12% coverage and C16:0-RGD at 10-12% coverage ($t=18$ h, blue: nucleus, red: actin, scale bars: 40 μm).

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 lipid-modified peptide affinity to induce morphological changes (Figure 3.13B-D).

Figure 3.13 | Cell spreading and shape as function of lipid-modified peptide affinity. 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 lipid-modified 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 18 h (red line represents the mean value, $n \geq 400$, each data point represents one cell, mean $\pm$ standard deviation, **: $p < 0.01$).

Contrarily, 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 lipid-

modified 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 3.13B-D).

Taken together, these results show that in response to an increase in the lipid-modified peptide affinity to SLBs from low to moderate, cells elongated more 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.

3.3. Conclusions

In conclusion, lipid-modified RGD ligands insert into supported lipid bilayers (SLB) with affinities that depend on the lipid length and structure. This provides a straightforward approach to fabricate dynamic bioactive surfaces, tunable both in ligand affinity (roughly ranging from 10^3 M^{-1} to over 10^5 M^{-1}) and ligand density (reaching densities over 30% with respect to the density of lipids in the top leaflet).

A pronounced effect could be observed of the affinity of these lipid-modified RGD ligands on hMSC cell binding and spreading behavior. Going from C12:0-RGD ($K_a = 9.3 \times 10^2 \text{ M}^{-1}$) to C14:0-RGD ($K_a = 8.0 \times 10^3 \text{ M}^{-1}$) enhanced focal adhesion formation, cell spreading and elongation. However, 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 while cell elongation and regulation of other morphological parameters remained mostly unaffected. Therefore, we envision that the 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.

In a broader perspective, we have demonstrated that surface affinity can be used as cue to guide cell behavior at material surfaces and that SLBs functionalized with lipid-modified RGD ligands can be used as biointerface to achieve the required tunable dynamicity.

A more detailed analysis of the behavior of these and other lipid-modified RGD ligands at very high densities at the SLB as well as the response of hMSC cells under those conditions is described in the next chapter.

3.4. Acknowledgements

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3.5. Experimental section

3.5.1. General methods

Chemicals were purchased from Sigma Aldrich or from Acros Organics. Protected amino acids were obtained from MultiSynTech and resin for solid phase peptide synthesis was obtained from Novabiochem. Lipids were obtained from Avanti Polar Lipids. Phosphate Buffered Saline (PBS) with pH 7.4 at 25 °C containing 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride was used as such or diluted one time (0.5XPBS) with Milli-Q water (MQ, Millipore, 18.2 mΩ) in order to maintain the same buffer solution throughout QCM-D experiments. Mass spectra were recorded with a Waters electrospray ionization time of flight mass spectrometer operated in positive ion mode (ESI(+)-ToF, Micromass LCT). Fluorescence spectroscopy was performed using a Perkin Elmer fluorimeter (LS 55) with a slit width of 2.5nm and $\lambda_{em} = 334$. HPLC was performed using a C₁₈ reverse phase column (Waters, Xbridge). Dynamic light scattering (DLS) analysis was carried out on an Anaspec particle analyser (Nanotrak operating with a Microtrac FLEX Operating Software)

3.5.2. Synthesis procedures

Peptides were synthesized using an automated solid phase peptide synthesis robot (Syro II, MultiSynTech) following standard Fmoc procedures on a Rink amide MBHA resin (100-200 mesh, loading 0.52 mmol/g). Fatty acids (lauric acid, myristic acid, palmitic acid, stearic acid, oleic acid, and hexyldecanoic acid) were coupled to the N-terminus of the peptide on resin in the presence of PyBOP and HOBt (5x molar excess over the resin loading). Deprotection of the peptide and cleavage from the resin was achieved using a 95:2.5:2.5 solution of trifluoroacetic acid (TFA), water, and triisopropylsilane. Purification was performed by repeated (3x) precipitation in cold diethyl ether and subsequent preparative HPLC using a C₁₈ reverse phase column. Preparative HPLC was operated at 4 ml/min, always in the presence of 0.1% TFA, for 10 min using 90:10 water:acetonitrile and then linearly in 65 min to complete acetonitrile, which was kept for another 25 min. The isolated peptide fractions were characterized using ESI(+)-ToF (Figure 3.14 - Figure 3.20).

Figure 3.14 | ESI(+)-ToF mass spectra of control peptide KGSGRGDSG (Bare-RGD).

Figure 3.15 | ESI(+)-ToF mass spectra of the lauroyl-KGSGRGDSG (C12:0-RGD).

Figure 3.16 | ESI(+)-ToF mass spectra of myristoyl-KGSGRGDSG (C14:0-RGD).

Figure 3.17 | ESI(+)-ToF mass spectra of palmitoyl-KGSGRGDSG (C16:0-RGD).

Figure 3.18 | ESI(+)-ToF mass spectra of stearyl-KGSGRGDSG (C18:0-RGD).

Figure 3.19 | ESI(+)-ToF mass spectra of oleoyl-KGSGRGDSG (C18:1-RGD).

Figure 3.20 | ESI(+)-ToF mass spectra of hexyldecanoyl-KGSGRGDSG (2xC8:0-RGD).

3.5.3. Surface binding monitoring

Quartz crystal microbalance with dissipation monitoring (QCM-D). SiO₂ coated QCM-D sensors (QSX303, Biolin Scientific) were used. Before each use, sensors were cleaned with a 2% sodium dodecyl sulfate solution, rinsed with Milli-Q water and ethanol, dried in a jet of nitrogen, and finally activated for 30 min using a UV/ozone chamber (Bioforce, nanosciences). Measurements were performed using a Q-sense E1 or E4 system (Biolin Scientific) under a 100 μ l/min flow and at 22 $^{\circ}$ C, unless stated otherwise. Throughout this work, the fifth overtone was used for the normalized frequency (F_5) and dissipation (D_5). All experiments were performed using a 5 mM phosphate-buffered saline solution of pH = 7.5 containing 70 mM sodium chloride (0.5x PBS).

In-situ combined spectroscopic ellipsometry and quartz crystal microbalance with dissipation monitoring (QCM-D/SE). *In-situ* combined QCM-D/SE measurements⁵⁸ were performed using SiO₂ coated QCM-D sensors (QSX335, Biolin Scientific) that had a thick Ti adhesion layer. Sensors were mounted in a 2.5 ml glass-walled open QCM-D chamber equipped with a stir bar. The chamber was placed in a vertically mounted Q-sense E1 QCM-D system which, as a whole, was incorporated

Figure 3.21 | Titrations of C12:0-RGD, C14:0-RGD, and C16:0-RGD to SLBs followed in combined QCM-D/SE. Titrations were performed *in duplo* and both individual results are plotted. Data points are fitted to the Langmuir model (see main text). In one instance, reliable fitting was not possible and a dotted line is given to guide the eye. Data points corresponding to concentrations $\leq 1 \mu\text{M}$ are presented but excluded during fitting because depletion of peptide from the open chamber setup were estimated to play a significant role at these low concentrations.

in an M2000 SE system (Woollam). The temperature was kept at 22 °C and samples were pre-heated to 22 °C before use. To wash away solutions from the chamber without exposing the sensor to air, 0.4 ml of the solution was replaced with buffer and

this washing was repeated 30 times. The chamber walls were passivated for 15 min using a 10 mg/ml BSA solution before each measurement followed by thoroughly rinsing.

For the SE measurement, polarized light at an angle of incidence of 70° is reflected on the flat sensor surface, resulting in changes to the ellipsometric angles Δ and Ψ . Both Δ and Ψ are functions of the wavelength of the light (λ) and are acquired over a range of 380-1000 nm. The obtained values are fitted to a multilayer model using CompleteEASE software (Woollman) as described previously⁴⁰ but with the following differences: firstly, A_{sol} of the buffer solution was now set to 1.324 and secondly, the areal mass (Γ) of the peptide layer was determined using de Feijter's equation in which the refractive index increment (dn/dc) for the peptide layer was assumed to be $0.18 \text{ cm}^3/\text{g}$ on the basis of literature values for proteins⁴².

In addition to the example shown for C14:0-RGD in the main text, all combined QCM-D/SE titrations of C12:0-RGD, C14:0-RGD, and C16:0-RGD to DOPC SLBs are presented in Figure 3.21.

3.5.4. Supported lipid bilayer (SLB) fabrication and characterization

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, $T_m = -20^\circ\text{C}$, Avanti Polar Lipids) was stored as a stock solutions under argon at -20°C in chloroform. An appropriate amount of the lipid solution was dried under a flow of nitrogen in a glass vial in order to create a film of lipid material at the glass wall. This film was further dried under vacuum for at least 1 h and subsequently hydrated by vortexing with Milli-Q water to form multilamellar vesicles at 1 mg/ml. The lipid suspension was extruded 11 times through a polycarbonate membrane (Whatman) with 100 nm pore size, resulting in large unilamellar vesicles (LUVs) as confirmed by dynamic light scattering (DLS, Nanotracc wave) measurements (Figure 3.22).

Figure 3.22 | Vesicle size. DLS size distribution of DOPC LUVs.

Alternatively, the lipid suspension was homogenized by five freeze-thaw cycles and sonicated in pulse mode for 30 min by tip-sonication (Branson) while keeping the lipid suspension on ice. The resulting vesicles were centrifuged (30 min, 60000g) to remove titanium particles. Vesicles were stored at 4 °C and used within two weeks. Immediately before use, the vesicles were diluted to a concentration of 0.1 mg/ml in 0.5x PBS. SLB formation was achieved by flowing this solution over a cleaned and activated surface.

QCM-D and combined QCM-D/SE for following SLB formation. The quality of SLBs fabricated via either technique was checked by the QCM-D (flow chamber or open chamber) response ($\Delta F = -24 \pm 1$ Hz and $\Delta D < 0.5 \times 10^{-6}$) and for combined QCM-D/SE measurements also by SE (expected change in coverage of 360 ng/cm²), see Figure 3.23.⁴⁰

Figure 3.23 | SLB formation. Assessment of the quality of SLBs, formed using two different methods, on SiO₂ coated QCM-D sensors. A good quality SLB is characterized by $\Delta F_5 = -24 \pm 1$ Hz and $\Delta D_5 \leq 0.5 \times 10^{-6}$. Buffer is 0.5x PBS throughout both measurements. **(A)** QCM-D (flow chamber) results when using 100 nm DOPC vesicles obtained by extrusion. Flow rate 100 μ l/min. **(B)** QCM-D (open chamber) and **(C)** SE (open chamber) results of a combined QCM-D/SE measurement when using DOPC vesicles obtained via tip-sonication. QCM-D/SE was performed in an open chamber set-up under continuous stirring.

Fluorescence recovery after photobleaching (FRAP). A DOPC SLB doped with 0.5 mol% of the green fluorescent lipid TopFluorPE was used for FRAP measurements. Using a confocal microscope (Nikon A1 CSLM), a spot of 10 μm in diameter was bleached (488 nm) and subsequently the fluorescence intensity in this bleached region was monitored. The intensity is normalized and corrected for acquisition bleaching by using the fluorescence intensity in a location not too close to the bleach spot. The FRAP protocol consisted of 11 imaging loops (1.16 seconds interval) before bleaching, 10 loops bleaching with 15% laser intensity and with no delay in between loops and 259 loops of recovery (1.16 seconds interval).

The results are presented in Figure 3.24. Analysis of the FRAP data revealed 100% recovery and a diffusion constant of $0.79 \pm 0.025 \mu\text{m}^2/\text{s}$, which is in agreement with literature.⁵⁹

Figure 3.24 | SLB mobility. FRAP experiment using a DOPC SLB doped with 0.5 mol% of the fluorescent TopFluorPE lipid. **(A)** Image recorded directly after bleaching (top) and after five minutes of recovery (bottom). **(B)** FRAP curve (black dots) fitted with modified Bessel functions (red line) as described by Soumpasis *et al.*⁶⁰ FRAPAnalyser (University of Luxembourg) was used for the fitting to determine the diffusion constant ($D = 0.79 \mu\text{m}^2/\text{s}$) and the degree of recovery ($A = 100\%$). The first 11 data points correspond to intensity before bleaching and are not included in the model.

3.5.5. Micelle formation

Micelle formation of the lipid-modified peptides was investigated using an established procedure.⁶¹ Briefly, a decrease in the ratio I_1/I_3 indicates a more hydrophobic environment of pyrene as a result of incorporation of pyrene in micelles (or other amphiphile assemblies). Using this method, micelle formation was not observed for C12:0-RGD, C14:0-RGD, C14:0-RGD, and C18:0-RGD in the concentration window used in this work (Figure 3.25). For sodium dodecyl sulfate (SDS), micelle formation between 5 and 10 mM could be observed using this method (not shown), in line with the reported CMC of SDS of 8.2 mM.⁶¹

Figure 3.25 | Critical micelle concentration (CMC). Plot of the ratio of the fluorescence intensity of the first and third vibronic band of pyrene (I_1/I_3) as a function of the concentration of given lipid-modified peptides in a 0.5x PBS buffer.

3

3.5.6. 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 vesicles 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 CaCl₂ and MgCl₂) was added to each well followed by addition of a vesicle suspension (to a final vesicle concentration of 0.1 mg/ml in the wells) and left to incubate for 1 h. 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 lipid-modified 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 μl/well) which were selected to achieve different coverage ranges (Figure 3.6, Table 3.2, concentrations are given in the presence of cells) of lipid-modified peptides in 1x DPBS were added to the SLBs and incubated at least for 2 h, based on QCM-D/SE results, at room temperature in order to reach an equilibrium in the interaction of the lipid-modified peptides with the SLB. No further washing was performed after the peptide incubation step and surfaces were used as such for cell experiments.

Table 3.2 | Corresponding concentrations and percent coverages for C12:0-RGD, C14-RGD, and C16:0-RGD peptides used in this chapter.

C12:0-RGD		C14:0-RGD		C16:0-RGD	
Conc. (μM)	Coverage range	Conc. (μM)	Coverage range	Conc. (μM)	Coverage range
-	-	50	18-20%	-	-
210	10-12%	28.6	10-12%	2.1	10-12%
-	-	14.3	6-8%	-	-
54	2-5%	5.7	3-5%	1	3-5%
21	0.2-1%	1.9	0.2-1%	0.7	1-3%

3.5.7. Cell culture on SLBs

Bone marrow derived human mesenchymal stem cells (hMSCs, Poietics, Lonza) were cultured in basic medium (αMEM (Gibco) containing 10% fetal bovine serum (FBS) (Sigma) and 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (Sigma)) and were incubated at 37°C and 5% CO_2 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/ cm^2 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, 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.

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.

Data analysis. All cell experiments were performed in triplicate 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.⁴⁷ 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.⁴⁷

3.6. References

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Chapter 4

Dense clusters of integrin-binding peptides on supported lipid bilayers

Supported lipid bilayers (SLBs) were fabricated and functionalized by insertion of lipid-modified Arginine-Glycine-Aspartic acid (RGD) peptides to produce a bioactive interface. This insertion was characterized using quartz crystal microbalance with dissipation monitoring (QCM-D), which showed Langmuir-type binding with anchor dependent affinity, except for a rhodamine labelled lipid-modified peptide that inserted in a positively cooperative fashion. At high peptide densities, lipid-modified peptides spontaneously assembled within the SLB into domains with increased peptide density, irrespective of the peptide's lipid anchor. Fluorescence recovery after photobleaching (FRAP) studies showed that these domains did not affect the lateral mobility of the bilayer, except for cholesteryl-anchored peptides in which case the mobility was reduced. hMSC cells did not adhere to bare SLBs while they readily adhered to and spread on RGD functionalized SLBs. No changes were observed in the amount of cells that adhered to the domains compared to the surrounding areas. By using a fluorescently labelled lipid-modified RGD peptide it was demonstrated that adhering cells were able to take up lipid-modified peptides from the SLB at a rate that was higher than the lateral mobility of the peptide within the SLB, while the SLB itself remained unaffected, as was visualized in live cell fluorescent imaging.

4.1. Introduction

Materials that present bioactive ligands can aid in the study or control of cell-extracellular matrix (ECM) or cell-cell interactions and are therefore of interest to research fields and applications such as tissue engineering, biosensing, and fundamental cell studies.¹ Besides the type of the ligand, many factors such as ligand spacing² and mechanical properties of the material³ have been shown to play a role in the way cells sense surfaces. Artificial systems provide useful insights but often lack the dynamic behavior sensed by cells in their natural environment.

For example, a static display of ligands with controlled ligand spacing generally possesses a 'threshold' (*i.e.* a spacing beyond which a significant increase in cell binding and spreading is observed) in ligand spacing of around 70 nm.⁴⁻⁷ However, for a dynamic display of ligands on, for example, fluid supported lipid bilayers (SLBs), threshold values of around 5-10 nm have been reported.^{8, 9} These differences may be related to in-plane (lateral) dynamics of ligands, as well as out-of-plane dynamics, *i.e.* cells are able to pull ligands off the surface. Lateral dynamics might make it more difficult for cells to achieve pulling forces involved in focal adhesion formation.¹⁰⁻¹³ In addition, lateral dynamics could also result in recruitment of ligands in response to (cell) binding,¹⁴ generating local ligand enrichments. For biological systems, physiological conditions might very well keep the membrane very close to the phase coexistence region, allowing small changes to result in the formation or dissolving of such enrichments, also referred to as rafts.¹⁵ Thus, the dynamic nature of biological systems is a key factor in the design of biointerfaces.

Existence and effects of rafts in natural membranes has been a subject for debate for many years, especially when concerning small (nanometer-sized) and transient enrichments.¹⁶⁻¹⁹ By now, these rafts have been quite well established as more and more reports demonstrate the apparent relevance of these rafts in a plethora of processes associated with both normal and diseased cell function.¹⁶⁻¹⁹ It is therefore of large interest to elucidate the governing principle behind raft formation on the one hand, and on the other hand to study the effect that rafts have on the way that cells respond to ligands displayed in clusters rather than freely dispersed ligands. A better understanding of these processes would be advantageous for increasing fundamental knowledge of cellular functioning but also for the development of drug delivery systems equipped with specific targeting functionality that can respond to ligand density at the cell surface.²⁰

In order to better mimic the natural environment, various methods have been described to achieve dynamic surfaces.^{1, 21} One method to generate dynamic biointerfaces featuring artificial rafts or domains is the generation of lipid bilayers consisting of a mixture of various lipids, often in the presence of cholesterol, resulting in phase separation into domains.^{22, 23} Functionalized lipids can then be added as they often have a tendency to partition in one of the phases, thus creating functional domains.^{24, 25} However, this phase separation using mixed lipid systems is generally achieved in giant vesicles and is more difficult to achieve in supported lipid bilayers, as it requires the use of the Langmuir Blodgett/Langmuir Schäfer method,²⁶ which is a tedious method that in addition hinders the use of microfluidic devices.

In this chapter, we describe a simple and robust lipid bilayer biointerface that consist of high density clusters of peptide ligands. Importantly, single component SLBs were made on glass and the subsequent addition of lipid-modified integrin-binding peptides, which spontaneously insert into SLBs, led to the formation of high density patches of peptide ligands, even though the host membrane consists of a single fluid phase. The approach provides an easy route to *in-situ* monitor cellular response to dynamic ligand domains.

Fluorescence microscopy was used to visualize the formation of the domains. In this case, either a small amount of dye-functionalize lipid was used that was found to partition into the peptide rich domains, or the lipid-modified peptide itself was functionalized with a fluorescent dye to conclusively confirm that domains consisted of peptide-rich phase. (Live) cell imaging reconfirms the excellent non-fouling properties of the bare SLB and specific binding to the RGD functionalized SLBs.

4.2. Results and discussion

The synthesis of a focused set of lipid-modified integrin-binding RGD peptides (KGSGRGDSG) is described in chapter 3. Their binding affinities to SLBs consisting of DOPC lipids (Figure 4.1C) were characterized using quartz crystal microbalance with dissipation monitoring (QCM-D). Furthermore, the effect of differences in binding affinity on hMSC cell adhesion and spreading behavior was studied. In addition to those results, we demonstrate in this chapter the remarkable spontaneous clustering behavior of these peptides, which occurred at high peptide densities at single-lipid fluid SLBs. Cell binding behavior on these SLBs with high density domains is investigated. Also, we optically traced the peptides both during insertion in the SLB and during cell binding by using a fluorescently labelled lipid-modified peptide.

Figure 4.1 | Structures of main molecules. Structures of **(A)** lipid-modified peptides Chol-RGD, C14-RGD, C16-RGD, oleic-RGD and **(B)** C16-Rho-RGD **(C)** as well as main lipids used in this chapter.

4.2.1. Insertion of lipid-modified peptides

The insertion and desorption behavior of myristoyl (C14), palmitoyl (C16), and cholesteryl (Chol) functionalized RGD peptides (Figure 4.1A) in DOPC SLBs was investigated by QCM-D (Figure 4.2). To allow inspection by fluorescence microscopy, a C16-lipid analogue was synthesized, which was functionalized with fluorescent rhodamine (C16-Rho-RGD, Figure 4.1B and see section 4.5.2 for synthesis). Interestingly, when flowing C14-RGD, C16-RGD, or Chol-RGD over SLBs, a Langmuir type binding profile was observed (Figure 4.2E-G), while the binding of C16-Rho-RGD to an SLB increased exponentially with the solution concentration up to approximately

5 μM (resulting in $\Delta F_5 = -20$ Hz) after which additional binding stopped, probably because of saturation of the surface (Figure 4.2H).

Figure 4.2 | Lipid-modified peptide interactions with SLBs. QCM-D traces, after DOPC SLB fabrication, recorded when flowing (100 $\mu\text{l/min}$) 0.5x PBS buffered (pH 7.4) solutions of **(A)** C14-RGD, **(B)** C16-RGD, **(C)** Chol-RGD, and **(D)** C16-Rho-RGD (*flow rate increased to 150 $\mu\text{l/min}$). Frequency shift plateau values for **(E)** C14-RGD, **(F)** C16-RGD, **(G)** Chol-RGD (note that in this case the plateau values are not fully stable), and **(H)** C16-Rho-RGD (*duplo*).

In addition, C16-RGD is relatively easily removed by washing with buffer, while C16-Rho-RGD desorption was slower and/or required higher flow rates. It seems that the presence of rhodamine on the lipid-modified peptide resulted in a positive cooperative insertion behavior, *i.e.* additional binding of C16-Rho-RGD is stimulated by already bound C16-Rho-RGD. Although the mechanism is not clear at this point, it has been reported that tetramethylrhodamine derivatives can readily form dimers and this could play a role here as well.²⁷

Chol-RGD desorption was not observed by washing with buffer alone, but desorption was achieved by flowing a solution of β -cyclodextrin (β -CD) over the surface (Figure 4.2C), as reported in literature.²⁸ The large frequency shift achieved for Chol-RGD might seem contradictory to the notion that the C16-Rho-RGD surface reaches saturation. This may be explained by the condensing effect that cholesterol is known to have on fluid lipid bilayers,²⁹ possibly facilitating higher densities.

4.2.2. Domain formation in single lipid systems

Mixing small amounts of fluorescently labelled lipids, such as a Texas Red functionalized lipid (TR, red) or a Bodipy functionalized lipid (TF, green) (Figure 4.1C), into SLBs allows for visualization of lipid bilayers using fluorescence microscopy. This is routinely done for mixed lipid systems to evaluate the phase behavior, as for instance TR is known to partition into the liquid disordered (l_d) phase.³⁰ In addition, these labelled lipids allow for the analysis of the lipid mobility in SLBs using fluorescence recovery after photobleaching (FRAP). Here, these techniques were used to investigate SLB stability and mobility in the presence of lipid-modified peptides at various concentrations and thus various surface densities as discussed above. Figure 4.3 shows the formation of regions with higher fluorescence intensity (domains) above certain peptide concentrations, depending on the lipid anchor. For 2 μ M C16-RGD, where the onset of domain formation was observed for this lipid-modified peptide, a surface coverage of >10% (100% is defined as one lipid-modified peptide per lipid in the top leaflet) was determined by combined QCM-D and spectroscopic ellipsometry as described in chapter 3. This density corresponds to a ligand spacing of <3 nm and can thus already be considered a high ligand density for cell adhesion, as discussed in section 4.1.

Figure 4.3 | Domain formation at high peptide densities. DOPC SLBs doped with fluorescently labelled lipids after 1 h incubation with increasing concentrations of (from top row to bottom) C14-RGD, C16-RGD, Chol-RGD and C16-Rho-RGD. For C16-Rho-RGD, excess peptide was washed away shortly before imaging. In the three top rows, red originates from TR emission, using epifluorescence microscopy. In the bottom row, both images are recorded at the same location using confocal fluorescence microscopy, showing emission from TF (green) or C16-Rho-RGD (red). The graph represents the intensity profile along the white line.

In agreement with the QCM-D results (Figure 4.2), the high intensity lipid domains of C14-RGD and C16-RGD disappeared when washing with buffer (shown for C14-RGD in Figure 4.4, top), but this was not the case for Chol-RGD (Figure 4.4, bottom). For Chol-RGD, domains disappeared when flowing a solution of β -cyclodextrin, a known binder of cholesterol, over the surface.²⁸

Figure 4.4 | Reversibility of domain formation. Top: C14-RGD and bottom: Chol-RGD domains formed after incubation of DOPC SLBs doped with 0.5 mol% TF for >1 h with 200 μ M C14-RGD or 20 μ M Chol-RGD. C14-RGD domains were removed upon washing with buffer while Chol-RGD domains remained in those conditions but disappeared when extracting Chol-RGD from the SLB using β -cyclodextrin.

4.2.3. Characterization of domain formation

The formation and growth of the domains over time was followed using fluorescence microscopy (for example Figure 4.5, using 200 μ M C14-RGD). These images showed that domains generally formed immediately after the lipid-modified peptide became available in solution, after which the domains steadily became larger. Similar behavior was observed (not shown) for the other lipid-modified peptides used in this chapter,

Figure 4.5 | Domain growth. Fluorescence microscopy images recorded on the same location of a DOPC SLB doped with 0.2 mol% TR before and at various time points after addition and incubation without flow of 200 μ M C14-RGD. Inset shows a magnification of the same region (scale bar, 40 μ m).

though at a lower rates, which is probably related to the lower concentrations that were used to achieve the same densities.

Domain formation in this system is surprising, considering the use of a single lipid type for the SLB, indicating that the lipid-modified peptides drive their own aggregation followed by the partitioning of the fluorescently labelled lipids into these domains. This was verified by domains of high intensity fluorescence emission observed after incubation of DOPC SLBs with the intrinsically fluorescent C16-Rho-RGD, which overlays with the high intensity regions of the fluorescently labelled lipid TF (Figure 4.3, bottom row). For non-labelled peptides, fluorescently labelled lipids TF and TR were observed to overlay in domains as well (Figure 4.6).

Figure 4.6 | TF and TR overlay. Domain formation in DOPC SLB after 1 h incubation of 20 μM of Chol-RGD, observed by partitioning in the domains of both (left) TF and (right) TR.

Assuming self-quenching does not play a significant role, the intensity profile in Figure 4.3 demonstrates the relative distribution of C16-Rho-RGD inside and outside domains, showing an approximately 60% higher coverage inside domain areas compared to outside. Interestingly, domain formation can also be recognized from the QCM-D results (Figure 4.2) where the dissipation increases (ΔD) with the decrease of the frequency (ΔF) only until a ΔF of approximately 5 Hz, at which point the dissipation does not increase further or even decreases slightly. This probably corresponds with the formation of peptide domains that are more rigid and devoid of water than “monomeric” peptides, which mainly translates to the change in observed dissipation. This is better visualized in Figure 4.7 by plotting the ΔD as function of $-\Delta F$.

The size of the domains was found to be highly dependent on the flow rate applied during the insertion of the lipid-modified peptides, either as a result of pipetting or controlled in a flow channel (see chapter 2 for specifications of the flow channel). Although no clear correlation could be deduced between domains size and flow rate, domain sizes remained small, below approximately 3 μm , under high flow conditions (100 $\mu\text{l/min}$), but many domains were formed (Figure 4.8A). This suggests that peptides were first inserted and subsequently clustered together, presumably facilitated by the lateral mobility of the lipids in the SLB. This is further supported by

Figure 4.7 | Lipid-modified peptide interactions with SLBs. $-\Delta F/\Delta D$ plot of various lipid-modified peptides of the stepwise insertion where data points represent the plateau values from QCM-D titrations shown in Figure 4.2, where the used concentration range is indicated as well. The solid line is only a guide for the eye.

insertion of lipid-modified peptides in gel-state (DPPC) lipid bilayers, which does not result in domain formation unless the bilayer is heated above its melting temperature of 41 °C (Figure 4.8B). So, for consistency, the lipid-modified peptides were added using very brief pipetting to achieve mixing but without further agitation in the remainder of this chapter.

Figure 4.8 | Domain formation under flow or at gel-state SLB. (A) Incubation of C16-RGD to fluid (DOPC) SLB in a microfluidic channel under flow (100 μl/min) or in a well-plate (no flow) for comparison. (B) Incubation, without flow, of C14-RGD to gel-state SLB (DPPC, $T_m = 41$ °C) and domain formation above T_m , all at the same location and in the inset an image at higher magnification.

In mixed-lipid bilayers displaying phase separated domains, it has been reported that lipid-modified peptides or proteins can partition into one of the domain types.³¹ One parameter that has been shown to play an important role in such phase sorting is the minimization of the difference in lipid tail and peptide anchor length, so-called lipid mismatch.³¹ To test if lipid mismatch induces the observed domain formation also here in the single lipid membranes, oleic-RGD was used. This oleyl anchor has the same length and structure as the phospholipid (DOPC) used for SLB formation. However, also the presence of oleic-RGD resulted in the observation of domain formation (Figure 4.9A).

The observed domain behavior appears similar to phase separation in mixed-lipid bilayers. To evaluate whether the lipid-modified peptide domains observed here also correspond to a change in phase behavior, FRAP analyses both outside and inside domains were performed. Surprisingly, Figure 4.9B shows a similar mobility of the dye-functionalized lipids in bare bilayers compared to inside C16-RGD and oleic-RGD domains. This observation suggests that the domain formation is not related to phase separation. Only domains formed by Chol-RGD showed a reduced mobility. It is indeed well established that cholesterol effects the phase behavior of lipid bilayers.²⁹ By performing FRAP on C16-Rho-RGD functionalized SLBs after washing excess peptide away from solution (to avoid that peptide insertion from solution would affect the FRAP analysis), the mobility of the lipid-modified peptide itself in the areas surrounding the domains was also found to be similar to the intrinsic mobility of DOPC membranes. The difference in phase behavior might explain that in the case of Chol-RGD, domains were observed with more than two different fluorescence intensities (Figure 4.3, Chol-RGD, right image), while in the case of C14-RGD and C16-RGD only two different fluorescent intensities were observed corresponding to domains (Figure 4.3, top two rows). Domains of two different intensities might be explained by domain formation in either one or both leaflets after (possibly defect mediated³²) flip-flop of the lipid-modified peptides. The existence of additional domains, as in the case for high concentrations of Chol-RGD, might be related to different phases of different densities.

Excluding changes in phase behavior or lipid mismatch as driving force for domain formation, the underlying mechanism could be related to a peptide crowding effect³³,³⁴ and/or a lipid anchor wedging effect.^{34, 35} Both mechanisms are based on steric effects, either of the peptide or the anchor, and are generally involved in membrane curvature and not necessarily in domain formation. However, membrane curvature is restricted for SLBs due to the interaction with the support, resulting in increasing

amounts of strain in the SLB for increasingly high densities of lipid-modified peptides. Therefore, the observed domains might form to minimize the overall strain.

Figure 4.9 | Evaluating the role of lipid mismatch, phase behavior, and support in domain formation. **(A)** Confocal microscopy image of oleic-RGD domains in DOPC SLB **(B)** FRAP analyses of a bare DOPC SLB, of C16-RGD, Chol-RGD, and oleic-RGD domain regions in a DOPC SLB using fluorescently labelled lipids (TF or TR) as well as FRAP analysis in a non-domain area of a DOPC SLB directly using the fluorescently labelled peptide C16-Rho-RGD. **(C)** Images corresponding to the FRAP experiments presented in (B) after bleaching and after 5 min recovery. **(D)** Representative fluorescence microscopy images after addition of 8 μM Chol-RGD showing domain formation at SLB (TR labelled, red) but no domain formation at GUV (TF labelled, green). All images are at the same location. GUV is immobilized at SLB using biotin-streptavidin interactions. Note that the structure inside the GUV is simply a lipid structure moving inside the GUV, already present before peptide incubation. **(E)** Collapse of GUV in presence of C16-RGD. Both images are of the same location (in between the displayed images, GUV collapsed in two integer steps before reaching the size shown at 12 min).

To further evaluate this hypothesis, giant unilamellar vesicles (GUVs) were supported on SLBs *via* biotin-streptavidin interactions and incubated with lipid-modified peptides (see experimental section 4.5.3 for details). The lipid bilayer of the GUV itself is not supported and therefore free to adapt its curvature. Incubation of such GUVs with 8 μM Chol-RGD did not result in any observable domain formation in the membrane of the GUV, while domain formation was observed in the directly underlying SLB (Figure 4.9D). However, incubation of GUVs with lipid-modified peptides seems to destabilize the GUVs as inferred from their rupture in the presence of 8 μM C16-RGD (Figure 4.9E). Destabilization of GUVs rather than domain formation supports the hypothesis that domains formed at SLBs may be a result of strain induced in the SLB when lipid-modified peptide are inserted. Such strain cannot be easily released by supported lipid bilayers while GUVs can more readily release strain by deformation or rupture, the latter being observed here.

4.2.4. Cell response to high density RGD surfaces

To investigate how cells respond to these high density RGD domains that reside in lower density (but still quite high, see section 4.2.2) RGD surfaces, the adhesion and spreading behavior of human mesenchymal stem cells (hMSCs) on these peptide functionalized SLBs was evaluated. SLBs presenting RGD peptides (C16-RGD, C16-Rho-RGD, and Chol-RGD) at high density were prepared as described above, with some minor adaptations to accommodate cell studies (see experimental sections 4.5.5 and 4.5.6). Mainly, lipid-modified peptides were always washed away from solution prior to cell seeding. Subsequently, cells were incubated for 18 h before fixation and immunostaining of the cytoskeleton, nucleus, and vinculin (Figure 4.10). Vinculin in particular is of interest here as it is part of the focal adhesion complex and thus provides insight in the binding behavior of the cells. Figure 4.10 shows increased spreading of cells on all RGD functionalized SLBs compared to the bare SLB. In addition, vinculin localization is observed for C16-Rho-RGD and Chol-RGD functionalized SLBs suggesting mature focal adhesion formation, while vinculin is diffuse for C16-RGD SLBs. This is probably because most C16-RGD is removed from the surface during the washing step before cell seeding, consistent with the earlier observation from QCM-D that this peptide binds with a lower affinity to the SLB (Figure 4.2). This observation is different from chapter 3 where the peptides were not washed away before cell seeding.

Figure 4.10 | Vinculin localization in hMSCs on DOPC SLBs at high ligand densities. hMSC adhesion and spreading on DOPC SLBs incubated with 10 μM C16-RGD, 10 μM C16-Rho-RGD, 5 μM Chol-RGD, without peptide (bare), and serum coated glass. Excess peptide was washed away prior to cell seeding. (Blue: nucleus, red: actin, and green: vinculin. Actin staining was performed with a far-red dye to exclude rhodamine signal and false colored as red, see experimental section, $t = 18$ h).

In addition, live cell imaging was performed to simultaneously and in real-time follow: cells in transmission mode, SLBs stained with green fluorescence from TF and inserted C16-Rho-RGD peptides (red fluorescence). This led to a number of interesting observations.

Firstly, both bare and peptide functionalized SLBs were found to be stable, *i.e.* no gaps in the green fluorescence originating from the TF lipid were observed, throughout the cell experiments (Figure 4.11). In addition, the C16-RGD and C16-Rho-RGD domains remained remarkably stable, probably because of the absence of flow, throughout the cell experiments. Chol-RGD domains however, seemed to slowly disintegrate. The latter was also observed for Chol-RGD domains after raising the temperature to 37°C in the absence of cells (Figure 4.12), demonstrating this was an effect of the temperature rather than the presence of cells.

Secondly, counting cells that were either in contact with domains or not, showed no significant preference of cells for domains 20 min and 60 min after seeding (Figure 4.13). This analysis was performed for the C16-Rho-RGD functionalized SLBs where domains were most pronounced and stable for this experiment.

Figure 4.11 | SLB (with or without lipid-modified peptides) stability over time. DOPC SLBs doped with TF (green) incubated with 10 μM C16-RGD, 10 μM C16-Rho-RGD, and 5 μM Chol-RGD at 20 min (top row) and 15 h and 20 min (bottom row) after cell seeding. After 2 h of peptide incubation and before cell seeding, excess peptide was washed away. Bare SLB was included as a control.

Figure 4.12 | Chol-RGD domains at 37°C. Domain formation in DOPC SLB doped with TF (green) after (left) 1 h incubation of 2 μM of Chol-RGD and (right) overnight incubation at 37°C. Locations of these pictures are not the same but representative for the behavior in the entire well.

Finally, the fluorescently labelled C16-Rho-RGD peptide could be traced during live cell imaging in a confocal microscopy setup (Figure 4.14). Regions of lower red fluorescence intensity were observed in areas where cells were in contact with the SLB during early stages of cell adhesion, suggestion local depletion of C16-Rho-RGD. No such dark regions were observed in the green channel from the TF (dye is attached to one of the lipid tails and head group is zwitterionic), demonstrating this is neither an optical effect nor nonspecific removal or uptake of any non-covalently bound material under the cells. These darker spots remained present during early stages of cell adhesion as long as the cells remained on the same place but immediately filled up again when cells moved away, as expected from laterally mobile SLBs. These

Figure 4.13 | Cell location with respect to domains. **(A)** Location of cells that are adhered but not yet spread 60 min after cell seeding. Domains are formed by C16-Rho-RGD as prepared and presented in Figure 4.11. **(B)** Quantification is performed 20 min and 60 min after cell seeding by counting cells that are in contact with domains and cells that are not. The domain area is defined as the area of domains including a rim with the size of the average cell diameter (highlighted in yellow in A) and the not-domain area is the rest. This definition was used to compensate for cells that only touch domains but that are still fully counted as “on domain”. A minimum of 72 cells was counted for each time point.

observations also mean that removal or uptake of C16-Rho-RGD by cells is at least as fast as the lateral mobility, which is already quite fast with almost complete back-filling of a 10 μm diameter bleach spot in approximately two minutes as was observed by FRAP (Figure 4.9B-C). Alternatively, the interaction of cells with the SLB might reduce the mobility of the SLB locally.

To take a closer look at what happens with C16-Rho-RGD after cells remove it from the SLB, cells were fixed after incubation on SLBs that present high densities of either C16-RGD, Chol-RGD, or C16-Rho-RGD (Figure 4.15). In this case immunostaining of the cytoskeleton (now green to allow imaging of the C16-Rho-RGD) and nucleus (blue) was performed. On the surfaces presenting C16-Rho-RGD, red fluorescence was observed in the cell, throughout the cell body (Figure 4.15, inset).

Figure 4.14 | Dynamics of peptide presentation in SLBs. Snapshots from live cell imaging using confocal microscopy at the early stages of cell adhesion on DOPC SLBs doped with TF (green) and presenting C16-Rho-RGD (red). Cells were visualized in transmission mode at the same time. All images show the same location. (t = time after cell seeding, fat arrow: dark spots colocalized with a cell disappearing after cell moved away from that spot, narrow arrow: following a dark spot colocalized with a cell remaining on the same place).

Figure 4.15 | Peptide uptake by cells. Fluorescence images from confocal microscopy of hMSCs after 18 h on different SLB surfaces. Note that red signal only originates from rhodamine and that peptides are washed away from solution before cell seeding. Inset: showing only the red emission, derived from C16-Rho-RGD, of the two cells in the left bottom corner of the main image. (Fixed cell imaging, blue: nucleus, red: C16-Rho-RGD, green: actin).

4.3. Conclusions

This chapter presents a facile surface functionalization method that enables the dynamic display of bioactive RGD ligands at high densities by using fluid supported lipid bilayers (SLBs) and lipid-modified peptides. Depending on the anchor type, the dynamicity is either in-plane (Chol-RGD) or both in-plane and out-of-plane (C14-RGD, C16-RGD, oleic-RGD, and C16-Rho-RGD). At high densities, the lipid-modified peptides are shown to assemble in domains within the SLB. After formation and growth, the domain size is quite stable but peptides within domains are still mobile. We tentatively conclude that this domain formation is the result of steric crowding of the peptide and/or wedging of the lipid anchor resulting in strain that is partially released by domain formation. We further demonstrate using immunostaining as well as live cell imaging that hMSC cells do not bind to bare SLBs while they readily bind and spread on RGD functionalized SLBs, although no differences were observed between cells (or cell parts) binding either onto or outside of domain areas. Moreover, we show that C16-Rho-RGD is locally removed from the SLB by adhered cells, though that lipid mobility results in replenishment of C16-Rho-RGD. Future work will be devoted to more in-depth studies of cell behavior and analysis on the high density RGD containing SLBs. Part of this will be presented and discussed by Gülistan Koçer in her PhD thesis.

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4.5. Experimental section

4.5.1. General methods

Chemicals were purchased from Sigma Aldrich or from Acros Organics unless differently specified and used without further purification. Protected amino acids were obtained from MultiSynTech and resin for solid phase peptide synthesis was obtained from Novabiochem. Phosphate Buffered Saline (PBS) with pH 7.4 at 25 °C containing 0.01 M phosphate buffer, 0.0027 M potassium chloride, and 0.137 M sodium chloride was used as such or diluted one time (0.5 x PBS) with Milli-Q water (MQ, Millipore, 18.2 mΩ) in order to maintain the same buffer solution throughout QCM-D experiments. TexasRed conjugated 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (TR, Molecular Probes, Thermo Fisher Scientific) and TopFluor-

phosphoethanolamine (TF, Avanti Polar Lipids) (dye-conjugated) lipids were introduced in DOPC vesicles at 0.2 mol% and 0.5 mol%, respectively. All other lipids were purchased from Avanti Polar Lipids. Polycarbonate membranes with 100 nm pore size were purchased from Whatman. Mass spectrometry (MS) was performed using a Waters ESI(+)-ToF spectrometer (Micromass LCT). Fluorescence microscopy was performed using an Olympus (1x71) microscope. For confocal fluorescence microscopy and FRAP analysis, a Nikon confocal (A1) microscope was used equipped with 488 nm and 561 nm lasers and 500-550 nm and 570-620 nm emission filters. Time lapse images were analyzed using ImageJ (NIH). Fluorescence microscopy pictures were enhanced in intensity in ImageJ for better visibility. Dynamic light scattering (DLS) analysis was carried out on an Anaspec particle analyzer (Nanotracs operating with a Microtrac FLEX Operating Software). Quartz crystal microbalance with dissipation monitoring (QCM-D) measurements were performed using a Q-sense E1 or E4 system (Biolin Scientific) under a 100 μ l/min flow and at 22 $^{\circ}$ C, unless stated otherwise using SiO₂ coated QCM-D sensors (QSX303, Biolin Scientific). Before each use, sensors were cleaned with a 2% sodium dodecyl sulfate solution, rinsed with Milli-Q water and ethanol, dried in a jet of nitrogen, and finally activated for 30 min using a UV/ozone chamber (Bioforce, nanosciences). Throughout this work, the fifth overtone was used for the normalized frequency (ΔF_5) and dissipation (ΔD_5). Vesicle and supported lipid bilayer preparation was performed as described in chapter 2. SLB fabrication and functionalization for cell culture is described below in section 4.5.5. The fabrication and use of the flow channel setup has been described in chapter 2.

4.5.2. Synthesis procedures

Peptide synthesis. Peptides were synthesized using an automated solid phase peptide synthesis robot (Syro II, MultiSynTech) following standard Fmoc procedures on a Rink amide MBHA resin (100-200 mesh, loading 0.52 mmol/g). Deprotection of the peptide and cleavage from the resin was achieved using a 95:2.5:2.5 solution of trifluoroacetic acid (TFA), water, and triisopropylsilane (TIS). Purification was performed by repeated (3x) precipitation in cold diethyl ether and subsequent preparative HPLC using a C₁₈ reverse phase column, HPLC programs are specified below.

Lipidation of the peptides (Figure 4.1). Fatty acids (myristic acid, palmitic acid, oleic acid, and cholesteryl hemisuccinate) were coupled to the N-terminus of the peptide on resin in the presence of a 5x molar excess over the resin loading of

benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) and hydroxybenzotriazole (HOBt). For these peptides (C14-RGD, C16-RGD, oleic-RGD, and Chol-RGD) preparative HPLC was operated at 4 ml/min, always in the presence of 0.1% TFA, for 10 min using 90:10 water:acetonitrile and then linearly in 65 min to complete acetonitrile, which was kept for another 25 min. The isolated peptide fractions were freeze dried to yield white solids and characterized using ESI(+)-ToF-MS, see Figure 4.17 for Chol-RGD and chapter 3 for other peptides.

Coupling of rhodamine to the lipid-modified peptide (Figure 4.16). For the coupling of tetramethylrhodamine (mixture of 5/6 isomer of the free carboxylic acid, TAMRA) to the peptide chain, the free N-terminus of the main peptide chain had already been used for coupling of the fatty acid. Therefore, an MTT protected lysine was used in the main peptide chain. The MTT group can be selectively removed under mild acid conditions without cleaving the peptide from the resin. MTT removal was performed 9 times for 5 minutes using 1% TFA + 2.5% TIS in DCM, at which point the yellow color due to the MTT removal was not observed anymore. After thorough washing, the coupling of TAMRA was performed overnight in DMF using TAMRA (eq. 1.5), *N,N'*-diisopropylcarbodiimide (DIC, eq. 1.7), and HOBt (eq. 1.7). For these peptides (C16-Rho-RGD) preparative HPLC was operated at 9 ml/min, always in the presence of 0.1% TFA, for 10 min using 50:50 water:acetonitrile and then linearly in 10 min to 80% acetonitrile and then linearly in 80 min to 100% acetonitrile. The isolated peptide fraction was freeze dried to yield a purple solid and characterized using ESI(+)-ToF-MS, see Figure 4.18.

Figure 4.16 | Solid phase synthesis route for the synthesis of C16-Rho-RGD.

Figure 4.17 | ESI(+)-ToF mass spectra of Chol-RGD.

Figure 4.18 | ESI(+)-ToF mass spectra of C16-Rho-RGD.

4.5.3. Giant unilamellar vesicle (GUV) preparation and immobilization

GUV fabrication was performed using the electroformation method following literature procedures.³⁶ In order to immobilized GUVs for imaging purposes DOPC GUVs were doped with 0.1 mol% 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-cap-biotinyl (DOPE-cap-biotin) and 0.5 mol% TF. In order to not rupture them, GUVs were subsequently immobilized on pre-formed SLBs doped with 1 mol% DOPE-cap-biotin after incubation of these SLBs with 0.5 μM streptavidin and subsequent rinsing of excess streptavidin.

4.5.4. Fluorescence recovery after photobleaching (FRAP)

The FRAP procedure is described in chapter 3, with in addition here the use of the 561 laser at 100% intensity for bleaching of TR and C16-Rho-RGD. For FRAP analysis in domain regions, a location was chosen with at least 2 large domains of the same intensity, of which one was used as reference to correct for acquisition bleaching.

4.5.5. SLB preparation and functionalization for cell culture

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 incubated as such for 1 h. After removing the solution, the wells were cleaned by rinsing 10x with sterile MQ water with gentle pipetting. Then, 50 μl of 1x Dulbecco's modified phosphate buffered saline (DPBS, without CaCl_2 and MgCl_2) was added to each well followed by adding 50 μl of a solution of LUVs (to 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 μl DPBS, 10x) in order to avoid damage to the SLBs (leaving 100 μl DPBS/well).

After DOPC SLB formation, but before incubation with lipid-modified 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 C16-RGD (at 10 μM), C16-Rho-RGD (10 μM), and Chol-RGD (5 μM) peptides were added to the wells (100 μl /well in DPBS), resuspended gently 2x (each time), followed by removal of 100 μl solution from the wells. This was repeated 3x where finally 200 μl solution was left in each well, for at least 2h incubation. Finally, the 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% TF were immediately used for live cell imaging experiments. SLBs prepared from DOPC LUVs without TF lipid were also studied in a side experiment for immunofluorescence analysis.

4.5.6. 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), 100 U/ml penicillin, 100 μ g/ml streptomycin (Sigma)), and incubated at 37 °C and 5% CO₂ 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 the 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 18 h and then fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature. After fixation, cells were washed 3x with PBS, left in PBS with 0.05% sodium azide (Sigma), and kept at 4 °C for immunofluorescence analysis.

4.5.7. 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 1 h 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-Rho-RGD presenting SLBs. For vinculin localization, in order to exclude the interference from TF dye, SLBs were formed with DOPC LUVs without TF lipid (Figure 4.10). 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 an OPERA high content screening system (Perkin Elmer) with corresponding objectives and filter combinations.

4.6. References

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Chapter 5

Superselective targeting probed in ligand-receptor interactions on supported lipid bilayers

Multivalent interactions are omnipresent in biological systems. Yet, their precise role in superselective targeting often remain elusive. In a model system we show experimentally how multiple weak interactions between nickel(II) complexing nitrilotriacetic acid (NTA(Ni)) displaying vesicles and histidine displaying supported lipid bilayers (SLBs) contribute to reach a strong overall binding. Through careful tuning of the display of ligands and receptors, this binding was not only specific, but shows a threshold receptor density, which was characterized by a more than linear increase in vesicle binding upon a linear increase in receptor density, i.e. superselective targeting. Furthermore, the lateral mobility of receptors on the SLBs led to receptor recruitment and depletion, presumably assisting in superselective targeting.

5.1. Introduction

The use of (nano)vehicles to solubilize drugs or imaging agents, to prolong drug circulation and to target drugs to desired locations in the body, has been an active but challenging field of research.¹ Targeting approaches are usually based on strong, monovalent interactions between ligands on the exterior of (nano)vehicles and receptors overexpressed at membranes of target, diseased cells. Unfortunately, the use of strong interactions will also result in undesired binding to healthy cells with normal expression levels of that same receptor. However, highly selective targeting does not require strong monovalent interactions.² It has been shown that multivalent targeting systems using individually weak interactions are not only able to target receptors of a specific type, but are also sensitive to the density of the receptor. This has been shown elegantly by Hyman *et al.* for the binding of a glycosaminoglycan biopolymer hyaluronan (HA) to CD44 cell receptors³ and also by Kiessling *et al.* who targeted tumor cells through strongly interacting with overexpressed $\alpha_v\beta_3$ integrins in order to decorate the cells with many oligosaccharide epitopes.⁴ These multivalent displays of epitopes tightly bind multivalent antibodies, which would only weakly interact when epitopes are displayed individually.⁴

While in both cases highly enhanced targeting was observed above a certain threshold receptor density, broadly applying the concepts underpinning these systems remains challenging. Recently, Frenkel *et al.* simulated *in silico* the binding of nanoparticles decorated with multiple ligands to a multivalent receptor surface. In these simulations, superselective binding was identified when a more than linear increase in binding of these particles occurred upon a linear increase in receptor density, only when the individual interactions were weak and multivalent.⁵ Superselective binding has been further evaluated numerically with various types of nanoparticles,⁵⁻⁷ polymers,⁸ and micelles.⁷ For solid nanoparticles, an optimal valency of around five was observed in order to provide a sharp threshold density, although the exact particle design will strongly influence this value.⁵ For solid nanoparticles with tethered ligands, an important balance exists between enthalpy gain from more interactions and entropy loss from limiting tether freedom as a result of binding.⁹ Combining functional and nonfunctional tethers could be used to tune the binding onset.⁶ For totally polymeric systems, higher valencies are more readily achieved due to chain flexibility and the possibility for interpenetration. Superselectivity is further aided for polymers since the entropy penalty is larger for the first few binding events.¹⁰ This can be further optimized by arranging ligands in blocks within the polymer.⁸

Only in few cases has superselective binding been experimentally reported. In a notable example, Richter *et al.* showed superselective binding of β -cyclodextrin grafted polymers to self-assembled monolayers (SAMs) of ferrocene or adamantane.¹⁰ Flexible polymer chains resulted in high ligand availability and entropic effects that favor superselectivity.¹⁰ Another example, from Meijer *et al.*, showed an increase in superselective behavior when the target platform displayed the receptors dynamically rather than statically, by using electrostatic interactions between ssDNA and supramolecular polymers.¹¹ The advantage of dynamic receptors for superselective targeting was also implied in work of Tomas *et al.* where laterally mobile receptors in lipid bilayers were shown to cluster as a result of the binding of divalent targeting molecules.¹² Additional modelling suggested that for higher valencies of the targeting system, an increasingly sharp threshold in receptor density would be achieved where clustering would occur.¹²

Here, we aimed for superselective targeting of lipid vesicles to supported lipid bilayers (SLBs) to serve as a relevant model for drug carrier vehicles targeting the cell membrane.¹³ Both lipid vesicles and SLBs provide readily tunable ligand or receptor densities and have low nonspecific interactions when consisting of zwitterionic lipids,¹⁴ which is of particular importance as superselectivity is based on multiple weak interactions and might therefore be negated by nonspecific interactions.

5.2. Results and discussion

We selected the interaction between hydrophilic nickel(II) complexing nitrilotriacetic acid (NTA(Ni)) and histidine containing peptides as a mobile model ligand-receptor pair on lipid bilayers,¹⁵ with binding constants that are typically ranging from nM to mM depending on the number and connectivity of histidines and NTA(Ni) groups.¹⁶ In this study, lipid anchored monovalent His₁ and divalent His₂ peptides were selected for their reported affinities for NTA(Ni) of ca. 10^{-3} M and 10^{-4} M, respectively,¹⁷ which are expected to be sufficiently weak to yield superselective binding (Figure 5.1).^{4, 5, 18}

While lipid anchored NTA(Ni) ligands were commercially available, lipid anchored His₁ and His₂ peptides (Figure 5.1B) were synthesized on solid phase and analyzed by ESI-ToF mass spectrometry (see experimental section for details). The oligopeptide between the histidine(s) and the lipid anchors was included to function as hydrophilic spacer and contains a single negative charge that, by charge repulsion, minimizes aggregation. Figure 5.1C shows the most likely binding modes of the histidine-NTA(Ni) interaction pairs.

Figure 5.1 | Superselective interaction platform. **(A)** Schematic presentation of the binding of NTA(Ni) functionalized vesicles to histidine functionalized SLBs, only for a sufficiently high histidine coverage, compared to “normal” binding referring to strong monovalent interactions. **(B)** Structures of the lipids, lipidated NTA(Ni), and lipidated peptides. **(C)** Most likely binding modes between NTA(Ni) and His₁ or His₂.

His₂ and NTA(Ni) bearing vesicles were made by lipid film hydration and extrusion. Formation of His₂ presenting SLBs (2 mol% His₂, *ca.* 6 nm interligand spacing, see experimental section 5.5.6 for the calculation and spacings at other used coverages) *via* vesicle fusion was followed *in-situ* by QCM-D, showing changes in frequency and dissipation that are in agreement with literature and indicative of high quality SLBs (step I, Figure 5.2A).¹⁹ The use of DOPC lipids resulted in liquid disordered SLBs with

laterally mobile ligands, which was verified both for lipids and lipidated ligands using fluorescence recovery after photo bleaching (FRAP, experimental section, Figure 5.18).

The binding of DOPC vesicles containing 2 mol% NTA(Ni) ligands to SLBs functionalized with 2 mol% His₂ was observed using QCM-D (step II, $\Delta F_5 = -161$ Hz, Figure 5.2A). The large increase in dissipation suggests that the NTA(Ni)-functionalized vesicles remained intact after binding to the His₂-functionalized SLB. Furthermore, the frequency shift is in agreement with reported values for forming a fully packed layer of 100 nm DOPC vesicles on a TiO₂ surface.²⁰ The layer of NTA(Ni) vesicles remained on the SLB when washing with buffer, suggesting that the binding occurred in a multivalent fashion since the monovalent interaction would be too weak to resist washing. However, the vesicles were washed off with 50 mM imidazole, which is a competing binding ligand for NTA(Ni), demonstrating specificity and reversibility of the interaction. When using 2 mol% monovalent His₁ instead of divalent His₂, nearly the same binding behavior of DOPC vesicles with 2 mol% NTA(Ni) ligands was observed ($\Delta F_5 = -132$ Hz, Figure 5.2B). Weaker binding was observed in the presence of excess NiCl₂ in solution (Figure 5.2B).

Figure 5.2 | Vesicle binding. (A) QCM-D trace starting from an oxidized SiO₂ sensor of flowing a solution of (step I) 0.1 mg/ml DOPC vesicles with 2 mol% His₂ resulting in a SLB, followed by (step II) 0.1 mg/ml DOPC vesicles with 2 mol% NTA(Ni), and (step III) the elution with 50 mM imidazole. (B) QCM-D traces starting from sensors coated with SLBs with 2 mol% of His₁. Binding and desorption of 0.1 mg/ml DOPC with 2 mol% NTA(Ni) vesicles in the presence and absence of 5 mM NiCl₂ in solution, throughout the whole measurement. Note that NTA(Ni) was commercially obtained as pre-formed Ni²⁺-complex. (A-B) Entire measurement is under flow (100 μ l/min), areas that are not highlighted in grey represent washing with HEPES buffer (pH 7.4).

As a next step, we took advantage of flow cytometry-based analysis of the receptor-ligand interactions (Figure 5.3) because this technique, compared to QCM-D, allows to investigate a much higher number of events of many different samples, and thus parameters, in a shorter time.

Figure 5.3 | SLB coated glass beads. (A) FRAP results of SiO₂ beads of 2.01 μm in diameter, coated with DOPC SLB containing 0.5 mol% of the fluorescently labelled lipid TopFluorPE, at various time points and over the area indicated in the inset. Intensities are normalized using the other side of the same bead to compensate for bleaching during FRAP analysis. (B) Confocal microscopy images of the bead used for FRAP before bleaching (left top), directly after bleaching (right top), after 6 seconds recovery (left bottom), and after 60 seconds recovery (right bottom). (C) Flow cytometry of glass beads without SLB with both side scatter (top) and fluorescence (bottom) channels as function of the forward scatter. (D) Flow cytometry of glass beads coated with SLB of DOPC with 0.5 mol% TopfluorPE after washing away excess vesicles. Note that TopfluorPE is added here to visualize the SLB but is normally not added to the vesicles that form the SLB.

To this end, fluorescently labeled fluid SLBs were successfully formed on 2 μm glass beads, as confirmed by fluorescence microscopy (Figure 5.17) and FRAP analysis (Figure 5.3A-B). The forward and side scatter as well as the fluorescent emission of these beads were, as expected,²¹ easily detected by flow cytometry (Figure 5.3C-D).

To avoid potential bead aggregation, diluted bead suspensions were used for vesicle binding studies and under constant stirring on a rotary bench. Indeed, forward and side scatter values remained unchanged after the incubation of beads presenting His₂ with vesicles displaying NTA(Ni) (compare Figure 5.4A to Figure 5.3C). Fluorescence intensities were recorded of SLB-coated beads with various His₂ densities after incubation with various concentrations of vesicles doped with fluorescent lipids and different NTA(Ni) densities (Figure 5.4C-E). NTA(Ni) vesicles bind in a vesicle concentration dependent manner to the His₂-functionalized SLB coated beads (Figure 5.4D) while controls without either His₂ in the SLB or without NTA(Ni) in the vesicles showed almost no interaction (Figure 5.4B,F). Receptor-ligand interactions were stable after 2 h of incubation (Figure 5.4F) and reversible in the presence of competitors (Figure 5.4G), both in agreement with observations made by QCM-D (Figure 5.2).

Figure 5.4 | Flow cytometry parameter screening. Flow cytometry results of glass beads coated with DOPC SLBs bearing His₂ after 2 h incubation on a rotary bench with vesicles displaying 2 mol% NTA(Ni), unless otherwise specified. A total of 10^4 events was analyzed per sample. Fluorescence was recorded using a 488 nm laser and a 530/30 nm emission filter. Note that values of individual measurement sets cannot always be compared quantitatively to each other due to differences in settings and lack of internal standard. **(A)** Scatter plot of DOPC SLB coated glass beads with 2 mol% His₂ after interacting with 0.02 mg/ml lipid vesicles with 2 mol% NTA(Ni) and 0.5 mol% of the fluorescent lipid TopfluorPE. Gate (grey area) was set based on control samples of bare beads (Figure 5.3). **(B)** Control for the nonspecific interaction of 0.02 mg/ml DOPC vesicles bearing 0.5 mol% NTA(Ni) and 0.5 mol% TopfluorPE on beads coated with a DOPC SLB without histidine (red circles) compared to beads coated with DOPC SLB bearing 2 mol% His₂ (green squares). **(C)** Overlay of fluorescence intensities of gated events after incubating beads coated with DOPC SLB containing 2 mol% His₂ with various concentrations of vesicles bearing 2 mol% NTA(Ni) and 0.5 mol% of TopfluorPE. **(D-E)** Mean fluorescence intensities of SLB coated glass beads at various His₂ coverages after incubation with various concentrations of vesicles with 2 mol% NTA(Ni) and 0.5 mol% of TopfluorPE, plotted as function of (D) the vesicle concentration or (E) the His₂ coverage. **(F)** Kinetics of the vesicle binding, using 0.1 and 0.01 mg/ml DOPC with 2 mol% NTA(Ni) vesicles at beads coated with DOPC SLBs presenting 2 mol% His₂. First data points are at 3 min incubation. **(G)** Binding of 0.02 mg/ml DOPC vesicles with 0.5 mol% NTA(Ni) and 0.5 mol% TopfluorPE to beads presenting His₂ at various coverages and the reversibility of the binding after one hour incubation with either EDTA (forming a high affinity complex with Ni²⁺) or imidazole (competitively binds to NTA(Ni)).

When plotting the gated mean fluorescence intensity for a range of mole percentages of His₂ on SLB coated beads that interact with different concentrations of 2 mol% NTA(Ni)-functionalized lipid vesicles (Figure 5.4D), the curves reflect the interaction between vesicles and beads for which overall association constants (K_{ov}) of $6.5 \times 10^9 \text{ M}^{-1}$ for 0.5 mol% and $1.6 \times 10^{10} \text{ M}^{-1}$ for 2 mol% His₂ were derived by fitting the Langmuir adsorption model to the data (Figure 5.5). The curves for lower His₂ coverages were too close to control signals to fit.

Figure 5.5 | Binding curves. Flow cytometry titrations of vesicles consisting of DOPC with 2 mol% NTA(Ni) and 0.5 mol% TopfluorPE to beads coated with a DOPC SLBs presenting **(A)** 0.5 mol% His₂ or **(B)** 2 mol% His₂. Each data point is recorded after 2 h of vesicles incubation on a rotary bench and reflects the mean fluorescence intensity of 10^4 events.

In order to derive the valency (n) of the overall interaction, the obtained overall binding constants can be related to the intrinsic binding constant (K_i) by defining a local concentration of ligands after vesicle binding, *i.e.* the effective molarity (EM). Assuming the term " $K_i \times EM$ " (the multivalent enhancement factor) is sufficiently larger than one, we can use the following simplified equation:²²

$$K_{ov} = K_i (K_i \times EM)^{n-1} \quad (\text{Eq. 5.1})$$

K_i is $8.9 \times 10^3 \text{ M}^{-1}$ for 1:1 NTA(Ni) to His₂ binding (Figure 5.1C).¹⁷ A rough estimate of the effective molarity of the NTA(Ni) ligands within the interaction interface can be performed by considering the ability of the ligands to freely move over the vesicle surface. Furthermore, the thickness of the interaction interface can be roughly estimated to be 1 nm considering the molecular structures of NTA(Ni) and the histidine peptide, assuming both ligands are flexible and can probe the entire interface. At, for example, 2 mol% of both NTA(Ni) and His₂, the volume per ligand (or receptor) becomes $36 \text{ nm}^3/\text{ligand}$ resulting in an EM of 42 mM. Note that $K_i \times EM$ is indeed significantly larger than one, as assumed above.

Using $K_{ov} = 1.6 \times 10^{10} \text{ M}^{-1}$ as experimentally determined for 2 mol% His₂, equation 5.1 gives a valency of $n = 3.4$. Considering a spacing of 6.0 nm between receptors at 2 mol%, this valency amounts to an interaction interface of approximately 123 nm² or 0.4% of the area of a spherical vesicles of 100 nm, which seems unrealistically low. A likely explanation is related to the low diffusion constant ($8 \times 10^{-16} \text{ m}^2/\text{s}$) for 100 nm vesicles.¹⁵ Since the diffusion distance depends inversely on the square root of this (small) diffusion constant and the diffusion time, significant local depletion of vesicles can be expected around the glass beads despite excess of vesicles in the bulk solution. For example, at a vesicle concentration of 0.1 mg/ml (1.6 nM), the total vesicle area is roughly 7600-fold higher than the total bead area. However, if we only consider vesicles reaching the beads by diffusion over the course of 2 h, there is only a 0.15-fold “excess” of vesicle area. At the lowest concentration of vesicles of 0.001 mg/ml, these values are 76 and 0.0015, respectively. Although the effect of stirring is not considered (while samples were stirred on a rotary bench), it still seems likely that the vesicle concentrations used in the titration is probably higher than the “effective” concentration probed by the surface of the beads over the course of 2 h. That would mean that the values for K_{ov} mentioned above should be considered as a lower limit and might actually be significantly higher, which would correspond to a higher valency. Note that the effect of ligand and receptor mobility and concomitant recruitment as discussed later is not taken into account in the above calculations, although it might play a significant role.

Based on the flow cytometry analysis, an interesting window of the His₂ densities was identified between 0.02 and 0.5 mol% as here the largest increase in fluorescent intensity upon binding was observed. This region was further probed with high-quality SLBs in QCM-D experiments that confirm the absence of significant nonspecific interaction of NTA(Ni) bearing vesicles to bare DOPC SLBs (Figure 5.6A, at 0 % His₂). The overlay of QCM-D results for selected His₂ densities (Figure 5.6A) shows that after 90 min, a maximum change in frequency was reached for all densities. Surfaces with 0.5 and 2 mol% His₂ were saturated at the same value, which indicates a physical saturation of the surface in agreement with flow cytometry data (Figure 5.4E). Moreover, a more than linear increase in frequency shift upon increasing the density of His₂ lipids in the SLB was observed going from 0.02 to 0.05 mol% His₂. When plotting the frequency change as function of the His₂ density (Figure 5.6B, black squares) a more than 9-fold increase of the frequency change was observed while only a 2.5-fold increase in His₂ density occurred. The observation was qualitatively reproduced for His₂ lipids (Figure 5.6C) while when monovalent His₁ was used, similar

superselective behavior occurred at an on average higher peptide density (between 0.02 and 0.5 mol% His₁, Figure 5.6D). This is also to be expected as K_i is smaller for His₁, meaning that more interactions are required to achieve the same overall binding constant. Individual measurement sets were performed with one and the same batch of NTA(Ni) bearing vesicles in order to minimize batch-to-batch differences. Such difference appeared quite relevant (compare Figure 5.6B and C), possibly because differences in vesicle size and vesicle concentration after extrusion as well as slight differences in NTA(Ni) densities, could add up. For this reason, individual measurement sets were kept separate and individual data points were not averaged.

Figure 5.6 | Superselectivity. (A) QCM-D frequency traces recorded when flowing (100 μ l/min) a HEPES (pH 7.4) solution of 0.02 mg/ml DOPC vesicles with 0.5 mol% NTA(Ni) lipids over sensors with DOPC SLBs with different densities of His₂ lipids, as indicated in the graph. The same batch of NTA(Ni) vesicles was used for each of these measurements. Formation of SLBs was monitored prior to each measurement. Areas that are not highlighted in grey represent buffer washing steps and imidazole washing was performed with 50 mM imidazole. (B) Plot of maximum change in frequency (black squares) and slope (at 20 min, red triangles) as function of His₂ density. Between 0.02 and 0.05% His₂, the increment in His₂ coverage and ΔF_5 is given. (C) Duplo results of results shown in (B) for the region of interest between 0 and 0.1 mol% His₂. (D) QCM-D results (*in duplo*) of the binding of 0.02 mg/ml DOPC with 0.5 mol% NTA(Ni) vesicles on SLBs of DOPC with various coverages of His₁. (A-D) Each individual measurement set is performed with one batch of NTA(Ni) vesicles to avoid batch-to-batch differences.

An important consideration is the possibility of a non-linear relationship between the amount of bound vesicles and the change in frequency measured by QCM-D. This can be caused by water trapped between vesicles, comparable to the hydration shell overlap described in chapter 3, and mainly plays a role at higher coverages of vesicles.²⁰ Therefore, we also evaluated the presence of superselectivity in the early stage of adsorbing NTA(Ni)-functionalized vesicles by plotting the slope during the onset of binding (red triangles in Figure 5.6B). The plotted data confirms the observation of superselectivity.

Interestingly, washing with buffer after binding of 0.5 mol% NTA(Ni) vesicle removes most vesicles for His₂ densities up to 0.1 mol%, but only a small fraction in the case of 0.5 mol% His₂ and no significant vesicle detachment was observed for 2 mol% His₂ (Figure 5.6A). A similar trend was observed when using 2 mol% NTA(Ni) vesicles (Figure 5.7). The observed partial (rather than complete or no) removal of the vesicles under continuous buffer wash might be explained by the size distribution of the vesicles: larger vesicles deform more readily²³ and might therefore bind with a higher valency. A representative size distribution from DLS of DOPC with 0.5 mol% NTA(Ni) vesicles is shown in the experimental section (Figure 5.16). Alternatively, increased chances for ligand rebinding²⁴ at decreased vesicle coverages might explain this effect.

Figure 5.7 | Reversibility of vesicle binding. (A) Overview of QCM-D results using DOPC SLBs with various densities of His₂ and the subsequent binding of 0.1 mg/ml DOPC vesicles displaying 2 mol% NTA(Ni) as well as (B) reversibility of the binding of these vesicles upon buffer wash (without imidazole). Reversibility is defined as the frequency shift for vesicle detachment under buffer flow divided by the frequency shift for the vesicle binding, multiplied by 100.

Taken together, these observations demonstrate the superselective character of the interactions between the NTA(Ni) lipid vesicles and histidine displaying lipids in the SLB. The His₂ threshold density is approximately an order of magnitude lower than

what has been found previously for β -cyclodextrin functionalized polymers targeting ferrocene or adamantine self-assembled monolayers.¹⁸ This difference might be related to His₂ or NTA(Ni) ligand recruitment to the contact area between vesicle and SLB. Especially when NTA(Ni) ligands are present in excess, His₂ ligands could continue to bind to NTA(Ni) after initial vesicle binding, thus locally creating a higher density of His₂ ligands in the SLB-vesicle interface, *i.e.* recruitment. This effect is of particular interest in this study since surfaces with low receptor density will be depleted faster than high density surfaces and in this way superselective targeting might be promoted.

To study this effect, parallel QCM-D experiments were performed in which, for a constant His₂ density of 0.1 mol% in the SLB, an increased binding was observed for vesicles presenting 0.1, 0.5, and 2 mol% NTA(Ni) (Figure 5.8). When increasing the NTA(Ni) ligand density further to 8 mol%, the frequency shift was drastically decreased to less than half of the frequency shift observed in the case of 2 mol% NTA(Ni). Despite the lower amount of vesicle binding in the case of 8 mol% NTA(Ni), these vesicles were stably bound when washing with buffer, while vesicles were detaching gradually in the case of 2 mol% NTA(Ni) and promptly in the case of 0.1 and 0.5 mol% of NTA(Ni) ligands. These data show that above a certain NTA(Ni) density, ligand recruitment significantly depletes the SLB of His₂ ligands preventing subsequent vesicle binding, despite the higher NTA(Ni) density. Size distributions obtained by DLS of the used NTA(Ni) vesicles were all similar (results not shown), showing that varying the NTA(Ni) coverages does not influence vesicle size.

Figure 5.8 | Receptor recruitment. QCM-D frequency traces recorded when flowing (100 μ l/min) a HEPES (pH 7.4) solution of 0.02 mg/ml DOPC vesicles presenting NTA(Ni) with various densities, indicated in the graph, over a sensors with DOPC SLBs with 0.1% His₂. Formation of SLBs was monitored prior to measurements. Areas that are not highlighted in grey represent buffer washing steps, last step (in grey) is a wash with 50 mM imidazole.

5.3. Conclusions

Specific binding of nickel(II)-complexing nitrilotriacetic acid presenting vesicles was achieved at supported model membranes functionalized with histidine bearing peptides. This interaction platform was designed in order to investigate the possibility of targeting mobile, membrane bound receptors based on receptor type, but also on receptor density. This so-called superselectivity was indeed observed both for peptides with one (His₁) or two (His₂) histidines and was found to occur at remarkably low histidine densities. One factor, that was hypothesized to play a role specifically for membrane targeting, is mobility and possible recruitment and depletion of receptors in the membrane upon multivalent targeting. These effects were demonstrated to play a role in this model system and should therefore also be taken into account in the biological context of cell membranes that are also highly dynamic. Looking ahead, this superselective targeting approach can be readily adapted for biological application because of the use of vesicles, which are well-established drug delivery vehicles that are easily prepared and functionalized by simply mixing in different ligands. The methodology shows that cell receptor targeting does not necessarily require the search for strong monovalent binding partners and that it can in fact be beneficial to rely on multivalent ligand displays of weaker monovalent interactions.

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5.5. Experimental section

5.5.1. General methods

Chemicals were purchased from Sigma Aldrich or from Acros Organics. Protected amino acids were obtained from MultiSynTech. Resin for solid phase peptide synthesis and the TAMRA dye were obtained from Novabiochem. Commercial lipids were obtained from Avanti Polar Lipids unless stated otherwise. HEPES buffer contained 0.01 M HEPES and 0.15 M sodium chloride was made using Milli-Q water (Millipore, 18.2 mΩ) and adjusted to pH 7.4 at 25 °C using sodium hydroxide. Non-porous glass (SiO₂) beads with 2.01 μm diameter (SS04N) were obtained from Bang Laboratories, Inc.

Mass spectra were recorded in positive ion mode on a Waters electrospray ionization time of flight mass spectrometer (ESI(+)-ToF, Micromass LCT). For high performance liquid chromatography (HPLC) a C18 reverse phase column was used (Waters, Xbridge). Dynamic light scattering (DLS) analysis was carried out on an Anaspec particle analyser (Nanotracs operating with a Microtrac FLEX Operating Software). QCM-D measurements were performed on a Qsense Analyser (Biolin Scientific) at 22 °C and operated with 4 parallel flow chambers, using 2 Ismatec peristaltic pumps with a flow rate of 100 $\mu\text{L}/\text{min}$, unless stated otherwise. Throughout this work, the fifth overtone was used for the normalized frequency (ΔF_5) and dissipation (ΔD_5). SiO_2 coated sensors (QSX303, Biolin Scientific) were used. Flow cytometry was carried out on a BD bioscience flow cytometer (FACS Aria II) equipped with a 488 nm laser operated with a 530/30 nm emission filter for detection. Epifluorescence microscopy was performed using an Olympus (1X71) microscope operated with 460-490 excitation filter and a 520/LP emission filter or with a 510-550 excitation filter and a 590/LP emission filter. For confocal microscopy, a Nikon confocal (A1) microscope was used equipped with a 488 nm laser and a 525/50 nm emission filter and with a 561 nm laser with a 595/50 nm emission filter. In microscopy images displayed in this work, contrast and brightness were adapted for using ImageJ. UV/ozone treatment was performed using a Bioforce chamber (Nanosciences).

5.5.2. Synthesis procedures

C16₂-Glu-PNP. The synthesis of C16₂-Glu-PNP (Figure 5.9) was done according to a 3-step procedure described in literature.²⁵ Briefly, the procedure involves a condensation between 1-hexadecanol and glutamic acid (Glu) in a 2:1 molar ratio, achieved by acid catalysis and constant water removal. The primary amine of the resulting ester is succinylated, and the free carboxy group is activated with p-nitrophenol. A white powder (3.7 g) was isolated with overall yield of 40%. ¹H-NMR (400 MHz, CDCl_3): δ = 0.81 (t, 6H, CH_3), 1.18 (m, 52H, CH_2), 1.53 (m, 4H, $\text{CH}_2\text{CH}_2\text{OCO}$), 1.94-2.13 (m, 2H, $\text{COCH}_2\text{CH}_2\text{CNH}$), 2.32 (m, 2H, $\text{COCH}_2\text{CH}_2\text{CNH}$), 2.60 (t, 2H, $\text{NHCOCH}_2\text{CH}_2\text{COO-}$), 2.87 (m, 2H, $\text{NHCOCH}_2\text{CH}_2\text{COO-}$), 3.98 (t, 2H, $\text{CH}_2\text{CH}_2\text{OCOCNH}$), 4.06 (t, 2H, $\text{CH}_2\text{CH}_2\text{OCOCCH}_2$), 4.55 (dt, 1H, $\text{CH}_2\text{CHCO,NH}$), 6.31 (d, 1H, NH), 7.23 (d, 2H, $\text{OCOC}_6\text{H}_4\text{NO}_2$), 8.19 (d, 2H, $\text{OCOC}_6\text{H}_4\text{NO}_2$).

Figure 5.9 | C16₂-Glu-PNP. (A) Structure and (B) ¹H NMR spectrum in CDCl₃.

Lipidated peptide synthesis. Peptides were synthesized using an automated solid phase peptide synthesis robot (Syro II, MultiSynTech) following standard Fmoc procedures on a Rink amide MBHA resin (100-200 mesh, loading 0.52 mmol/g). Lipid anchors as well as fluorescent dyes were coupled on the resin as described further on. Cleavage and deprotection of the peptide were achieved using a solution of 2.5 vol% water and 2.5 vol% triisopropylsilane (TIS) in trifluoroacetic acid (TFA). Additionally, 2.5 vol% 1,2-ethanedithiol (EDT) was added in the cocktail for cysteine containing peptides. Purification routes are specified further on. When possible, purification was performed by repeated (3x) precipitation in cold diethyl ether and subsequent preparative HPLC using a C18 reverse phase column. Peptides were characterized using ESI(+)-ToF.

His₁ and His₂ peptides. The synthesis of the main peptide chains (GDSNSNGGHG, His₁ and GDSNGGHAHG, His₂) and the cleavage are described above. C16₂-Glu-PNP was coupled on resin to the N-terminus of the peptide. For this, C16₂-Glu-PNP (4 eq.) and HOBt (4 eq.) were dissolved in 1:1 NMP:DCM and reacted for 4 h or overnight. After cleavage, the His₁ and His₂ peptides (see structures in Figure 5.10 and Figure 5.11) were precipitated in cold Milli-Q water. Subsequently, the peptides were

trituated 3x with methyl tert-butyl ether (MTBE), based on reported purification of various lipidated peptides of Bertozzi *et al.*²⁶ The products were freeze dried to yield white solids. The peptides were analyzed using ESI(+)-ToF mass spectrometry (Figure 5.13 and Figure 5.14)

Figure 5.10 | Chemical structure of His₁.

Figure 5.11 | Chemical structure of His₂.

Cys-Rho-His₂ peptide. The synthesis of the main peptide chain (DCSSGGHAHG) and the cleavage are described above. The coupling of tetramethyl rhodamine (mixture of 5/6 isomer of the free carboxylic acid, TAMRA) was done on the N-terminus as the last step (Figure 5.12). This coupling was performed overnight in DMF using TAMRA (eq. 1.5), DIC (eq. 1.7), and HOBT (eq. 1.7). HPLC purification was performed starting with 10% acetonitrile in water for 5 minutes and a subsequent linear gradient to 100% acetonitrile for 35 minutes. All solvents contained 0.1% TFA and the flow rate was 10 ml/min. The product was freeze dried to yield a purple solid. The peptide was analyzed using ESI(+)-ToF mass spectrometry (Figure 5.15).

Figure 5.12 | Synthesis of Cys-Rho-His₂.

Figure 5.13 | ESI(+)-ToF mass spectrum of His₁.

Figure 5.14 | ESI(+)-ToF mass spectrum of His₂.

Figure 5.15 | ESI(+)-ToF mass spectrum of Cys-Rho-His₂.

5.5.3. Large unilamellar vesicles (LUV) and supported lipid bilayer (SLB) formation

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidomethyl)cyclohexane-carboxamide] (MCC), 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] nickel salt (NTA(Ni)), and the tail modified lipid dye conjugate 1-palmitoyl-2-(dipyrrometheneboron difluoride)undecanoyl-sn-glycero-3-phosphoethanolamine (TopfluorPE) were stored in chloroform at -20 °C. Synthesized (lipidated) peptides were stored as lyophilized powder or in 3/1 chloroform/methanol at -20 °C. Dissolved lipids and lipidated peptides were mixed in desired molar ratios before use and dried under a flow of nitrogen in a glass vial in order to create a film of lipid material at the glass wall. This film was further dried under vacuum for at least 1 h and subsequently hydrated by vortexing with Milli-Q water to form multilamellar vesicles at 1 mg/ml. The lipid suspension was extruded 11 times through a polycarbonate membrane (Whatman) with 100 nm pore size, resulting in large unilamellar vesicles (LUVs) that were stored in the fridge and used within two weeks. NTA(Ni) bearing vesicles were instead hydrated in HEPES buffer and used within two days. The vesicle size was

verified using dynamic light scattering (DLS) before use (see Figure 5.16 for a representative example). For SLB fabrication, the vesicle suspension was diluted to a concentration of 0.1 mg/ml in HEPES directly before use. SLB formation was achieved by flowing or incubating this solution on a cleaned and activated surface. For glass beads, this is described in more detail further on. For flat QCM-D sensors or glass bottom well plates, cleaning was performed using a 2 wt% sodium dodecyl sulphate (SDS) solution and thoroughly rinsing with Milli-Q water. Activation was performed using a 30 minute UV/ozone treatment (for QCM-D sensors) or overnight incubation in 2% Hellmanex and again thoroughly rinsing with Milli-Q water (for well plates). The quality of SLBs was monitored by fluorescence recovery after photobleaching (FRAP) or *in-situ* by QCM-D (where high quality SLB are defined as $\Delta F = -24 \text{ Hz} \pm 1 \text{ Hz}$ and $\Delta D < 0.5 \times 10^{-6}$).²⁷

Figure 5.16 | Vesicle size. Dynamic light scattering (DLS) size distribution of DOPC with 0.5 mol% NTA(Ni) vesicles extruded 11x through 100 nm membrane.

5.5.4. SLB coating of glass beads and flow cytometry

SLB coating of glass beads has been previously optimized and characterized by flow cytometry.²¹ This procedure was adapted for our purpose and for glass beads with 2.01 μm diameter. Here, 13.6 μl of the 10 wt% stock suspension of beads was incubated for 1 h in a 2% Hellmanex solution on a rotary bench. These beads were centrifuged (500 rcf, 5 min) and washed 3x with 2 ml Milli-Q water and finally suspended in 1 ml Milli-Q water and sonicated for 1 min. 100 μl of this suspension was added to 900 μl of a vesicle solution (generally functionalized with up to 2 mol% His₂) that was freshly diluted to 0.1 mg/ml in HEPES. This corresponds to a 15x surface area excess of vesicles over beads which is reported to be the optimal ratio for SLB formation on glass beads.²¹ SLB formation was now allowed to take place for 1 h on the rotary bench. This mixture was either used directly or stored overnight under argon in the fridge,

although it can be stored up to more than a month under these conditions according to literature.²⁸ To remove excess of vesicles, the suspension was gently centrifuged at 100 rcf for 5 min. The supernatant was replaced with 2 ml Milli-Q water and the process was repeated 2 more times and briefly vortexed after the last washing step. Care was taken to not dehydrate the bead pellet during the washing steps.

For binding measurements with NTA(Ni) bearing vesicles labelled with the fluorescent lipid TopfluorPE, the suspension of SLB coated beads was diluted another 100x into, for instance, a 0.1 mg/ml vesicle solution in HEPES, providing a large excess of vesicle surface area compared to the coated bead surface area. Vesicle binding was usually performed for 2 h on a rotary bench, unless otherwise stated. Subsequently, this mixture was used without removing of excess vesicles for flow cytometry analysis, as centrifugation would now compact the beads presenting His₂ together with the vesicles presenting NTA(Ni), which would result in large aggregates and disturb proper analysis. However, free vesicles are much smaller than beads and can therefore be simply excluded during flow cytometry analysis by using only the forward scatter signal corresponding to the beads. For each sample, 10⁴ events corresponding to the bead signal were recorded.

Vesicle concentrations in mg/ml were converted to (nano)molar concentrations using a vesicle molar mass of 6.2×10^7 g/mol, assuming that all vesicles are exactly 100 nm in diameter, using a surface area of 0.725 nm² per DOPC lipid,²⁹ and a DOPC molar mass of 768.1 g/mol.

Figure 5.17 | Epifluorescence microscopy images of 2.01 μm diameter glass beads coated with DOPC SLB containing 2 mol% His₂ and 0.5 mol% of fluorescently labelled lipid (TopFluorPE) at 100x magnification. **(A)** Bright field and **(B)** green fluorescence microscopy images.

5.5.5. Fluorescence recovery after photobleaching (FRAP)

A DOPC SLB was doped with 0.5 mol% of the green fluorescent lipid TopfluorPE and/or with a fluorescently labelled lipidated peptide (for details of the peptide, see

synthesis Cys-Rho-His₂). Using a confocal microscope, a spot of 10 μm in diameter was bleached in case of flat substrates (Figure 5.18) or a small part of the edge of the glass bead was bleached in case of SLBs on glass beads (Figure 5.3B). Subsequently, the fluorescence intensity in the bleached regions was monitored. The intensity was normalized and corrected for acquisition bleaching by using the fluorescence intensity in a location not too close to the bleached spot for flat surfaces and a location on the opposite side of the same bead for the SLBs on glass beads. For flat substrates, the FRAP protocol consisted of 11 imaging loops (1 second interval) before bleaching, 10 loops bleaching with no delay in between loops, and 300 loops of recovery (1 second interval). For SLBs on glass beads, the FRAP protocol consisted of 2 imaging loops (2 second interval) before bleaching, 1 loop bleaching, and 30 loops of recovery (2 second interval).

Figure 5.18 | FRAP on SLBs presenting reacted Cys-Rho-His₂. The thiol of the cysteine of Cys-Rho-His₂ (100 μM) was reacted for 1 h to an SLB consisting of DOPC with 1 mol% maleimide lipid (MCC) and 0.5 mol% of a fluorescently labelled lipid (TopFluorPE). Inset: red fluorescence originating from Cys-Rho-His₂ recorded directly after bleaching (top) and after 5 min recovery (bottom).

5.5.6. Spacing between ligands or receptors in DOPC lipid bilayers

The surface area per lipid is 0.725 nm² for DOPC lipid,²⁹ meaning 1.38 lipids/nm² per leaflet. Assuming a homogeneous distribution, mixing in of 2 mol% of a certain receptor or ligand corresponds to a spacing between receptors or ligands of $\sqrt{\frac{1}{\frac{2}{100} \times 1.38}} = 6.0$ nm. Other coverages used in this work are 0.02 mol%, 0.05 mol%, 0.1 mol%, and 0.5 mol%, corresponding to 60.2 nm, 38.1 nm, 26.9 nm, and 12.0 nm respectively.

5.6. References

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Chapter 6

Studying the interaction of influenza virus particles at the interface

Influenza virus threatens global health causing millions of human infections every year. However, quantitative insight in the multivalent interactions between influenza viruses and sialic acid residues at the cell surface is lacking and required to better understand the mechanism of virus infection and the role of multivalent binding therein. In this chapter, we report the development of a platform that allows the study of the multivalent interaction of influenza A viruses. The use of biotinylated supported lipid bilayers (SLBs) permits the mimicking of cell membranes and the loading of different amounts of sialic acid receptors on a surface, followed by quartz crystal microbalance with dissipation monitoring (QCM-D). In combination with the use of hemagglutinin (HA) protein clusters as virus model, this system allowed the investigation of selectivity for human or avian receptors as well as quantitative determination of affinities and the role of the multivalency.

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6.1. Introduction

Influenza virus threatens global health causing millions of human infections every year.¹ Influenza A viruses are subtyped based on the antigenic properties of the two glycoproteins hemagglutinin (HA) and neuraminidase (NA).² HA is responsible for binding the virus to sialic acids (SAs) on cell membranes and signifies the first stage of cell infection. The binding of the influenza virus to cells is regulated by multivalent interactions between homotrimeric HA and SAs.³ The final overall affinity of the virus binding depends on the virus strain in combination with the specific form of SA presented at the membrane. This diversity is an important factor that determines what species can be infected. For example, avian influenza viruses bind preferentially to 2,3-sialyl-(*N*-acetyl-lactosamine) residues (2,3-SLN) while human influenza viruses prefer binding to 2,6-sialyl-(*N*-acetyl-lactosamine) residues (2,6-SLN).^{4, 5} Mutated binding sites of a particular virus species could alter the binding specificity, which can lead to interspecies transmission of the virus.⁶ This phenomenon contributes to serious outbreaks of influenza. For this reason, it is important to be able to study the preferred interaction of various virus strains for different SA residues. Some examples of sensor platforms bearing surface modifications for the study of the interaction of viruses with model cell receptors have been already reported.⁶⁻⁸ Although these approaches allow the determination of the selectivity of different virus strains or mutants over specific SA residues, they generally lack control over either the SA density or the HA density, and thus, limited information on the valency of the binding can be gained. Quantitative insight in the interactions between influenza viruses and sialic acid residues is lacking and required to better understand the mechanism of virus infection and the role of multivalent binding therein.

Various methods have been developed to achieve control over the surface densities, using self-assembled monolayers (SAMs) to obtain static layers, or fluid supported lipid bilayers (SLBs) to obtain laterally mobile layers.^{9, 10} For example, the density of NTA(Ni) could be controlled using SAMs for His₆-tag protein immobilization,¹¹ the density of arginine-glycine-aspartic acid (RGD) peptide was controlled using SAMs to investigate the effect on cell binding,¹² and the density of biotin was controlled both using SAMs and SLBs to investigate the multivalent binding of streptavidin.¹³

In this chapter, a SA-receptor platform with improved control over the SA density is described based on the aforementioned biotinylated SLBs. Together with the use of HA protein clusters as virus models, this enables the control and the quantification of the binding valency. The surface modification is based on a supported lipid bilayer

(SLB), which is a well-known cell membrane mimic that provides ease of preparation, controlled functionalization and excellent nonfouling properties.¹⁴ Surfaces were then functionalized with a tunable amount of human or avian receptors. HA protein clusters, which selectively bind to receptors, are used for a better prediction of the number of proteins involved in the interaction. The tunable sugar density together with the controlled valency of the HA cluster enables the use of a simple binding model allowing the quantification of the multivalent interaction at a cell surface.

6.2. Results and discussion

6.2.1. Design and characterization of the interaction platform

In this chapter, a platform was designed to study the interaction between HA clusters and 2,3- or 2,6-SLN functionalized surfaces. Due to the size and polydispersity of influenza viruses, it is very difficult to quantitatively study how valency controls the overall binding. For this reason, smaller HA clusters are used in this chapter as a model of the influenza virus, providing a controlled size and valency. These clusters consist of trimeric HA that formed clusters containing an average of 12 trimers. Therefore it is possible to make clusters of HA, originating from different strains of viruses with affinities for different sialic acid residues, namely those that are of human or avian origin. The advantage of using such protein clusters lies in a better prediction of the number of proteins involved in the binding to the receptors. In this chapter, three different HA protein clusters were used, *i.e.* A/California/07/2009, A/New Caledonia/20/99 and H1 A/Brisbane/59/07, all reported to bind to human sialic acid residues.¹⁵⁻¹⁷

The SLB-based interaction platform is assembled in a number of steps as schematically presented in Figure 6.1. Unilamellar vesicles consisting of both DOPC lipids and biotinylated lipids (biotin-PE) were prepared by extrusion, using a polycarbonate membrane with 100 nm pore size and analyzed for their size distribution by dynamic light scattering (DLS, Figure 6.6). Such vesicles are known to bind and rupture on oxidized glass substrates, resulting in SLBs that functionally display biotin moieties at the water interface.^{13, 18} SLBs consisting of zwitterionic DOPC lipids have been demonstrated to effectively repress nonspecific interactions.¹⁴ This is of particular importance here, considering that the overall binding is based on multiple weak interactions, meaning that even weak nonspecific interactions can cause problems. Furthermore, the density of biotin displayed at the SLB was controlled simply by

mixing-in the desired ratio of biotin-PE lipids during vesicle preparation. Subsequently, streptavidin (SAv) was bound by exploiting the strong biotin-SAv interaction. The surface-bound SAv still presents free binding pockets and these were used to bind a polyacrylamide (PAA) polymer presenting both biotin and SLN moieties (biotin-PAA-SLN). By controlling the biotin density, the SAv and finally the SLN density can be tuned. The sugar density at cell membranes is expected to influence the binding characteristics of the influenza virus by influencing the valency of the overall interaction.^{19, 20}

Figure 6.1 | Design of SLN presenting platform. Schematic representation of the platform as well as structure of the molecules used in this chapter to study the interaction between HA clusters and SAs.

In this chapter, quartz crystal microbalance with dissipation monitoring (QCM-D) was used to monitor the step-by-step formation of the SLB-based interaction platform (Figure 6.2A) as well as the interaction of HA clusters with surface-bound SA residues. All steps were followed *in-situ* for all measurements, allowing quantitative interpretation of different experiments. An important consideration when using QCM-D for (quantitative) analysis of biological entities at surfaces is the contribution of hydrated mass to the QCM-D output, *i.e.* frequency (F) and dissipation (D). As the water fraction is generally not known, the amount of bound molecules can be estimated from the QCM-D output but not determined exactly. However, when binding of the same molecule is concerned, it is possible to quantitatively relate individual QCM-D results. Note that in this case, the relative contribution of hydrated water can still depend on the packing density of the adhered molecule, especially at high packing densities where hydration shells can significantly overlap.²¹

In Figure 6.2A, an example of four parallel QCM-D measurements is presented, where a different biotin-PE density (0.1, 0.4, 1 and 5 mol%) was used in each of the measurements. As first step, high quality SLBs (*i.e.* $\Delta F = -24 \pm 1$ Hz and $\Delta D < 0.5 \times 10^{-6}$) were formed on SiO₂ coated sensors.¹⁸ Subsequently, all four surfaces were washed with buffer and incubated with SAV (0.5 μ M). The adsorption of SAV for different concentrations of biotin in the SLB is reported in Figure 6.2B, showing a linear trend between the biotin-PE density and the binding of SAV up to 1 mol% biotin-PE. This observation suggests that above 1 mol% biotin-PE, the surfaces approaches physical saturation with SAV, which is in agreement with previously reported high packing densities of SAV at SLBs that were functionalized with 5% or 10% biotin.^{22, 23} The subsequent binding of biotin-PAA-2,6-SLN (4 μ g/ml) was found to be linearly related to the amount of SAV that was bound in the previous step (Figure 6.2C). These results show that this platform is very suitable to tune the density of the sialic acid residues, with densities ranging between 3.7 and 28 pmol/cm² (respectively 6.7 and 2.4 nm spacing between SA residues) depending on the initial biotin density. These values are based on QCM-D frequency changes during the binding of biotin-PAA-SLN and the assumption that the Sauerbrey model is valid for this step (a reasonable assumption considering the small increase in dissipation for the polymer binding step). Furthermore, we used a 20% SLN grafting density (provided by the company) of the biotin-PAA-SLN and an estimated 80% water content of biotin-PAA-SLN (based on experimental QCM-D values ranging from 70-90% obtained for other large biomolecules)²¹. Note that these are average sugar densities, not taking into account

the probable inhomogeneous SA distribution over the sensor surface which is related to the use of a polymer, discussed in more detail later (Figure 6.4C, section 6.2.3).

An intriguing property of cell membranes is the lateral mobility of the individual constituents. DOPC based SLBs with 1 mol% biotin-PE retain this property and had a similar lateral mobility of bound SAV as compared to that of the bare SLB as was verified by fluorescence recovery after photo bleaching (FRAP) measurements (see details in experimental section 6.4.3, Figure 6.7).

Figure 6.2 | Assembly of SLN presenting platform. (A) Example of four QCM-D measurements showing the formation of an SLB using DOPC vesicles (0.1 mg/ml) with 0.1–5 mol% biotin-PE followed by the binding of SAV and then biotin-PAA-2,6-SLN, all in HEPES saline buffer at pH 7.4 and under flow. Grey areas indicate the binding steps and white areas indicate washing with buffer. (B) Plot of the shifts in frequency of binding of SAV as a function of the biotin-PE density. (C) Plot of the shifts in frequency of binding of biotin-PAA-2,6-SLN to the SAV-displaying SLB as a function of the shifts in frequency for binding of SAV. (B-C) Data points are averages of 2 or 3 measurements and error bars reflect the 95% confidence interval. Dashed lines are linear fits of the data points within the range of the fit.

6.2.2. Selectivity of the HA cluster binding

Next, the selectivity of the interaction between HA clusters and the SLN displaying platform was investigated. After verifying the formation of biotin-functionalized SLBs, subsequent binding of SAV and biotin-PAA-2,3-SLN or biotin-PAA-2,6-SLN, two different concentrations (0.14 and 0.56 nM) of California HA clusters were flown over

SLBs modified with biotin-PAA (without SLN) or biotin-PAA-2,6-SLN. Figure 6.3A shows the binding of HA clusters to surfaces that were functionalized with 2,6-SLN, while no binding was observed without 2,6-SLN on the SLB. This result shows that nonspecific interaction are negligible on this SLB-based interaction platform.

Figure 6.3 | HA clusters selectivity for 2,3- and 2,6-SLN. (A) QCM-D data of the binding of California HA clusters to pre-formed biotin-PAA-2,6-SLN or biotin-PAA presenting SLBs (both starting from 0.4 mol% biotin-PE). (B) QCM-D data of the binding of Brisbane HA clusters to pre-formed biotin-PAA-2,6-SLN or biotin-PAA-2,3-SLN presenting SLBs (both starting from 1 mol% biotin-PE). Grey areas indicate the binding steps and white areas indicate washing with buffer. All steps are under flow and using HEPES saline buffer at pH 7.4. Surface functionalization prior to the HA cluster binding was monitored but not shown here.

Various influenza virus strains are known to selectively bind to specific glycan structures. Therefore, the selectivity of the binding of Brisbane HA clusters (0.28 nM) to 2,6-SLN and 2,3-SLN functionalized SLBs was evaluated. A much higher QCM-D response was measured for 2,6-SLN modified SLBs indicating a preference for binding the Brisbane HA strain to human sialic acid residues (Figure 6.3B). This difference was not due to a different amount of SA residues that was bound to the sensor surface since the amount of binding of biotin-PAA-SLN to the biotin-SAv-modified SLB was comparable ($<10\%$ difference in ΔF_5). Subsequently, binding of the Brisbane HA cluster at a higher concentration (1.4 nM) showed a higher amount of binding, but with a similar preferred binding to the 2,6-SLN-modified SLB. Even more selective binding was observed in the case of binding of California and New Caledonia clusters to 2,6-SLN-modified SLBs: no significant binding was observed for California and New Caledonia HA clusters on 2,3-SLN surfaces while significant binding was observed at 2,6-SLN surfaces (Figure 6.4A and Figure 6.5B).

6.2.3. Quantifying the overall binding affinity and effect of sugar density

To determine the overall dissociation constant (K_d) of the interaction of California HA clusters with the 2,6-SLN-modified SLBs, solutions of California HA clusters over a

range of concentrations between 0.14 and 4.2 nM were titrated to the surface and changes were monitored with QCM-D (Figure 6.4A). Figure 6.4B shows the resulting binding data when plotting equilibrium ΔF values corresponding to each new concentration, versus the concentration of the HA cluster. $K_d = 5.2$ nM was found from fitting these results with the Langmuir model, when starting from 0.4 mol% of biotin-PE. In correspondence with this relatively strong binding, very limited dissociation was observed at the measurement time scale when washing the surface with buffer after the titration (Figure 6.4A). When the same type of titration was performed on a 2,6-SLN-modified SLB with 5 mol% biotin-PE, which results in a three times higher surface coverage of 2,6-SLN (based on Figure 6.2C), $K_d = 9.4$ nM was found (Figure 6.4B). These titrations were also performed for New Caledonia (at 0.4 mol% biotin-PE only) and Brisbane clusters (at 0.4 mol% and 5 mol% biotin-PE). The titrations and binding curves are presented in Figure 6.5 and the resulting overall dissociation constants are summarized in Table 6.1. Comparing the results for the three strains, very similar dissociation constants for 2,6-SLN presenting surfaces were found (Table 6.1). To the best of our knowledge, overall dissociation constants of the HA clusters used in this study for a multivalent 2,6-SLN displaying interface have not been reported before. For reference, concentrations of 100 pM of whole influenza virus were used by Gamblin *et al.*¹⁹ to achieve either partial or full virus coverage of surfaces coated with the same biotin-PAA-2,6-SLN polymer as used in this chapter.¹⁹

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As mentioned, the relative contribution of hydrated water can depend on the packing density of the adhered moiety, in this case the HA cluster. For this reason, we generally worked at modest biotin-PE densities which should prevent dense packing of the HA clusters. To evaluate whether the hydration shell overlap influences the observed binding curves, we evaluated the dissipation as function of the frequency shift for all titrations. Figure 6.4D shows that in these $-\Delta F/\Delta D$ plots, largely linear behavior was observed when starting from 0.4 mol% biotin-PE. At 5 mol% biotin-PE, the corresponding titrations show nonlinear behavior, which is probably related to increasingly overlaying hydrations shells of the HA clusters.

As expected, the absolute amount of binding is higher for the higher sugar densities (Figure 6.4B (note the different axes) and Figure 6.5). However, these results do not show the expected increased affinity for the higher sugar density. Instead, the affinity is more or less unaffected suggesting that the *sensed* sugar density does not change with increasing amounts of biotin-PAA-SLN. It is possible that the abovementioned hydration shell overlap during HA cluster binding has some influence on the QCM-D

Figure 6.4 | Affinity of California HA cluster for 2,6-SLN surfaces and effect of sugar density. (A) QCM-D titration of HA cluster to 2,3- and 2,6-SLN-modified SLB (0.4 mol% biotin-PE). Grey areas indicate the binding steps and white areas indicate washing with HEPES saline buffer at pH 7.4. All steps are under flow. Surface functionalization up to binding of HA cluster was monitored using QCM-D but not shown. (B) Binding curves from QCM-D titrations for California HA clusters at 2,6-SLN surfaces starting from DOPC SLBs with 0.4 mol% (red circles) and 5 mol% (black squares) biotin-PE. Langmuir model fitted (solid lines) to the binding data. (C) Schematic presentation of how the used PAA-based system results in an effectively constant local SLN density regardless of the total biotin-PAA-SLN coverage. (D) ΔD as function of $-\Delta F$ for the HA cluster titration step for the three tested clusters at surfaces starting from either 0.4 mol% or 5 mol% biotin-PE which are further functionalized with SAv and biotin-PAA-2,6-SLN.

Table 6.1 | Binding constants. Overall dissociation constants for HA clusters of the three different stains at 2,6-SLN presenting surfaces starting from 0.4 mol% or 5 mol% biotin-PE

Biotin-PE (mol%)	K_d (nM) California	K_d (nM) Brisbane	K_d (nM) New Caledonia
0.4	5.2	3.2	3.4
5	9.4	20	-

Figure 6.5 | HA cluster binding results. QCM-D titration experiments to pre-formed interaction platforms of the **(A)** New Caledonia HA cluster for 0.4 mol% biotin-PE and 2,6-SLN surface, **(B)** New Caledonia HA cluster for 0.4 mol% biotin-PE and 2,3-SLN compared to 2,6-SLN surface **(C)** Brisbane HA cluster for 0.4 mol% biotin-PE and 2,3 as well as 2,6-SLN surfaces and **(D)** Brisbane HA cluster for 5 mol% biotin-PE and 2,6-SLN surface. Grey areas indicate the binding steps and white areas indicate washing with HEPES saline buffer at pH 7.4. All steps were under flow. **(E-G)** The Langmuir binding model is fitted (red lines) to binding data. Dissociation constants are given.

output for the higher biotin-PE density, but this cannot explain that there is no increased affinity for the higher sugar density, as it would only enhance the apparent affinity (at higher packing density, more hydration shell overlap would result in an earlier levelling off of the binding curve and a concomitant increased apparent affinity, *i.e.* decrease in K_d).

Instead, we attribute this insensitivity of HA cluster binding to the sugar density to the use of the polymeric biotin-PAA-SLN. This will result in a fixed *local density* of SLN, regardless of the PAA-SLN coverage. It is only this local density that is probed by the HA cluster because of the similar size of the biotin-PAA-SLN (diameter of 15 nm)¹⁹ and the HA cluster (diameter of 28 nm, assuming a diameter of twice the size of one HA protein).²⁴ This also explains the seemingly contradictory coverage-dependent virus binding observed in literature, as the whole virus has a diameter of approximately 100 nm and can therefore interact with multiple biotin-PAA-2,6-SLN polymers simultaneously.¹⁹

For all the measurements, the highest concentration of HA cluster that was used was 4.2 nM. At this point, full saturation was not yet achieved, however higher concentrations of HA clusters were technically not feasible. Still, the concentration range was sufficient to reliably determine the affinity.

6.2.4. Insight in virus binding

The use of the HA protein clusters helps with the formulation of a model of the binding of the virus with a cell surface. The overall binding of multivalent ligands to surfaces developed earlier in our group²⁵ can be described as follows, assuming the $K_i \times EM$ factor is sufficiently larger than one:

$$K_{ov} = K_i (K_i \times EM)^{n-1} \quad (\text{Eq. 6.1})$$

With K_{ov} the overall association constant, K_i the monovalent association constant, EM the effective molarity and n the multivalence. The factor $K_i \times EM$, *i.e.* the multivalent enhancement factor, indicates which effect an increase in the valency has on the overall binding constant. In our case, considering the HA cluster and the steric considerations, we can estimate that three HA trimers can bind simultaneously to a flat surface, giving a valency n of nine. Filling in the equation 6.1 with the K_{ov} values obtained from the measurements at 0.4 mol% biotin-PE (Table 6.1), the value calculated for $K_i \times EM$ is approximately 6, indicating that increasing the valency has only a moderate effect on the overall affinity. Note that this value is sufficiently larger than one, confirming that the simplified equation 6.1 can indeed be used.

6.3. Conclusions

We report the development of a platform that allows the study of the multivalent interaction of influenza A viruses. The use of biotinylated supported lipid bilayers (SLBs) permits the mimic of cell membranes and the loading of different amount of receptors on a surface. Selectivity for human sialic acid residues compared to avian sialic acid residues was established as expected from the strains of hemagglutinin (HA) protein clusters used as virus model. Low nanomolar affinities of the HA cluster binding to the SA presenting surfaces was established. Due to the small size and limited valency of the HA cluster compared the whole virus, this allowed us to determine a quite low multivalent enhancement factor of about 6, indicating that each additional binding event only moderately contributed to the overall binding of influenza A viruses.

6.4. Experimental section

6.4.1. General methods

Chemicals were purchased from Sigma Aldrich and Acros Organics. Commercial lipids were obtained from Avanti Polar Lipids unless stated otherwise. Streptavidin labelled with Alexa Fluor® 488 (SAv488) was obtained from ThermoFisher Scientific. HEPES saline buffer contained 0.01 M HEPES and 0.15 M sodium chloride was made using Milli-Q water (MQ, Millipore, 18.2 mΩ) and adjusted to pH 7.4 at 25 °C using sodium hydroxide. Biotin-PAA-SLN was obtained from Lectinity. QCM-D measurements were performed using a Qsense Analyser (Biolin Scientific). Measurements were performed at 22 °C and operated with 4 parallel flow chambers, using 2 Ismatec peristaltic pumps with a flow rate of 100 µl/min. Throughout this chapter, the fifth overtone was used for the normalized frequency (ΔF_5) and dissipation (ΔD_5). SiO₂ coated sensors (QSX303, Biolin Scientific) were used. For confocal microscopy, a Nikon confocal (A1) microscope was used equipped with a 488 nm laser and a 525/50 nm emission filter and with a 561 nm laser with a 595/50 nm emission filter. In microscopy images displayed in this work, contrast and brightness were adapted for using ImageJ. UV/ozone treatment was performed using a Bioforce chamber (Nanosciences). Size of extruded vesicles was checked using Dynamic light scattering (DLS) analysis carried out on an Anaspec particle analyser (Nanotracs operating with a Microtrac FLEX Operating Software)

6.4.2. Large unilamellar vesicles (LUV) and supported lipid bilayer (SLB) formation

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl), sodium salt (biotin-PE) were stored in chloroform at -20°C. The head group modified lipid-dye conjugate, Texas Red-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (TR, ThermoFisher Scientific) was stored in methanol at -20 °C. Dissolved lipids were mixed in desired molar ratios before use and dried under a flow of nitrogen in a glass vial in order to create a film of lipid material at the glass wall. This film was further dried under vacuum for at least 1 h and subsequently hydrated by vortexing with Milli-Q water to form multilamellar vesicles at 1 mg/mL. The lipid suspension was extruded 11 times through a polycarbonate membrane (Whatman) with 100 nm pore size, resulting in large unilamellar vesicles (LUVs, Figure 6.6) that were stored in the fridge and used within two weeks. For SLB fabrication, vesicles were diluted to a concentration of 0.1 mg/ml in HEPES saline directly before use. SLB formation was achieved by flowing this solution on a cleaned and activated surface. For flat QCM-D sensors or glass bottom well plates, cleaning was performed using a 2 wt% sodium dodecyl sulphate (SDS) solution and thorough rinsing with Milli-Q. Activation was performed using a 30 minute UV/ozone treatment (for QCM-D sensors) or overnight incubation in 2% Hellmanex and again thorough Milli-Q rinsing (for well plates). The quality of SLBs was monitored by fluorescence recovery after photobleaching (FRAP) or in-situ by QCM-D (where high quality SLB defined as $\Delta F = -24 \text{ Hz} \pm 1 \text{ Hz}$ and $\Delta D < 0.5 \times 10^{-6}$). After SLB formation, care was taken to keep the surface submerged in buffer and without bubbles.

Figure 6.6 | Dynamic light scattering (DLS) size distribution of DOPC vesicles containing 20 mol% biotin-PE, extruded 11x through a membrane with 100 nm pore size.

6.4.3. Fluorescence recovery after photobleaching (FRAP)

A DOPC SLB was doped with 0.2 mol% of TR and 1 mol% of biotin-PE. Subsequently, 0.2 μM SAV488 was incubated for 1 h and for at least 15 times, while care was taken not to dehydrate the SLB. Using a confocal microscope, a spot of 10 μm in diameter was bleached and subsequently, the fluorescence intensity in this bleached region was monitored (Figure 6.7). The intensity is normalized and corrected for acquisition bleaching by using the fluorescence intensity in a location not too close to the bleach spot. The FRAP protocol consisted of 11 imaging loops (1 second interval) before bleaching, 10 loops bleaching with no delay in between loops and 300 loops of recovery (1 second interval).

Figure 6.7 | SLB mobility. (A) Confocal FRAP recovery curves on a DOPC SLB with 0.2 mol% TR and 1 mol% biotin-PE after 1 h incubation of 0.2 μM SAV488 and subsequent rinsing with HEPES saline buffer at pH 7.4. (B) Red channel from TR after bleaching (top) and after 5 minutes recovery (bottom). (C) Green channel from SAV488 after bleaching (top) and after 5 minutes recovery (bottom).

6.5. References

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Chapter 7

Epilogue

Section 7.2 was performed in collaboration with Ralph Bosmans and Prof. Luc Brunsveld and is based on published work: R. P. G. Bosmans, W. E. Hendriksen, M. Verheijden, R. Eelkema, P. Jonkheijm, J. H. van Esch and L. Brunsveld, Chemistry – A European Journal, 2015, 21, 18466-18473. “Supramolecular Protein Immobilization on Lipid Bilayers”

Section 7.3 was performed in collaboration with Lukasz Poltorak and Prof. Ernst Sudhölter and is based on the manuscript: Poltorak, L., Verheijden, M.L., Bosma, D., Jonkheijm, P.; de Smet, L.; Sudholter, E.; “Lipid bilayers cushioned with polyelectrolyte-based films on doped silicon surfaces”

Section 7.4 was performed in collaboration with Afroditi Nanou, Dorothee Wasserberg, Anouk Mentink - Leusink and Prof. Leon Terstappen

7.1. Introduction

Throughout this thesis, interactions with supported lipid bilayers (SLBs) have been studied and used in various settings. In this epilogue, we expand on that by showing an example that demonstrates that SLBs can be easily adapted for devices and applications that rely on immobilized proteins (Section 7.2), by presenting an easy route to generate SLBs on a more broader range of material surfaces (Section 7.3) and by employing supramolecular functionalization of cell membranes, which is of interest for medical diagnostics (Section 7.4).

7.2. Supramolecular protein immobilization on lipid bilayers

Surface-immobilized proteins have great potential in biotechnological research. By developing strategies for the controlled immobilization of proteins on surfaces, biosensors can be made for use in, for example, biomarker detection and drug discovery.¹ These protein-covered surfaces are also of great interest for the development of cell growth in or on biomaterials. Such biomaterials can also be applied for tissue engineering and the development of medical implants.^{2, 3} One of the main challenges for protein immobilization strategies is to find chemical methodologies that allow for an efficient immobilization of proteins while ensuring that their functionality is retained. Moreover, there is interest in molecular strategies that allow for the immobilization of the proteins through singular and defined molecular handles, rather than, for example, approaches that are based on random amide bond immobilization.⁴

In this thesis, lipid bilayers have proven to be a versatile and powerful platform for immobilization of a variety of (supramolecular) functionalities. An interesting topic here is the search for techniques that control the introduction of proteins on these bilayer surfaces. This generally requires the introduction of a hydrophobic anchor to the proteins of interest. As a result, many covalent chemical modification strategies have been developed to synthesize membrane-anchored proteins.⁵⁻⁸ Although successful, synthetic access to these protein constructs is typically troublesome and only amenable to a limited set of proteins.

Specifically, for the immobilization of proteins on lipid bilayers, supramolecular chemistry offers the potential advantage that there is no need for modification of the proteins through potentially cumbersome chemical protein lipidation methods. Additionally, the use of noncovalent interactions also provides a way to ensure that

the immobilization occurs site-specifically and under milder conditions than required for many covalent immobilization strategies. Finally, supramolecular protein immobilization also enables reversible release of the proteins from the lipid bilayer, for example upon addition of noncovalent competitor molecules.¹

In chapter 2 of this thesis, we demonstrated how supported lipid bilayers (SLBs) functionalized with methyl viologen (MV) can be used in conjunction with the supramolecular host molecule cucurbit[8]uril (CB[8]) for supramolecular immobilization of naphthol functionalized cells. Here, we have adapted that strategy for supramolecular protein immobilization. CB[8] is used to immobilize proteins by formation of a ternary complex of CB[8],⁹ MV presented at the SLB and N-terminal tryptophan displayed by the protein as schematically shown in Figure 7.1A.

Fluorescent protein concentrations up to 2 μM , both in the presence and in the absence of equimolar cucurbit[8]uril, did not result in any detectable adsorption on the bare DOPC SLB. Similarly, cucurbit[8]uril at high concentrations (75 μM) did not show any affinity towards the bare SLB. These results show that there is negligible nonspecific interaction of the protein and cucurbit[8]uril with the lipid bilayer, which bodes well as a surface for the selective immobilization of proteins.

To anchor the supramolecular complex on the SLB, 5 μM of a the cholesterol functionalized methylviologen (Chol-MV, see publication¹⁰ for synthesis details) was inserted in a bare DOPC lipid bilayer, in the presence of 50 μM CB[8]. Subsequently, 2 μM of yellow fluorescent protein modified with an N-terminal Trp-Gly-Gly motif (WGG-YFP) was incubated, again in the presence of 50 μM CB[8]. The entire process was monitored using quartz crystal microbalance with dissipation monitoring (QCM-D), see Figure 7.1B. Both the insertion of Chol-MV (as complex with CB[8]) and the subsequent binding of WGG-YFP was clearly observed, showing that this platform can indeed be used for the supramolecular immobilization of proteins at surfaces.

Some, though clearly less, binding was also detected for a control protein where the tryptophan is replaced by a methionine (MGG-YFP). This is probably elicited by binding events of solvent-exposed in-chain aromatic amino acids. Both the binding of WGG-YFP and MGG-YFP is largely reversible by rinsing with buffer. Binding and washing was repeated two more cycles. The decrease in the amount of protein immobilized on the bilayer after each binding cycle might the result from a partial extraction of the Chol-MV during these cycles. The possibility of dissociation of cholesterol anchored moieties from lipid bilayers has been previously reported¹¹ and has been observed in chapter 4 of this thesis as well, in the presence of β -cyclodextrin.

Figure 7.1 | Supramolecular protein immobilization on lipid bilayers. (A) Schematic presentation of the molecules and supramolecular interaction platform. **(B)** Overlay of two QCM-D measurements showing the formation of the SLB using 0.1 mg/ml DOPC vesicles, the insertion of 5 μ M Chol-MV in the presence of 50 μ M CB[8] and the interaction of 2 μ M of the target protein WGG-YFP as well as the control protein MGG-YFP, also in the presence of 50 μ M CB[8]. Areas that are not highlighted (in grey) represent buffer washing steps.

7.3. Lipid bilayers cushioned with polyelectrolytes

The development of a bio-interface that mimics biological membranes is important from both the application and fundamental point of view.^{12, 13} Supported lipid bilayers (SLBs) have been used widely and successfully for this purpose. The fastest and most convenient method to fabricate SLBs consists of vesicle adhesion and fusion at hydrophilic surface like freshly cleaved mica or oxidized glass. However, vesicle fusion turns out to be quite surface specific and does for instance not (or not as easily) occur on oxidized gold or TiO₂.¹⁴ Moreover, the support may affect the fluidity of the proximal leaflet and restrict the accommodation of bulky functional groups like transmembrane proteins. A number of different methods have been developed to overcome these problems such as the use of tethered lipid bilayers¹⁵ patterned surfaces that allow the formation of semi-supported lipid membranes¹⁶ and the use of polymeric cushions to support lipid bilayers.¹⁷

A fast, simple and reproducible method that allows the formation of polymeric cushions is based on the alternating deposition of oppositely charged polyelectrolytes which has emerged as field of research on its own.¹⁸ Recently, the use of polyelectrolyte multilayers (PEMs) for the generation of cushioned lipid bilayers has showed some promising results.^{19, 20} The importance of carefully tuning the charge

interactions between the PEM and the vesicles used in the SLB formation is also demonstrated.^{19, 20}

Here, we show that by using PEMs consisting of three layers, high quality SLBs can be fabricated. The mobility of these PEM supported bilayers was somewhat reduced compared to “regular” zwitterionic SLBs on glass.

Specifically, poly(ethyleneimine) (PEI, $\overline{M}_w = 25,000$ g/mol), poly(allylamine hydrochloride) (PAH, $\overline{M}_w = 17,500$ g/mol) and poly(sodium-4-styrene sulphonate) sodium salt (PSS, $\overline{M}_w = 70,000$ g/mol) were used to produce PEMs simply by successive incubation and washing of 1 mg/ml of each polymer in HEPES buffer with 150 mM KCl on activated glass surfaces. Polymer structures are presented in Figure 7.2A. Vesicles composed of zwitterionic DOPC or DOPC mixed 1:1 with negatively charged DOPS (DOPC/DOPS) were doped with 0.5 mol% of a Texas Red labeled lipid. The surface charge of these vesicles was measured using ζ -potential (Table 7.1.)

Table 7.1. | Surface charge of vesicles. ζ -potential measurements of extruded vesicles of various consistencies. Errors are calculated based on three independent measurements.

Lipid composition	DOPC	DOPS	DOPC/DOPS (1/1)
ζ-potential (mV)	-5.0 (+/- 0.2)	-41.8 (+/- 1.0)	-22.0 (+/- 2.0)

Fluorescent recovery after photobleaching (FRAP) was used to evaluate SLB quality and mobility. We investigated the interaction between DOPC as well as DOPC/DOPS vesicles and activated bare glass or glass modified with three polyelectrolyte layers in the order PEI (at glass), PSS and PAH. As expected, due to charge repulsion, no fluorescence emission was detected after incubation of overall negatively charged DOPC/DOPS vesicles on activated, negatively charged glass surfaces (data not shown). Figure 7.2A-B shows that DOPC vesicles at an activated glass surface results in high quality SLBs with high lateral mobility ($0.29 \mu\text{m}^2/\text{s}$), in line with reported values.²¹ However, DOPC did not form SLBs at PEM surfaces, based on the observed absence of lateral mobility, despite the adsorption of vesicles which is visible from the fluorescence emission. This suggests the formation of a vesicle layer. Finally, the formation of SLBs on a PEM coated surface was possible by using oppositely charged DOPC/DOPS vesicles. The high quality of the SLB was concluded from the recovery, which approached 100%. This PEM supported SLB shows a mobility ($0.079 \mu\text{m}^2/\text{s}$) that is significantly smaller than for a DOPC SLB at glass. The reduction in mobility is not fully understood. Possible explanations are related to increased affinity of lipids to the

polyelectrolyte layer or to an increased surface roughness of the supporting PEM compared to glass. Increased affinity of lipids for the surface might reduce mobility by simply holding lipids in place or by compacting the SLB which could result in increased lipid interdigitation and, thus, lowering lateral diffusivity. Roughness of the PEM layer could lower the apparent mobility as the lateral distance within the lipid bilayer following the surface roughness is effectively higher than the cross-section distance assumed in the model. It is not expected that the latter effect could fully account for the observed reduction in mobility, as a large surface roughness was not observed in confocal microscopy images.

Figure 7.2 | Layer mobility on glass and polyelectrolyte multilayers (PEMs). (A) Structures of the used polyelectrolytes. (B) Fluorescence emission from the adlayer directly after, 10 s after and 5 min after bleaching. (C) Corresponding FRAP plots.

7.4. Supramolecular functionalization of cells

The lipid vesicle field has experienced significant progress in the last decades with particularly promising applications in the biomedical field such as drug or enzyme carriers²²⁻²⁷ as well as vehicles that allow for molecularly engineering of the cell surface.²⁸⁻³⁰ The main feature of vesicles that make them promising carriers, is their relatively high stability in the bloodstream leading to prolonged circulation and higher treatment efficiency compared to free molecule administration.^{26, 31}

The effect of vesicle size on the interaction with blood cells or cells in target tissue has been studied thoroughly in animal studies, in the case of intravenous administration. Vesicles with a diameter range between 2-5 μm have the tendency to

localize to the vascular wall. Hence, they are good candidates as gene carriers to treat cardiovascular diseases or blood vessel disruption.³² Smaller vesicles of 100-500 nm diameter remain in the core of the bloodstream and follow the blood flow pattern together with the red blood cells (RBCs) with minimal adhesion to the wall,³³ allowing the use of these smaller vesicles for the targeting and functionalization of certain cell types that can then be isolated and subjected to downstream analysis.

Although the biodistribution to various tissues of vesicles of different sizes has been well-studied after intravenous administration, there are few studies exploring the uptake of such vesicles by different cell types in suspension. Herein, we explored three different vesicle systems in terms of their incorporation into membranes of different cell types in suspension. Specifically, we used vesicles doped with supramolecular handles. By using, for example, fluorescently labeled binding partners, this strategy enables the quantification of the efficiency of display of the supramolecular handles at the cell surface. Moreover, incorporation of supramolecular handles into recipient cells can serve as reversible anchors, potentially enabling their isolation and further downstream analysis. In the present study, we used phospholipid or cholesteryl anchored biotin or phospholipid anchored desthiobiotin (desbiotin) as supramolecular handles. While phospholipid anchored biotin serves as positive control, the use of desbiotin as well as cholesteryl anchored biotin additionally provides reversibility, thus the possibility for isolation and release, by using β -cyclodextrin³⁴ or excess free biotin,³⁵ respectively. The reversible interaction of the cholesteryl anchor with SLBs in presence of β -cyclodextrin has also been demonstrated in chapter 4.

In the present study, we compared the different (des)biotin vesicles (Figure 7.3) in terms of their fusion with different cell types in suspension. For this purpose, a desthiobiotin functionalized lipid (DOPE-desbiotin) and a cholesteryl modified biotin molecule with a short peptide and triethylene glycol spacer (Chol-EG₃-biotin) were synthesized as described in section 7.5.2. The biotin functionalized lipid (DOPE-biotin) was commercially obtained.

Vesicles made up of 100% of these molecules readily precipitated and DLS measurements showed a broad range in size distribution (not shown). Instead, zwitterionic DOPC vesicles containing up to 20 mol% of DOPE-biotin, 20 mol% of DOPE-desbiotin or 5 mol% of Chol-EG₃-biotin of approximately 100 nm were near monodisperse and stable for more than one month (Figure 7.4). Note that DOPC vesicles with 5 mol% Chol-EG₃-biotin were used since 20 mol% Chol-EG₃-biotin vesicles were polydisperse and not stable. The use of 20 mol% of the other biotin and

desbiotin ligands was maintained in order to achieve the highest possible labeling efficiency.

Figure 7.3 | Supramolecular cell functionalization. Schematic presentation of the vesicle-based system for the supramolecular cell functionalization and structures of the main molecules.

Figure 7.4 | Vesicle sizes. Overlay of DLS size distribution of DOPC vesicles with 20 mol% DOPE-biotin, 20 mol% DOPE-desbiotin or 5 mol% Chol-EG₃-biotin **(A)** directly after extrusion and **(B)** after > 1 month storage in the fridge.

To compare the three different (des)biotin vesicles in terms of their ability to fuse with different cell types and functionally display (des)biotin ligands, the white blood cells (WBCs) subsets lymphocytes, granulocytes, and monocytes and platelets obtained from whole blood and cells from the breast cancer cell line MCF-7 were analyzed by flow cytometry. Cells were identified by their light scattering properties as well as their expression of cell-specific receptors, using fluorescently labeled antibodies. To determine the incorporation of biotin into the cell membrane, cells were incubated with allophycocyanin-labeled streptavidin (SAV-APC). Figure 7.5 shows the flowcytometric analysis of the cells incubated with SAV-APC but not incubated with (des)biotin vesicles. The histograms show the fluorescence intensity of the cells with SAV-APC, as control for nonspecific binding.

Figure 7.5 | Flow cytometric analysis of cell populations. left column: Forward and side scatter of granulocytes (blue), monocytes (red), lymphocytes (green), platelets (orange) and MCF-7 cells (cyan). Middle column: Hoechst (DNA) staining versus CD45-PE antibody staining (top), CD61-PE antibody staining (middle) and EpCAM-PE antibody staining (bottom). Right column: negative control for nonspecific binding of SAV-APC to all cell types. SAV-APC was detected using a 633 nm laser with 660/20 nm emission filter, PE was detected using a 488 nm laser with 585/42 nm emission filter and Hoechst was detected using a 375 nm laser with 450/40 nm emission filter.

To investigate the ability of the (des)biotin vesicle systems to functionally label various cell types, cells were incubated for 1 h with one of the three vesicle types (0.1 mg/ml) after which cells were washed and stained with SAV-APC, in addition to the aforementioned antibody staining. Using the same flow cytometry settings as in Figure 7.5, these cells were analyzed for SAV-APC fluorescence emission intensity (Figure 7.6). These measurements were repeated (at least once) and the mean fluorescence intensities from SAV-APC were averaged and summarized in Figure 7.7.

From the individual (Figure 7.6) and averaged (Figure 7.7) results, it follows that all the cell types can indeed be functionalized with one or more of the ligand types, however large differences exist in labeling efficiency both depending on the cell type and the type of vesicle used for (des)biotin labeling. Note that the “labeling efficiency” is here defined by the amount of SAV-APC binding, which will be determined both by the uptake of the (des)biotin vesicles and the functional display of (des)biotin at the cell surface. The 5 mol% Chol-EG₃-biotin vesicles resulted in the highest labeling efficiency for all the tested cell types. On the contrary, DOPE-desbiotin did not show significant labeling for any of the cell types. DOPE-biotin showed labeling of all cells except for the lymphocytes, though to a lesser extent than Chol-EG₃-biotin. Both Chol-EG₃-biotin and DOPE-biotin showed the highest labeling efficiency in MCF-7 cells followed by the monocytes, granulocytes, lymphocytes and platelets. This relation may partially be explained by the differences in size, as MCF-7 cells (16 μm) and monocytes (12–20 μm) are the largest, followed by granulocytes (10–18 μm), lymphocytes (10–18 μm) and platelets (roughly 3 μm).^{36, 37}

The large differences in labeling efficiency between the three (des)biotin vesicle systems might be explained by various reasons. Desbiotin is expected to show less SAV binding as the affinity is about four orders of magnitude lower, but that does not by itself explain that no significant binding is observed at all to DOPE-desbiotin labeled cells as the affinity of desbiotin for streptavidin is still very high (10^{-11} M).³⁸ An additional cause might be the slightly shorter linker that connects desbiotin to DOPE compared to DOPE-biotin, possibly sterically hindering proper binding of desbiotin in the SAV binding pocket. The labeling using Chol-EG₃-biotin is consistently more efficient, contradictory to the lower percentage of this ligand in the labeling vesicles. Partially, this might again be explained by the larger linker that connects biotin to the Cholesteryl anchor. In addition, this linker is more hydrophilic, which can aid in the functional display of the cholesterol at the lipid membrane.³⁹ Finally, Chol-EG₃-biotin is overall neutral at physiological pH, while DOPE-(des)biotin displays a negative charge. The resulting negatively charged vesicles might bind and fuse less efficiently

Figure 7.6 | (des)biotin tagging of different cell types. Distribution of the SAV-APC fluorescent intensity of granulocytes, lymphocytes, monocytes, platelets and MCF-7 cells after incubation with (left column) 20 mol% DOPE-desbiotin vesicles, (middle column) 20 mol% DOPE-biotin vesicles and (right column) 5 mol% Chol-EG₃-biotin vesicles. After 1 h vesicle incubation, samples were washed to remove excess vesicles, incubated with SAV-APC and washed again before analysis. The same flow cytometry settings were used for each of these measurements. Each graph is represents 1000-10,000 events.

Figure 7.7 | Overview of labeling efficiency. Averages of mean fluorescence intensities of SAV-APC from two to six flow cytometry measurements of granulocytes, monocytes, lymphocytes, platelets and MCF-7 cells after incubation with vesicles displaying 20 mol% DOPE-desbiotin, 20 mol% DOPE-biotin, or 5 mol% Chol-EG₃-biotin. After vesicle incubation, samples were washed to remove excess vesicles, incubated with SAV-APC and washed again before analysis. Errors are standard deviations based on two to six individual measurements and for each experiment 1000-10,000 cells were measured.

with cell,^{40, 41} further explaining their lower labeling efficiency. This was tested by the addition of 20 mol% of positively charged 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) lipids in the 20 mol% DOPE-desbiotin vesicles that indeed increased the vesicle uptake by the cells as seen by an increased fluorescence related to a fluorescent lipid that was used in this measurement (not shown). Still, no significant SAV-APC binding was observed in this case, further suggesting that DOPE-desbiotin is not suitable to bind streptavidin to cells.

Further analysis of this system will be aimed at investigating the reversibility of the streptavidin binding to the Chol-EG₃-biotin functionalized cells, in the presence of β -cyclodextrin. To optimize the desbiotin system, the same, longer, spacer as in the Chol-system will be used. Finally, the ability of this system to functionalize and reversibly isolate (tumor derived) extracellular vesicles (tdEVs) will be evaluated, which would be of great interest for medical diagnostics to identify the presence of tumor-related genes.

7.5. Experimental section

7.5.1. General methods

Chemicals were purchased from Sigma Aldrich or from Acros Organics. SAV-APC was obtained from Bioconnect. aCD45-PE (clone H130) was obtained from Biolegend. aCD61-PE (VIPL2 clone) was obtained from Exbio. Hoechst (33342) was obtained from Thermo Fischer Scientific. Protected amino acids were obtained from MultiSynTech except for Fmoc-lys(biotin)OH and Fmoc-NH-(PEG)₂-COOH which were obtained from MERCK. Resin for solid phase peptide synthesis was obtained from Novabiochem. Commercial lipids were obtained from Avanti Polar Lipids unless stated otherwise. HEPES buffer contained 0.01 M HEPES and 0.15 M sodium chloride was made using Milli-Q water (MQ, Millipore, 18.2 mΩ) and adjusted to pH 7.4 at 25 °C using sodium hydroxide.

Mass spectra were recorded with a Waters electrospray ionization time of flight mass spectrometer operated in positive ion mode (ESI(+)-ToF, Micromass LCT). High performance liquid chromatography (HPLC) was performed using a C18 reverse phase column (Waters, Xbridge). Dynamic light scattering (DLS) analysis was carried out on an Anaspec particle analyser (Nanotrac operating with a Microtrac FLEX Operating Software). Zeta (ζ) potential measurements of the lipid vesicles were performed at 20 °C with a ZetaSizer instrument (NanoZS, Malvern). QCM-D measurements were performed using a Qsense Analyser (Biolin Scientific). Measurements were performed at 22 °C and operated with 4 parallel flow chambers, using 2 Ismatec peristaltic pumps with a flow rate of 100 μ l/min, unless stated otherwise. The fifth overtone of the normalized frequency (ΔF_5) and dissipation (ΔD_5) is presented. SiO₂ coated sensors (QSX303, Biolin Scientific) were used. Flow cytometry was carried out on a BD bioscience flow cytometer (FACS Aria II) equipped with a 375 nm, a 488 nm and a 633 nm laser. For Hoechst excitation, the 375 nm laser was used and fluorescence was detected with a 450/40 nm emission filter on a photomultiplier. For PE excitation, a 488 nm laser was used with a 585/42 nm emission filter. For APC excitation, a 633 nm laser was used with a 660/20 nm emission filter. For confocal microscopy, a Nikon confocal (A1) microscope was used, equipped with a 488 nm laser with a 525/50 nm emission filter and with a 561 nm laser with a 595/50 nm emission filter. In microscopy images displayed in this work, contrast and brightness were adapted for clarity using ImageJ. UV/ozone treatment was performed using a Bioforce chamber (Nanosciences). FRAP curves were fitted with the modified Bessel functions as described by Soumpasis

*et al.*⁴² FRAPAnalyser (University of Luxembourg) was used for the fitting to determine the diffusion constant and the degree of recovery. YFP protein constructs featuring an N-terminal tryptophan or methionine were obtained as described in the full publication.¹⁰

7.5.2. Synthesis procedures

DOPE-desbiotin (Figure 7.8). 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE, 24 mg, 0.32 μ mol) was dissolved in 1 ml chloroform and methanol in a 3:1 ratio. To this solution, *N*-hydroxysuccinimide-desthiobiotin (NHS-dtbiotin, 10 mg, 0.32 μ mol) and *N,N*-diisopropylethylamine (DIPEA, 6.7 μ l, 0.39 μ mol) were added under argon and stirred overnight at room temperature. The solution was then diluted with chloroform and extracted with 5% aqueous ammonia. The aqueous layer was extracted again with chloroform and the combined layers were concentrated. Further purification using silica gel chromatography (1% aqueous ammonia, 10% methanol in dichloromethane) gave, after freeze drying, pure product as white powder. Yield: 8 mg (27%); ¹H-NMR (400 MHz, CDCl₃, Figure 7.9): δ = 0.81 (t, 6H, CH₃CH₂), 1.10 (d, 3H, CH₃CH) 1.2-1.82 (m, 52H, CH₃CH₂CH₂ + CH₂CH₂CH₂ + NHCHCH₂) 1.93 (m, 8H, CHCHCH₂) 2.24 (m, 6H, OCOCH₂) 3.42-3.75 (m, 4H, NHCH₂CH₂ + NHCH) 3.92-4.32 (m, 7H, POCH₂ + CHCH₂OP + CHCH₂OP + OCOCH₂CH) 5.16-5.27 (m, 4H, CHCH), 7.6 (bs, 1H, CH₂NHCO), 8.21 (bs, 2H, CHNHCO). ESI(+)-ToF MS (Figure 7.10): [M+2H]⁺ = 940.6486 (calc. 940.6750), [M+H+Na]⁺ = 962.6315 (calc. 962.6569), [M+2Na]⁺ = 984.6144 (calc. 984.6388).

Figure 7.8 | Synthesis route for the synthesis of DOPE-desbiotin.

Figure 7.9 | ^1H -NMR of DOPE-desbiotin.

Figure 7.10 | ESI(+)-ToF mass spectrum of DOPE-desbiotin. Reserpine was used as internal reference.

Chol-EG₃-biotin (Figure 7.11). Chol-EG₃-biotin was synthesized using automated solid phase peptide synthesis (Syro II, MultiSynTech) following standard Fmoc procedures on a Rink amide MBHA resin (100-200 mesh, loading 0.52 mmol/g) using HBTU, HOBT and DIPEA. The oligo ethylene glycol linker was introduced using Fmoc-NH-(PEG)₂-COOH (MERCK) and biotin was introduced using Fmoc-lys(biotin)OH (MERCK). Cholesterol hemisuccinate (Chol) was coupled manually in the last step using PyBOP, HOBT and DIPEA. Cleavage and deprotection of the peptide were performed by adding to the resin a solution of 2.5vol% water and 2.5vol% triisopropylsilane in trifluoroacetic acid (TFA) and stirring for 4h. Purification was performed by repeated (3x) precipitation in cold diethyl ether and subsequent preparative HPLC using an XBridge C18 reverse phase column. The HPLC program consisted of 10 min flow with 1% acetonitrile in water and then a 40 min gradient up to 100% acetonitrile, always in presence of 0.1% TFA and using a 7 ml/min flow rate. The functionalized peptide was characterized using ESI(+)-ToF (Figure 7.12).

Figure 7.11 | Solid phase synthesis route for the synthesis of Chol-EG₃-biotin.

Figure 7.12 | ESI(+)-ToF mass spectrum of Chol-EG₃-biotin.

7.5.3. Fabrication of vesicles and SLBs

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl), (biotin-PE), tail modified lipid dye conjugate 1-palmitoyl-2-(dipyrrometheneboron difluoride)undecanoyl-sn-glycero-3-phosphoethanolamine (TopFluorPE) as well as the synthesized lipids DOPE-desbiotin and chol-EG₃-biotin were stored in chloroform at -20°C. Dissolved lipids were mixed in desired molar ratios before use and dried under a flow of nitrogen in a glass vial in order to create a film of lipid material at the glass wall. This film was further dried under vacuum for at least 1 h and subsequently hydrated by vortexing with Milli-Q water to form multilamellar vesicles at 1 mg/mL. The lipid suspension was extruded 11 times through a polycarbonate membrane (Whatman) with 100 nm pore size, resulting in large unilamellar vesicles (LUVs) that were stored in the fridge and used within two weeks. For supported lipid bilayer (SLB) fabrication, vesicles were diluted to a concentration of 0.1 mg/ml in HEPES directly before use. SLB formation was achieved by flowing this solution on a cleaned and activated surface. For flat QCM-D sensors or glass bottom well plates, cleaning was performed using a 2 wt% sodium

dodecyl sulphate (SDS) solution and thorough rinsing with Milli-Q. Activation was performed using a 30 minute UV/ozone treatment (for QCM-D sensors) or overnight incubation in 2% Hellmanex and again thorough Milli-Q rinsing (for well plates). The quality of SLBs was monitored by fluorescence recovery after photobleaching (FRAP) or in-situ by QCM-D (where high quality SLB defined as $\Delta F = -24 \text{ Hz} \pm 1 \text{ Hz}$ and $\Delta D < 0.5 \times 10^{-6}$). After SLB formation, care was taken to keep the surface submerged in buffer and without bubbles.

7.5.4. Cell culture

Cells from the breast cancer cell line MCF-7 breast were obtained from the American Type Culture Collection ATCC (Rockville, MD). The cell line was cultured at 37 °C and 5% CO₂ in Dulbecco's modified Eagle medium, RPMI 1640 with L-glutamine (Thermo Fischer Scientific, 11875) supplemented with 10% v/v fetal bovine serum, 10 units/mL penicillin and 10 µg/ml streptomycin. The medium was refreshed every second day. The initial cell density was 10-15,000 cells/cm², as recommended by ATCC and their culture lasted until they reached 80% confluence, when they were trypsinized and passaged.

7.5.5. Blood samples

Blood samples from anonymous healthy donors were collected in EDTA blood draw tubes. The donors provided informed consent prior to blood donation, in accordance to the study protocol approved by the METC Twente ethics committee.

7.5.6. Leukocyte isolation

Two different ways were used for the isolation of white blood cells from whole blood, namely density gradient separation using Ficoll-Paque Plus (GE Healthcare) and red blood cell lysis using ammonium chloride. The protocol suggested from the manufacturer of the Ficoll-Paque Plus was followed for the isolation of lymphocytes from whole blood. In case of the red blood cell lysis, blood was diluted 20x in filtered with 0.2 µm freshly made ammonium chloride (155 mM ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM ethylene dinitrilo tetraacetic acid disodium salt, pH=7.25) and incubated in ice for 20 min until full red blood cell lysis. Remaining white blood cells were centrifuged at 300xg for 10 min, the pellet was washed twice with filtered PBS with 1% BSA and cells were resuspended in PBS with 1% BSA of the same volume as the whole blood volume used. The two protocols were compared in regards

to their effect on the fusion of the differently isolated cells with the vesicles and no differences were observed.

7.5.7. Isolation of platelets

EDTA blood was centrifuged at 300 xg for 10 min without brake and platelet-rich plasma was collected without disturbing the buffy coat. Platelet-rich plasma was centrifuged at 1730 xg for 7 min, the supernatant was discarded and the pellet containing most of the platelets was collected and resuspended in 0.2 μ m filtered PBS with 1% BSA for further use.

7.5.8. Fusion of (des)biotin vesicles with cells and staining procedures

Different cell types were investigated regarding their fusion with the different vesicle systems. In the case of cancer cell lines (MCF7) and white blood cells, 9 aliquot of 150,000 - 200,000 cells were pipetted in properly labeled FACs tubes. In the case of platelets, 500,000 cells were pipetted. Cells were centrifuged at the correct speed (500xg for 5 min in case of cell lines and white blood cells and 1730 xg for 7 min in case of platelets) and their pellets were resuspended in 180 μ l of 0.2 μ m filtered PBS with 1% BSA. 20 μ l of 1 mg/ml vesicles was added to the correct samples to a final vesicle concentration of 0.1 mg/ml following 1 h incubation at RT. Cells were washed once with PBS with 1% BSA and stained for 30 min at RT with staining solution (CD45-PE, CD61-PE and EpCAM-PE) and SAV-APC. One more washing step was followed and finally, cells were stained with 4 μ g/ml of the nuclear dye Hoechst for 10 min at 37 °C and kept afterwards on ice until measurement with flow cytometry on the same day.

7.6. References

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Summary

With the aim to improve our understanding of biological interactions involving viruses, vesicles and cells, it is worthwhile to reconstitute them on surfaces. This can facilitate the incorporation of such biological entities in bioanalytical and medical diagnostic devices. Examples of such applications range from organ-on-a-chip to sensing devices for pathogens, viruses, protein-protein interactions, etc.

Although much progress has been achieved in the reconstitution of biological interactions on surfaces, it remains a formidable task to additionally incorporate the dynamicity that is found in biological systems in the natural context, for example in (bacterial) cell adhesion, in virus infection and in laterally mobile cell membranes. In such systems, dynamic behaviour often goes hand in hand with multivalent interactions, together providing control over affinity and kinetics.

In this thesis, artificial cell membranes in the form of supported lipid bilayers (SLBs) were used to reconstitute biological interactions involving dynamic behavior. In addition to the intrinsic lateral dynamics of biological ligands that can be presented at these SLBs, out-of-plane dynamics of the biological ligands was controlled and studied by the choice of the interaction motifs and/or by tuning the overall valency. To this end, various types of supramolecular interactions were used. Host-guest chemistry was used to bind cells and proteins to surfaces. Tunable surface affinity was achieved by insertion of bioactive peptides with various lipid chains to SLBs and employed to study stem cell adhesion and spreading. The number of metal-ligand interactions between lipid vesicles and the SLB determined the extent of vesicle binding and provided a threshold density for vesicle binding. Finally, carbohydrate-protein interactions were reconstituted on SLBs and used to study virus binding in more detail.

Learning from nature to combine dynamics and multivalency is not a straightforward endeavor; however, each of the studies in this thesis can potentially be used to improve the functionality and thus the performance of devices that rely on biological interactions.

Samenvatting

Met het doel voor ogen om biologische interacties, zoals die van virussen, vesicles en cellen, beter te begrijpen, is het nuttig om deze te kunnen reconstrueren op oppervlaktes. Dit kan de opname van dergelijke biologische entiteiten in analytische en medisch-diagnostische apparaten faciliteren. Voorbeelden van zulke toepassingen variëren van orgaan-op-chip technieken tot apparaten voor de detectie van pathogenen, virussen, eiwit-eiwit interacties, etc.

Ondanks dat er veel vooruitgang is geboekt in de reconstructie van biologische interacties op oppervlaktes, blijft het een grote uitdaging om daarin ook het dynamisch gedrag na te bootsen dat essentieel is in biologische systemen in een natuurlijke context, bijvoorbeeld in (bacteriële) celadhesie, virus infectie en lateraal mobiele celmembranen. In dit soort systemen gaat dynamisch gedrag vaak hand in hand met multivalente interacties. Samen verschaft dit controle over de affiniteit en kinetiek van de interacties.

In dit proefschrift zijn artificiële celmembranen, in de vorm van ondersteunde lipide dubbellagen (OLDs), gebruikt om biologische interacties, waarbij dynamisch gedrag een belangrijke rol speelt, te reconstitueren. Naast het lateraal dynamische karakter dat biologische liganden in OLDs kunnen vertonen, werd ook het dynamisch gedrag van zulke liganden uit het vlak gereguleerd en bestudeerd door middel van variaties in het interactiemotief en/of door het bepalen van de multivalentie. Daartoe zijn verschillende typen supramoleculaire interacties gebruikt. Host-guest chemie werd bijvoorbeeld gebruikt om cellen en eiwitten aan oppervlaktes te binden. In een ander voorbeeld, werd controle over de affiniteit van liganden voor het oppervlak bereikt door het inbrengen van bioactieve peptiden met verschillende lipide ketens in OLDs. Dit type oppervlakken werd toegepast om stamceladhesie en -spreiding te bestuderen. Een volgend voorbeeld was het variëren van het aantal metaal-ligand interacties tussen lipide vesicles en OLDs om inzicht te verschaffen in de drempelwaarde van de receptordichtheid voor het binden van vesicles. Tot slot werden koolhydraat-eiwit interacties gereconstitueerd op OLDs en gebruikt om virusbinding te bestuderen.

Het leren om dynamisch gedrag en multivalentie te combineren zoals dat gebeurt in biologische systemen, is geen eenvoudige uitdaging. Echter, de studies beschreven in dit proefschrift kunnen potentieel gebruikt worden om de functionaliteit en prestatie te verbeteren van apparatuur gebaseerd op zulke biologische interacties.

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