Supramolecular surfaces for cell adhesion

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Chapter 1

Supramolecular surfaces for biodevices

Supramolecular chemistry offers diverse opportunities for the fabrication and improvement of biodevices. Starting with various examples of supramolecular self-assembly in solution, this chapter continues by setting the base for the experimental chapters making use of RGD peptides. Applications in supramolecular chemistry can be pursued both on surfaces and in solution, for example via the functionalization of biomolecules (peptides, proteins, DNA, etc) with supramolecular tags for selective and reversible binding, immobilization and orientation. The targeted fields of application range from diagnostics for the detection and quantification of biomarkers to biomaterials that mediate controlled interactions with eukaryotic cells or bacteria, hence, promoting the subsequent development of these biomaterials towards biosensors or implants. Thus, supramolecular concepts such as host-guest interactions offer dynamic platforms and a high versatility. The dynamicity and reversibility of supramolecular host-guest assemblies open the door to acquiring self-healing materials.

1. Introduction

1.1. Smart biomaterials

Biomaterials that allow for specific control of cellular adhesion are desired in biomaterial engineering. A specific ligand, such as a peptide or a protein, can be immobilized on the material to enhance its biocompatibility, performances or specificity. Conventional methods include covalent attachment via polymers or direct reaction with a surface, but lack versatility. On the other hand, non-covalent approaches such as via supramolecular chemistry introduce dynamicity and potential reversibility to the ligand at the surface (Figure 1.1). The ability to control the presence and behavior of the ligand via a simple switching mechanism is attractive, while the dynamic character of the interaction is reminiscent of non-covalent interactions found in nature such as the assembly of actin or tubulin network in the cell.

![Figure 1.1 Scheme depicting the desired elements for the design of smart biomaterials: a dynamic surface responsive to external trigger to control the ligand at the surface, and markers promoting a specific action.](image)

New developments in smart biomaterials are occurring rapidly, of which only a handful of selected examples are described below. Biomaterial design frequently includes elements to prevent adhesion at their surface. Introduced in the body, within seconds proteins will start to adsorb at the surface of the material and potentially trigger cascade responses within the body which ultimately lead to the materials rejection. Coating of surfaces with poly(ethylene glycol), superamphiphobic fluorinated based or bioinert polymers can help circumvent such issues. Alternatively, materials displaying specific biomolecules such as peptides, proteins or DNA are also of interest in the biomedical field, as these can selectively recruit the desired cellular environment to the biomaterial. Coatings promoting cellular adhesion and integration are used for example in hip or knee prosthesis with the aim being to avoid painful and costly replacement surgeries by increasing the lifetime of the prosthesis.
Coatings with antimicrobial properties are also of utmost interest in the biomaterials field. Microorganisms form biofilms at the surface of many materials and thus increase their resistance to antibiotics. As such preventing bacterial adhesion in the first place is crucial. Polymers complexing silver, an efficient antimicrobial heavy metal, or functionalized with antibiotics such as vancomycin are two new approaches in use to prevent bacterial adhesion and the formation of a biofilm on the surface.

Unlike coating that can be applied on a large variety of materials, hydrogels are a specific subtype of biomaterial and are composed of hydrophilic polymers capable of retaining large amounts of water via hydrogen bonding. The mechanical properties, such as its stiffness or elasticity, can be modulated by changing the composition of the polymer structure. Applications of hydrogels include tissue engineering, contact lenses or biodegradable drug delivery system. A next level of control is gained through self-healing polymeric gels. These materials utilize supramolecular non-covalent interactions, e.g. hydrogen bonding or host-guest chemistry between the polymeric chains (Figure 1.2), to adapt to the changing environmental conditions. These materials respond to changes in pH, temperature or light. One example shown in figure 1.2 makes use of two polymers substituted with methylviologen or naphthalene guests, capable of forming a ternary complex upon addition of cucurbit[8]uril, thereby crosslinking the polymer chains and increasing the stability and mechanical properties of the material.

Figure 1.2. Example of polymers modified with supramolecular elements for cross-linking. Adapted with permission from reference 11.

Trigger-responsive biomaterials, in particular to electrochemical stimuli, can induce cellular responses in certain cells types, such as in neurons, osteoblasts or cardiomyocytes. Polyaniline is a conducting and biocompatible polymer used in photovoltaic cells and light emitting diodes, but also more recently in biosensors and to stimulate DNA and protein
secretion (Figure 1.3 left).\textsuperscript{17} The addition of end groups can make the material biodegradable and improve its biocompatibility. Fibroblasts are a type of cells responding to small current (20 mA), which favors the formation of actin filaments in the cells. A stronger current (200 mA) has the opposite effect and in this case cells don’t develop (Figure 1.3 right).

\textbf{Figure 1.3.} (left) Different oxidation states of polyaniline. A: completely reduced base, B: completely oxidized base. (right) Culture of NIH-3T3 fibroblasts on nanofiber scaffolds of polyaniline and poly(L-lactide-co-\(\varepsilon\)-caprolactone) for 2 days under electrical stimulation of 20 mA (a), and 200 mA (b). Fluorescence micrograph on the left shows F-actin filament formation in the cells, which is absent in cells stimulated at a higher current value. Adapted with permission from references 17 and 18.

\subsection*{1.2. Molecular self-assembly mimicking nature}

Ever since the discovery of crown ethers in the 1960s, and the subsequent Nobel Prize awarded to Cram, Lehn and Pedersen in 1987 for their work on cryptands and crown ethers, a significant effort has been invested in designing and capitalizing on supramolecular systems. While at first this effort was more from the viewpoint of material sciences,\textsuperscript{19} nowadays, the focus has shifted towards biomedical applications.\textsuperscript{20} When combined, the fields of materials and biomedical sciences generate new and exciting opportunities for developing biodevices based on supramolecular functionality.

Supramolecular chemistry comprises chemical systems composed of a certain number of assembled molecules, interacting reversibly and non-covalently.\textsuperscript{21,22} Molecules can for example interact via hydrophobic interactions, be stabilized by charge interactions or self-assemble via e.g. \(\pi\)-\(\pi\) stacking, resulting in dynamic rods that can be functionalized.\textsuperscript{23} These molecular characteristics are often found in nature: dynamic, modular, complex and large systems such as the organization of a cell or complex RNA-protein assemblies like the ribosome. Importantly, all these biological supramolecular systems function in aqueous environments. Applications of synthetic supramolecular systems at the interface with biology similarly require compatibility with the physiological environment and can be pursued both at surfaces as well as in solution.
Interactions in nature in general and in the cell in particular are mostly of a non-covalent nature. From a set of building blocks such as amino acids, sugars, lipids and minerals, nature has evolved and self-assembled these elements to create life and complex structures. In particular, the assembly of identical molecules or monomers results in structures that can be a few orders of magnitude larger than the monomer and possesses different functions and properties. Actin monomers can assemble into microfilaments that compose in part the cytoskeleton of cells and are involved in cell migration and cell contraction during cell division.\(^2^4\)\(\alpha\) and \(\beta\) tubulins form microtubules that play a role in intracellular transport together with kinesin and dynein, and during mitosis. Actin and tubulin are globular proteins, water soluble as monomer but forming long and stiff fibers upon self-assembling.\(^2^5\) When the monomers undergo modifications, dramatic consequences such as impossible fiber formation or excessive cell proliferation take place, resulting in diseases like Parkinson or Alzheimer. Control over the assembly of the monomer and over their integrity is therefore of utmost importance.

The assembly and disassembly mechanisms found in nature are a source of inspiration for chemists. Self-assembly of peptides amphiphiles to form fibers in water have been reported by Stupp and co-workers.\(^2^6\) Aggregation in nanotubular structures, thanks to the hydrophobic tail linked to the peptides, enable micrometer size structure formation that can find application in medicine (Figure 1.4).\(^2^0,2^7,2^8\) Indeed, the peptide sequence can be chosen for a specific goal. IKVAV peptide sequence from laminin was studied and showed a thousand-fold increase in ligand density compared to laminin itself, as well as reduced cell death. Furthermore, these effects were only evident with the peptide amphiphile structure, and not the peptide sequence by itself.\(^2^8\)

![Figure 1.4. Peptide amphiphile design and self-assembly. A hydrophobic tail (1) is attached to four cysteines (2) that play a role in covalent locking of the self-assembled structure under oxidizing conditions. A RGD peptide (5) ensures cell adhesion function. Reproduced with permission from reference 26.](image)

Synthetic systems such as benzene-1,3,5-tricarboxamide (BTA) have been extensively studied.\(^2^9\) Thanks to its hydrophobic core and amide group available for hydrogen bonding,
rod-like structures can be formed in various solvents depending on the side chain substituent. Chiral substituents enable control over the conformation of the helical structure – left or right handed – in the same way as the polarity of the solvent.\textsuperscript{30–32} Substitution by ethylene glycol chains makes the rod water soluble.\textsuperscript{33} They can also be substituted with gadolinium (III) ions and act as supramolecular MRI contrast agent,\textsuperscript{34} or with Cy3 and Cy5 dyes to study the mechanism of assembly and exchange between rods in water.\textsuperscript{35}

The bis-urea motif has been widely used in different molecular structures. Steed and coworkers used urea – C4 – urea or urea – C6 – urea motifs that pair with H-bonding to form gels in various solvents.\textsuperscript{36,37} Formation of hydrogels in water can be challenging, but the addition of hydrophobic units protects the urea moiety and allows the self-assembly in polar solvents, and in particular in water (Figure 1.5). The 2,4-bis(2-ethylhexyl-ureido)toluene (EHUT) motif was reported by Bouteiller and coworkers. In a similar manner, the hydrophobic alkane chains and toluene unit allow the H-bonding to take place while the PEG tail improves the solubility of the molecule in polar solvents (Figure 1.5).\textsuperscript{38}

Cucurbit[n]urils (CB[n]) and cyclodextrins (CD) are two representative classes of host molecules that are frequently employed to form supramolecular systems. By forming inclusion complexes with hydrophobic guests, such as ferrocenes,\textsuperscript{39} azobenzenes\textsuperscript{40} or methylviologen\textsuperscript{41} in aqueous environment, various binding affinities can be covered to tailor specific applications. Depending on the size of the cavity, one or two guests can be accommodated inside the host, sometimes in a controllable manner allowing responsive switching using electrochemical or optical stimuli.\textsuperscript{42,43}

![Figure 1.5. (left) Bis-urea structures described by Steed and coworkers. (right) Gels of alanine-derived gelator in various solvents (water, acetonitrile, THF, chloroform, ethyl acetate and toluene). (bottom) Bis-urea structure reported by Bouteiller and coworkers. The design allows for a solubility in a wide range of solvents. Adapted with permission from references 36 and 38.](image-url)
Polymers relying on host-guest chemistry can also be prepared. Cyclodextrin-based polymers have been reported by Bouteiller and Harada. The first group reported the formation of a supramolecular 2D-assembly based on the interaction between cyclodextrins modified with two azide groups (Figure 1.6 left). Strands are formed via primary interactions (azide acting as a guest within the cyclodextrin cavity by hydrophobic inclusion) and via secondary and tertiary interactions, either within the strand itself via azide-azide dipole-dipole interaction or between the strands via azide-hydrogen bonding array. However, this equilibrium is subtle and the design of the component is crucial: an overly rigid linker might result in insolubility of the components, while an overly flexible linker would favor the formation of intramolecular host-guest assembly over intermolecular assembly desired in the case of polymer formation. Harada and coworkers make use of the interaction between beta-cyclodextrin and adamantyl poly(acrylamide) functionalized polymers to visualize the self-assembly at the macroscopic scale. Small pieces will assemble in water with each other and the multivalency character of the interaction allows for a self-assembled material that can resist gravity (Figure 1.6 right). Simultaneous self-sorting of two different host-guests pair was also achieved.

Figure 1.6. (left) 2D assembly of cyclodextrin-azide modified monomer. Adapted with permission from 45. (right) Self-assembly recognition of polymer functionalized with host (αCD or βCD) or guest (adamantyl, n-Bu or t-Bu). Adapted with permission from 47.

For the design of substrates promoting cell adhesion, well defined surface orientation of the protein or peptide is required. For this goal, host-guest chemistry appears to be an ideal candidate to provide the orientational display of the bioactive functionalities (Figure 1.7). Surfaces supramolecularly coated with an active peptide can e.g. promote cell adhesion, and depending on the host-guest system chosen, the controlled release of cells from such surfaces. Proteins can also be specifically immobilized on cyclodextrin-coated surfaces via polymer coating or direct surface functionalization. Surfaces modified with peptides and protein in a supramolecular fashion are described extensively in section 3 of this chapter.
Figure 1.7. Overview of the most applied supramolecular host-guest systems in biodevices and their respective dissociation constants.39,41,55–58

1.3. Covalent vs. Supramolecular approaches

Immobilization of biomolecules is of great interest for a number of applications such as biosensors, medical coatings or biochips. Functionalization of surfaces with RGD peptides (vide infra) promoting cell adhesion can be achieved in several ways: e.g. attachment on gold via thiol bond,59 immobilization on glass via polymer coating and streptavidin-biotin tag60 or polymer functionalization and subsequent polyelectrolyte multilayer membrane coating.61 While these techniques allow for site-specific immobilization of peptides, the same can not be said of protein immobilization due to their large size and the multiple repetitions of the same chemical functionalities, making it near to impossible to address specifically one particular amino-acid.

Adsorption is a random coating method that induces partial loss of activity due to statistical misorientation of the protein on the surface, which obstructs the active site. Covalent attachment is possible via thiol-maleimide62, thiol-methylsulfonyl-functionalized heteroaromatic derivatives63 or reaction of amines of the biomolecules with NHS esters of the surface material.64 However in all these cases, the reactive biomolecule functional side-chains might be located at an active site or patch of the protein. Engineering of an orthogonal functionality is possible but costly and challenging to perform practically.65,66
Conventional covalent approaches are commonly used and are well-established. However, they present major drawbacks: for example, there is no possibility of dynamicity and reversibility. Cellular phenomenons are highly dynamic and therefore a surface presenting dynamic properties could be of great interest to study cell behavior. For example, receptors present at the cell surface such as the EGF receptor or integrins cluster upon binding to their ligand and trigger intracellular responses. Supramolecular surfaces coated with their ligand may allow the tracking of the receptor rearrangement at the surface with fluorescent molecules or adjustment of the surface topologies upon rearrangements in the cellular membrane. A number of different supramolecular surfaces have been functionalized with fluorescent proteins, and coupling of EGFR ligands to fluorescent proteins could allow for clustering tracking at the cellular surface with the appropriate microscopy techniques.

With some of these systems, it is even possible to control the release of the protein from the surface. Electrochemical stimuli reduces Fc into Fc⁺, thereby diminishing its affinity for the βCD cavity, while reduction of methylviologen disrupts the complex with naphtol or tryptophan, releasing it from the CB[8] cavity. Azobenzene undergoes a conformational change from trans to cis upon UV radiation, which prevents its binding into βCD cavity for spatial reasons. The supramolecular approach provides therefore an alternative to approaches based on covalent protein immobilization.

Most of these systems require some form of biological functionalization to be biologically active; therefore this chapter will start by exploring the extra-cellular matrix (ECM) composition as well as a well-known and commonly used peptide sequence known to promote cell adhesion: the RGD sequence. Afterwards, attention will turn on the potential of supramolecular host-guest chemistry in biodevices, with highlights from recent literature on its utilization in controlled non-covalent immobilization of biological components. The targeted applications range from diagnostics to biomaterials and their subsequent development towards biosensors or implants.

2. Extracellular matrix and RGD

2.1. Extra-cellular matrix

The environment of cellular tissues, the extra-cellular matrix (ECM), determines cell growth, adhesion, differentiation and survival. Cells are able to produce and release a variety of molecules via exocytosis, mostly polysaccharides and proteins, to sustain themselves in their environment. Malfunctions and composition modifications in the ECM have been associated with cancer and fibrosis, therefore it plays an essential role in sustaining vital functions in the human body. Glycosaminoglycans are attached to ECM proteins and form proteoglycans that can accommodate growth factors and attract positively charged ions due
to their overall negative charge. Another notable polysaccharide is hyaluronic acid, capable of absorbing large amounts of water, thus helping to keep the ECM hydrated and can be found in interstitial gel and in joints. Characteristic proteins, such as collagen and elastin, form fibers. While collagen is the most abundant protein in the human body, especially in the bone matrix and provides structure to cells, elastin provides elasticity to tissues and thus is found in organs such as lungs, skin and blood vessels. Finally, two proteins assist particularly the cells in their adhesion process: laminin and fibronectin. Laminin forms networks in the basal laminae and help with cell adhesion, through binding to other ECM elements, collagen amongst others (Figure 1.8.). In a similar manner, fibronectin connects the ECM via its collagen fibers to integrin receptors present at the cell surface. In this way, cells interact with the ECM, attach and reorganize their cytoskeleton allowing migration to occur.

![Figure 1.8. Schematic of the vascular basement membrane, showing interactions between collagen, laminin, fibronectin and integrins at the surface of endothelial cells. These elements interact with each other via non-covalent interactions to form a functional interface. Adapted with permission from reference 82.](image)

### 2.2. Integrins

Integrins are cell transmembrane receptors that interact with the ECM. They play a critical role in the cell interaction with its environment and between cells themselves. Processes such as cell proliferation and differentiation, wound healing, cell-matrix adhesion and immune system response depend on these interactions. More specifically, they are critical for cell adhesion and migration via the formation of focal adhesion at the location where they interact with the ECM.

These receptors consist of α and β subunits, which form heterodimers. Together, 18 α and 8 β known subunits form 24 different integrins, which determines their ligand binding
specificity (Figure 1.9). Fibronectin binds to half of the known integrins (Figure 1.9) and is therefore involved in many processes, especially in cell adhesion and migration.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Integrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus penton base protein</td>
<td>α₂β₃, α₅β₁</td>
</tr>
<tr>
<td>Bone sialoprotein</td>
<td>α₁β₁, α₅β₁</td>
</tr>
<tr>
<td>Collagens</td>
<td>α₁β₁, α₂β₁, α₃β₁, α₂β₂, α₁β₄</td>
</tr>
<tr>
<td>Denatured collagen</td>
<td>α₁β₁, α₂β₁, α₅β₃</td>
</tr>
<tr>
<td>Cytotactin/tenascin-C</td>
<td>α₂β₁, α₃β₁, α₂β₂, α₂β₃</td>
</tr>
<tr>
<td>Disintegrins</td>
<td>α₃β₁, α₂β₂, α₇β₁</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>α₂β₁, α₅β₁</td>
</tr>
<tr>
<td>HIV Tat protein</td>
<td>α₁β₁, α₅β₁</td>
</tr>
<tr>
<td>Invasin</td>
<td>α₂β₁, α₃β₁, α₂β₂, α₁β₃</td>
</tr>
<tr>
<td>Laminin</td>
<td>α₂β₁, α₃β₁, α₂β₂, α₁β₃</td>
</tr>
<tr>
<td>Prothrombin</td>
<td>α₂β₁, α₅β₁</td>
</tr>
<tr>
<td>Sperm fertilin</td>
<td>α₂β₁, α₅β₁</td>
</tr>
<tr>
<td>Thrombospondin</td>
<td>α₂β₁, α₅β₁</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>α₂β₁, α₅β₁</td>
</tr>
<tr>
<td>von Willebrand factor</td>
<td>α₂β₁, α₅β₁</td>
</tr>
</tbody>
</table>

Figure 1.9. Overview of integrins α₂β₃ and α₅β₁ extracellular ligands.

2.3. From fibronectin to RGD

Fibronectin is a large protein active as an antiparallel dimer stabilized by disulfide bridges located near the C-terminus of the protein. It can be found both in the plasma as a soluble fraction and in the ECM as an insoluble fraction. Coded by a single gene, alternative splicing of the mRNA results in over 20 different forms of fibronectin. Each monomer is circa 220 to 250 kDa and homologous in structure, composed of three repeats, type I, II and III (Figure 1.10).

The discovery of the RGD sequence in 1984 by Pierschbacher and Ruoslahti has been groundbreaking for the creation of biomaterials, allowing the coating of materials with a tripeptide instead of the full length FN proteins, which is difficult to isolate, express or manipulate in sterile conditions without being degraded or subject to proteolysis. The RGD motif is located in the type III domain, the structure of which has been elucidated by NMR. More specifically, RGD is positioned between β-strands, engendering a more rigid and oriented structure to the solvent exposed loop region, thus favoring binding to integrins (Figure 1.11 left). The arginine and aspartic acid residues of RGD form a charge clamp, which interacts with two Asp on the α subunit of the integrin for Arg and with a metal cation located in the β subunit for Asp. Furthermore, the Gly is located close to the integrin surface. Extra hydrogen bonds are formed between the backbone of the ligand and the
integrin (NH of Asp with carboxy group of (β)-Arg 261 and Asp side chain with (β)-Gln 215) (Figure 1.11 right).

Figure 1.10. Structure of the disulfide cross-linked fibronectin (FN) dimer and location of key binding sites. The synergy site and the RGD-loop are located on the 9th and 10th type III modules, FN-III 9 and FN-III 10, respectively. Adapted with permission from reference 89.

Figure 1.11. (left) Ribbon diagram of the FN7–10 structure in the type III domain is shown. The RGD loop (residues 1493–1497) is shown in red. Adapted with permission from reference 93. (right) crystal structure of c(RGDf[NMe]V) in αvβ3. Adapted with permission from reference 86.

In fibronectin, the GRGDSP sequence was found to promote cell adhesion, with the glycine residue functioning as a linker and serine increasing the binding (affinity/selectivity) to the receptor. Flanking amino acids modify the conformation and thus the binding to integrins. For example, the serine can be replaced by an alanine, a valine, a cysteine or a threonine without loss of activity. But replacing it with a lysine residue kills the activity.

Other proteins display the RGD motif, but don’t display cell adhesion properties: the binding sequence might not be accessible or in a correct conformation for optimal integrin recognition. The conformation of RGD influences its binding selectivity. Cyclic RGD peptides compared to linear RGD peptide display a higher affinity for integrin and an increased selectivity. The reason for this is that cyclization constrains the conformation of the peptide to the correct folding, as we can imagine looking at the structure of the RGD loop in fibronectin (Figure 1.11. left). Furthermore, a frequent cyclic RGD peptide used in research and in this thesis, c(RGDFk), is further stabilized by hydrophobic interaction.
between D-Phe and the protein, while the last amino acid lysine has no effect or interaction.\textsuperscript{86,102}

Animal studies where the RGD sequence has been mutated out showed a loss of activity and a potentially lethal phenotype. For example, mutation of the RGD sequence into RGE sequence in mice resulted in death after 10 embryonic days,\textsuperscript{103} though without perturbing the formation of the fibronectin matrix.\textsuperscript{104}

We have seen that RGD sits tightly in the binding pocket on the integrin. Even small modification in the amino acid residues typically result in a loss of activity and can thus be used as negative controls for cell experiments. The RAD sequence, which adds a methyl group to the glycine, is commonly used as a negative control.\textsuperscript{98,105} RGE (extra carbon in the side-chain) results in the same effect, as well as the reverse sequence DGR.\textsuperscript{98} The ligand, maintained too far from the binding interface of the integrin due to the extra methyl group, is then not properly positioned.

Examples of covalent attachment of RGD on surface to promote cell adhesion are numerous,\textsuperscript{97,101,106} however this attachment is non-reversible. Supramolecular chemistry allows for a dynamic immobilization with potential for controlled reversibility to manipulate cell adhesion and migration. Furthermore, the dynamicity is one of the characteristics of the ECM and might influence the way cells sense the ligand at the surface, and therefore their response and behavior.

3. Supramolecular chemistry in biodevices

3.1. Protein biochips

Protein biochips are of great interest for the detection of protein-protein binding events or drug profiling.\textsuperscript{73} Such protein biochips allow for the use of minute amounts of expensive compounds. However, often multiple steps are necessary to immobilize the proteins, while control over orientation is challenging. This limits processability and may result in protein denaturation or other unwanted effects. Hence, to immobilize proteins it is crucial to prevent the loss of protein functionality upon surface attachment. Efficient protein immobilization requires attachment at a non-active site, and oriented immobilization on the substrate in such a manner that the functional binding site of the protein remains accessible. Covalent approaches for protein immobilization include coupling via carbodiimide chemistry, but this typically results in random orientation of the protein at the surface and potential loss of biological activity. Several non-covalent approaches have been developed, including via the streptavidin-biotin interaction, NiNTA-His\textsubscript{6} tag\textsuperscript{107} or DNA-protein interactions.\textsuperscript{108} However these methods are typically sensitive to small temperature or pH changes, are sometimes lacking in specificity, or do not allow reversible switching.
Supramolecular protein tagging and protein immobilization via host-guest chemistry potentially overcome these limitations.

A typical example of host-guest mediated protein immobilization was shown using glucose oxidase (Gox), which was randomly labeled with ferrocene as supramolecular guest and immobilized on CB[7] modified surfaces (Figure 1.12 a). The proteins retain sufficient activity to perform glucose sensing experiments.\textsuperscript{109} The activity of the multiply ferrocenylated enzyme was lowered compared to that of a non-labeled Gox, similar to the covalent protein immobilization techniques based on random amide bond formation between the protein and surface. In this regard, recently developed site-specific protein labeling techniques constitute an interesting alternative to random techniques.\textsuperscript{110} Proteins can be site-selectively labeled using expressed protein ligation between a C-terminal protein thioester of interest and a cysteine labeled supramolecular immobilization tag.\textsuperscript{39,70} For conceptual studies, fluorescent proteins have frequently been used as they are easy to detect using fluorescence spectroscopy and microscopy, and are relatively stable. Upon incubation with a CB[7] monolayer on gold, fluorescent proteins, site-selectively labelled with ferrocene, formed a stable and homogeneous monolayer. Because of the strong interaction between ferrocene and CB[7], a monovalent labeling ensures functional protein immobilization (Figure 1.12 b).\textsuperscript{70}

![Figure 1.12. a) Unspecific vs. b) site-specific labeling of protein with a ferrocene tag for immobilization on a CB[7] monolayer.](image)

For various applications, control over the patterning and total surface area coverage of the immobilized proteins is critical. Microcontact printing of supramolecular protein conjugates allows various patterns to be printed on different surfaces, resulting in microstructures visible by microscopy. Another useful property of these supramolecular protein surfaces is their reversibility. The supramolecular nature of the interaction and the electrochemical responsiveness of ferrocene allow for reversible immobilization of ferrocene tagged proteins on βCD monolayers.\textsuperscript{39} Noteworthy, in this case, a protein tagged with two ferrocene moieties is necessary to obtain a sufficiently high affinity.
Another strategy for protein labeling of interest is to fuse them with a SNAP tag (enzyme alkylguanine transferase) which reacts bioorthogonally with a small O6-benzylguanine tag.68,69 The proteins thus obtained are site-specifically labeled and their orientation at the supramolecular surface can be precisely controlled, ensuring optimal activity.

The larger cavity of CB[8] allows the formation of a ternary complex with two guests, for example with methylviologen and naphthol. The complex can be covalently anchored to a surface via one of the guests. Here, a fluorescent protein was labeled with naphthol and immobilized via the ternary complex on surfaces.111 The complex responded to electrochemical stimuli to release the protein from the surface.

3.2. Biosensors for quantification and detection of biomarkers

Supramolecular surfaces and immobilization techniques provide ample opportunities for improving the sensitivity of biosensors. Ortiz et al. made use of a polymer coating displaying supramolecular βCD units to design a supramolecular ELONA (Enzyme Linked OligoNucleotide Assay). For this, first a βCD covered surface was created, which was used to capture a polymer bearing ferrocene or adamantane (ada) guests and probe DNA. Upon incubation with the target DNA and a Horseradish Peroxidase (HRP) labeled secondary DNA, an optical signal could be detected. The level of detection corresponded to concentrations close to 1 nM of DNA, which is comparable to other DNA capture methods (Figure 1.13).112

![Figure 1.13. Principle of supramolecular ELONA. a) βCD polymer immobilized on surface via maleimide chemistry. b) adamantane or ferrocene-polymer displaying DNA. c) incubation with the target DNA and HRP-labelled secondary DNA probe, for subsequent color development with tetramethylbenzidine (TMB).](image)

Immobilization of enzymes requires mild reagents, buffered conditions, specificity, stability, and a good packing. Moreover, the enzymes should retain their kinetic properties. A supramolecular immobilization approach might exactly fit these criteria. Supramolecular assembly of enzymes on gold surfaces was performed using the βCD/bisadamantane
supramolecular complex as well as the Streptavidin/biotin interaction. The assembly is reversible and permits simultaneous immobilization of multiple enzymes, as well as recovery of the chips/microchannels.\textsuperscript{113} Graphene (GR) displays excellent mechanical properties as well as a high electrical conductivity. Lu et al functionalized graphene with $\beta$CD and immobilized it on a bare glassy carbon (GC) electrode. After immersion in HRP-Ada, a HRP-Ada/CD-GR/GC electrode was obtained, which was used to detect H$_2$O$_2$ levels in real waste-water sample.\textsuperscript{114} The same technique could be used to immobilize redox enzymes for biosensing applications. In an example to selectively immobilize antibodies reported by Ludden et al., $\beta$CD was immobilized in microchannels, and supramolecularly labeled biotin (bisadamantane-biotin) and streptavidin flown subsequently in the microchannels were detected using fluorescence spectroscopy. Via protein A-biotin or protein G-biotin (proteins that bind to antibodies), fluorescently labeled antibodies could be immobilized.\textsuperscript{115} Using the same technique, the same group developed a CD3+ lymphocytes read-out system which was able to provide an estimate on how many lymphocytes are present in a sample. By modifying the molecule present at the surface (different antibody or biomarker), this technique could be generalized to a cell count system.\textsuperscript{116}

Wan et al. prepared an azobenzene self-assembled monolayer (SAM) that allows reversible interaction with poly(acrylic acid)-$\beta$CD polymer (PAA-$\beta$CD). Cyctochrome c (a heme-containing protein), which is positively charged at pH 7.2, could then be immobilized on the negatively charged PAA. Release of cytochrome c was achieved either by decreasing the pH to 4 or upon shining UV-light at the surface. Indeed, the azobenzene structure undergoes a conformational change (trans to cis) upon UV irradiation, resulting in a loss of affinity for $\beta$CD \textsuperscript{117} and release of PAA-$\beta$CD from the surface.

The extraction of membrane proteins is of great interest to understand the behavior of cells. Current techniques include density gradient centrifugation or aqueous-phase partitioning, but these techniques are harsh and as such there is a need for milder methods. Kim et al. labeled plasma membrane proteins with a ferrocene tag. After cell lysis, in the presence of CB[7] modified beads, the labeled proteins were extracted from the membrane and isolated from the bead through competition with a ferrocene guest or upon heating.\textsuperscript{118} This technique allows the capture and the detection of small amounts of membrane proteins in a reversible manner.

\textbf{3.3. Biological coatings for implants}

Small molecule drugs are generally poorly soluble in water. The use of coatings capable of encapsulating them to improve their water solubility via slow release is therefore of great interest for biological applications. The diffusion of active molecules and their kinetics is also a parameter of importance. Layer-by-layer films containing $\beta$CD display such properties
towards several drugs and anti-inflammatory compounds (Figure 1.14). Layer-by-layer films also allow the formation of bifunctional films: close to the surface, a drug release film with βCD as drug carrier, and close to the material, an antimicrobial film for long-term implantation. In this example, even after two weeks, no bacterial film was formed, and cells were able to attach and divide normally. Benkirane-Jessel et al. described the formation of a polypeptide copolymer (poly(L-lysine) and poly(L-glutamic acid)) coating functionalized with a charged βCD, improving the complexation with an anti-inflammatory drug. Depending on the polymer ratio, release of the drug resulting in the diminution of TNFα levels in cells could be observed between 1h and 12h.

Figure 1.14. Formation of a layer-by-layer film (Figure adapted from reference 119 with the permission of Wiley-VCH).

Adhesion of cells to biomaterials is important for the integration of implants or scaffolds in vivo. Bioactive peptides (here promoting cell adhesion) were immobilized via a supramolecular host-guest coating on a surface making use of the strong CB[7] – ferrocene interaction. Endothelial cells formed a monolayer on this coating and a wound in the cell monolayer could recover within hours. Electrochemistry is a non-invasive method to probe the response of cells to stimuli and allows for real-time monitoring. The ternary complex methylviologen - CB[8] - tryptophan was immobilized on surfaces and modified with the bioactive peptide RGD, allowing an electrochemical control over the release of cells cultured on the supramolecular surface. Subcellular resolution was achieved, to release only part of the cell from the substrate.

Polymer coatings are used as biomaterials, for example in non-fouling surfaces based upon poly(ethylene glycol) (PEG). A poly(ε-caprolactone)-βCD coating for vascular grafts was developed by Ji et al. allowing its modification upon incubation with an adamantane
functionalized peptide promoting endothelial progenitor cells (EPC) recruitment. Since proteins adsorb on the coating during the first minutes after implantation, a non-fouling surface is of importance: the authors used PEG for that purpose. Li et al. report the synthesis of an adamantane-terminated polystyrene-b-poly(ethylene oxide) polymer and its non-covalent interactions with βCD peptide conjugates, leading to a controlled density of ligand at the surface, as well as the ability to perform cell culture on these materials. This material enhanced cell spreading in the presence of adhesive peptides such as RGD and IKVAV, though it made no difference in initial cell adhesion.

CB[7] can also be functionalized, allowing a covalent attachment on surfaces. This offers new development possibilities, for example for drug-eluting stent and other types of implants containing small molecules. These sometimes need a particular environment to stabilize the drug. Cao et al. described a CB[7] container to which they covalently attach biotin to improve its targeting towards leukemia cancer cells. Oxaliplatin was encapsulated in the hydrophobic pocket of CB[7], protecting it from degradation reactions. Other hydrophobic small molecules could possibly be encapsulated in the same way.

4. Aim and outline of this thesis

Supramolecular chemistry provides a new line of development for biodevices. Labeling of peptides or proteins with a supramolecular tag at a specifically chosen position ensures that there is no loss of activity, through precise control on their orientation, resulting in high potential for protein biochips and biosensors. Furthermore, the systems are intrinsically dynamic and can rearrange, as nature does e.g. in the case of cell-membrane-bound proteins. Some of the interactions are reversible upon external stimuli (electrochemistry, UV-light). This opens the door to greater control over the immobilization and release of molecules on surfaces. Applications in biosensors are promising, and the systems presented here are capable of detecting small quantities of proteins or peptides. Finally, the development of supramolecular coatings for implants or biomedical devices creates opportunities for promoting selective cell attachment and drug delivery applications.

The aim of this thesis is to apply host-guest chemistry to control cell adhesion on surfaces. By conjugating biologically relevant compounds such as RGD peptides or RGD-modified fluorescent proteins to supramolecular tags, a practically straightforward immobilization on surfaces is then possible. Studies were performed at the molecular level to characterize the surface interaction, as well as at the cellular level to evaluate the efficiency of the supramolecularly modified biocompounds.

In Chapter 2, a proof of principle for the use of CB[7] – ferrocene as a host-guest pair to immobilize RGD peptides in a spatial resolution was performed. Complex formation was
characterized and first cell studies were carried out to find appropriate conditions for selective attachment on RGD- vs RAD-coated surfaces.

Chapter 3 continued with the same system, looking in more detail at the affinity of ferrocene-peptide derivatives for CB[7] surfaces, and comparing it to affinities obtained in solution. Furthermore, a library of ferrocene-peptide with various linker lengths, multivalency and peptide sequences was evaluated using three different methods, in static and flow conditions. Possibilities for reversible surface immobilization were also investigated.

Chapter 4 makes use of a more well established host-guest pair, β-cyclodextrin – ferrocene. As in chapter 3, the characterization of the affinity was performed via QCM experiments, and ferrocene-peptide derivatives were evaluated on βCD-coated glass surfaces. The complex is known to be reversible upon electrochemical reduction of the ferrocene, and possible release of cells from such surfaces was investigated.

In chapter 5, carborane was used as a guest molecule for βCD. Although this interaction has already been reported in solution, we tried to gain a better understanding of the complex formation both in solution and on surfaces. The affinity in solution was studied using ITC and FRET, and with QCM-d on surfaces. Finally, cell adhesion and morphology was analyzed on surfaces, and some specific parameters could be identified that corroborate the qualitative observation from cell images with the data analysis.

A fluorescent protein modified with an active RGD sequence as well as a supramolecular tag was used in chapter 6. First results regarding selective supramolecular immobilization of this protein via the ternary complex methylviologen – CB[8] – tryptophan were obtained, as well as regarding cell adhesion. The results here suggest that the controlled release of cells from such surfaces should be possible with simultaneously monitoring of the release of the RGD fluorescent protein from the surface.

5. References

Chapter 2

Supramolecular control over cell adhesion via ferrocene-cucurbit[7]uril host-guest binding on gold surfaces

Supramolecular control over adhesion of cells is demonstrated using synthetic integrin binding RGD peptide-ferrocene conjugates that were immobilized via host-guest chemistry onto cucurbit[7]uril coated gold surfaces. Ferrocene-peptide conjugates were synthesized using copper-catalyzed click chemistry and their immobilization of CB[7]-coated surfaces was characterized using IR-RAS, water contact angle and XPS. Appropriate conditions for specific cell adhesion were determined by introducing a blocking spacer. The effect of RGD-vs. RAD-coated surfaces was demonstrated over 48 h. Finally, spatial resolution could be achieved showing specific adhesion of endothelial cells on RGD-coated surfaces. The system show promise as a general method to functionalize surfaces with bioactive molecules such as peptides or proteins, to generate e.g. biosensors.

1 Introduction

Supramolecular construction processes provide innovative platforms for the study of fundamental aspects of cell biology, as a basis for the engineering of biomaterials with self-adaptive cell interface properties. On surfaces, structured interfaces can be formed with molecular precision – on a scale most relevant to cellular function – through the spatial or temporal localization of ligands. With the appropriate combination of ligands, the surface functionality can be tuned to specifically manipulate and monitor cell adhesion and migration. This offers unique opportunities for the development of biomaterials for cell-growth engineering. The use of semi-synthetic proteins, growth factors or peptides in various patterns can direct adhesion and migration of e.g. endothelial cells. Supramolecular chemistry would allow for potentially reversible or stimuli-responsive surfaces with interesting properties, currently being integrated into biological systems and biomaterials with intended regenerative medicine applications.

To investigate supramolecular control over endothelial cell adhesion, the ferrocenylamine-CB[7] based host-guest system was selected (Figure 1). The (ferrocenylmethyl)-trimethylammonium cation binds to cucurbit[7]uril (CB[7]) in solution with a $K_d$ in the range of $10^{11}$ M$^{-1}$. CB[7] spontaneously adsorbs onto gold surfaces and forms a stable self-assembled monolayer (SAM). Such supramolecular CB[7]-SAMs have been used for the site-selective immobilization of fluorescent proteins that were monovalently labelled with ferrocenylamine. Although binding affinities to CB[7]-SAMs might differ from those in solution, strong and reversible binding to CB[7]-coated gold surfaces was still observed. This surface immobilization system bodes well for exploration of cellular applications such as cell adhesion studies, and is potentially attractive due to the ease of CB[7] SAM formation and the low toxicity of gold.

Here, important progress towards such supramolecular-based bio-interfaces is reported, based on the non-covalent immobilization of ferrocene-labelled cyclic RGD (Fc-cRGD) peptides to gold surfaces coated with a CB[7] (cucurbit[7]uril) monolayer using host-guest interactions. The synthesis and purification of such ferrocene-peptide conjugates is reported, as well as the characterization of their binding on CB[7] coated surfaces. Adequate conditions for cellular experiments to avoid unspecific attachment of the cells on gold are studied. Only surfaces featuring the correct supramolecular host-guest combination, displaying the RGD motif, enable enhanced molecular-induced endothelial cell adhesion and wound-recovery in a controlled manner.
Figure 2.1. Coating of gold surfaces with a CB[7]-monolayer, pre-incubation with Fc-cRGD 9 and reference compound Fc-cRAD 8, and subsequent endothelial cell adhesion to the supramolecularly functionalized surfaces. Structure of cucurbit[7]uril 1.

2 Results and discussion

2.1. Design and synthesis of the ferrocene guest molecule

Contrary to CB[7] which adsorbs to the gold surface through contact with the carbonyl oxygens, ferrocenes have to be functionalized with bioactive peptides promoting cellular adhesion and still be able to bind CB[7] with high affinity. Crucial to the high affinity of this system is the amino functionality present in the ferrocene guest molecule. While the ferrocene ring system occupies the lipophilic CB[7] cavity, the protonated amino functionality protrudes from the cavity, where it makes stabilizing electrostatic interactions with the polar carbonyl groups located at the CB[7] rim.12 To enable studies of endothelial cell adhesion on such CB[7]-coated surfaces, RGD peptides16–19 were attached to the key ferrocenylamine moiety via an oligoethylene glycol (OEG)-based linker (3). The use of OEG was suitable for the envisioned purposes due to its biocompatibility and inertness to gold surfaces.20 The choice of linker length was based on previous investigations regarding the immobilization of ferrocenylamine-labelled fluorescent proteins onto CB[7]-SAMs.15

It was anticipated that a suitable strategy for attaching the ferrocenylamine guest molecule (5) to RGD peptides21 would require special consideration of the specific design of the guest molecule. Firstly, the ferrocenylamine group was initially developed for peptide synthesis as an amine protective group, and is typically cleaved under acidic conditions (TFA/β-thionaphthol/CH₂Cl₂, 2-4 h).22 Furthermore, compatibility with standard amide coupling chemistry23 would in all likelihood require further modification (at least one additional step) or laborious protection-deprotection steps of the ferrocenyl secondary amine functionality.
Thus, an approach that introduced the ferrocenylamine guest into a fully unprotected peptide sequence was preferred to enable a divergent late stage attachment of the ferrocenylamine moiety. Therefore a strategy based on copper-catalyzed azide-alkyne click chemistry was chosen. The novel ferrocenylamine-PEG-azide \( \text{5} \) (Figure 2.2) was readily prepared in one pot, via \textit{in situ} imine formation followed by reduction using NaBH\(_4\). Cyclic RGDfK, and RAdfK\textsuperscript{25} as reference, were synthesized using standard solid-phase peptide synthesis methods\textsuperscript{26} and then coupled to pentynoic acid to form the alkyne derivatives \( \text{6} \) and \( \text{7} \). Ligation of \( \text{5} \) to either \( \text{6} \) or \( \text{7} \) was achieved using CuSO\(_4\) and sodium ascorbate in the presence of copper. This afforded the guest molecules Fc-cRAD (8) and Fc-cRGD (9), respectively, in useful yields after purification by reversed-phase-HPLC.

**Figure 2.2. Synthesis of ferrocene-peptide conjugates 8 and 9 via copper-catalyzed azide-alkyne cycloaddition of azide 5 to alkynes 6 or 7. Reagents and conditions: i. 5 (1.0 eq.), CuSO\(_4\) (30 mol\%) sodium ascorbate (50 mol\%) copper ribbon, 40 °C, 24 h.**

### 2.2. Surface preparation and characterization

CB[7] coated gold surfaces were prepared according to previous methods\textsuperscript{11,13–15} Shortly, surfaces were cleaned for 10 sec in piranha solution, rinsed extensively with milliQ and incubated 4 h in a 0.1 mM aqueous solution of CB[7]. Immobilization of the ferrocene-peptide conjugates onto the CB[7] coated surfaces was performed by immersion in aqueous
solutions (50 μM) of 8 or 9 for 3 h. After washing, the surfaces were characterized using infrared reflection absorption spectroscopy (IR-RAS), water contact angle measurements (WCA) and X-Ray photoelectron spectroscopy (XPS). The morphological properties of the CB[7]-coated gold surface monolayer have been previously described. Cyclic voltammetry (CV) measurements and dynamic force spectroscopy (DFS) suggested that CB[7] covers ca. 40-50% of the gold surface, and atomic force microscopy (AFM) showed that the surface adsorbed CB[7] molecules are accessible for the immobilization of ferrocene-labelled proteins via a specific monovalent CB[7]-ferrocene interaction.

The water contact angle (WCA) of the CB[7]-SAMs, indication of the general hydrophilicity character of the surface, decreased upon incubation with CB[7] from 92° to 32° (Table 2.1), and increased upon incubation with Fc-cRGD 9 from 32° to 58°, which is indicative of the immobilization of CB[7] and subsequently of the ferrocene-peptide conjugate and is in agreement with previously reported values for similar surfaces.

The IR-RAS spectrum of the CB[7]-coated gold surface reveals characteristic peaks for CB[7] at 1477 (C-N) and 1740 (C=O) cm⁻¹ (Figure 2.3). After incubation with Fc-cRGD 9, peaks at 1430, 1540, 1577 (side chains amino acids) and 1670-1700 (amide bonds) cm⁻¹ can be observed which are characteristic for the presence of the peptide-ferrocene conjugate. Finally, after subsequent blocking with (ethyleneglycol)₆-thiol (OEG₆-SH) additional peaks at 1116 cm⁻¹ (C-O-C) were observed.

XPS (Table 2.2) shows the expected C/N/O ratio of 3/2/1 for CB[7]-SAMs, and the appearance of an Fe signal upon incubation with Fc-cRGD 9. The surface characterization data thus demonstrate the successful immobilization of the ferrocene-peptide conjugates onto the CB[7]-SAMs via a ferrocene-CB[7] host-guest interaction.
Table 2.1. Water contact angle values at the different steps of the surface assembly (n=3).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Average WCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gold substrate</td>
<td>92°</td>
</tr>
<tr>
<td>CB[7]</td>
<td>32°</td>
</tr>
<tr>
<td>CB[7] + Fc-cRGD 9</td>
<td>58°</td>
</tr>
<tr>
<td>CB[7] + Fc-cRGD 9 + OEG(_{6})SH 10</td>
<td>55°</td>
</tr>
</tbody>
</table>

Table 2.2. Percentage of elements on the CB[7] and the CB[7] + Fc-cRGD 9 SAMs, theoretically calculated and experimentally determined values.

<table>
<thead>
<tr>
<th>Plate/peak area</th>
<th>C1s</th>
<th>N1s (%)</th>
<th>O1s (%)</th>
<th>Fe2p (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB[7] (exp)</td>
<td>51.5</td>
<td>30.7</td>
<td>17.8</td>
<td>-</td>
</tr>
<tr>
<td>CB[7] (theory)</td>
<td>50.0</td>
<td>33.3</td>
<td>16.7</td>
<td>-</td>
</tr>
<tr>
<td>CB[7] + Fc-cRGD 9 (exp)</td>
<td>57.29</td>
<td>25.08</td>
<td>17.19</td>
<td>0.45</td>
</tr>
<tr>
<td>CB[7] + Fc-cRGD 9 (theory)</td>
<td>59.6</td>
<td>26.3</td>
<td>13.5</td>
<td>0.6</td>
</tr>
</tbody>
</table>

2.3. Cell experiments

Human umbilical vein endothelial cells (hUVECs) were used as a model system to study cellular response to supramolecularly immobilized peptides 8 and 9. Cells were seeded on CB[7]-SAMs and on CB[7] with 8 or 9 using different chemical surface functionalizations and high cell density was observed in all conditions, including the controls. This could be due to the cells adhering unspecifically to the positively charged gold. To prevent unspecific binding, different blocking protocols were tested. After unsuccessful trials with BSA, a blocking step using OEG\(_{6}\)-SH 10 was evaluated at different steps in the surface preparation protocol against unspecific interactions and the cell response was monitored at different time points over a period of 48 h (Figure 2.4, 24 h).

To determine the optimal blocking conditions, cells were seeded on different substrates. After 3 h, the medium was removed, each well was washed once with 2 mL PBS buffer and transferred into a new well with fresh medium. Images were recorded at different time points. Different blocking steps were tested: no blocking (blocking 0), blocking with OEG\(_{6}\)-SH 10 before CB[7] and Fc-cRGD 9 incubation (blocking 1), blocking between CB[7] and Fc-cRGD 9 incubation (blocking 2) and blocking as the last step, after CB[7] and Fc-cRGD 9 incubation (blocking 3). Significant differences were observed between the different surfaces (Figure 2.4 for 24 h). The two controls, cells seeded on a CB[7] surface and on a CB[7] and Fc-PEG surface, were performed without any further blocking. No blocking results in overall high cell adhesion, whether on the controls or on the CB[7] and Fc-cRGD 9 surfaces, no discrimination between the different conditions was possible. Blocking 1 and 2 resulted in an overall low cell density and cell remaining round and non-spread, even after 24h. It is
possible that the OEG$_6$-SH 10 prevents the formation of the CB[7] monolayer at the surface (CB[7] control surface shows high cell adhesion) in the case of blocking 1. After formation of the CB[7] monolayer, it could be that the OEG$_6$-SH hinders the formation of the CB[7]-Fc complex (blocking 2). Blocking 3 seems most efficient to allow the cells to adhere specifically to the peptide, by preventing them to interact with gold. Indeed, cells are spread out and dense on the surface coated with Fc-cRGD, whereas they remain mostly round and did not proliferate on the Fc-cRAD surface.

![Figure 2.4](image)

**Figure 2.4. 24 h incubation time. Scale bar = 200μm. Top lines: substrate was incubated with CB[7], then 8 or 9, and blocked at different time points. Lines 3 and 4: controls with CB[7] only or with CB[7] and Fc-PEG.**

Addition of the short ethylene glycol chain 10 directly after the incubation of the surface with the ferrocenylamine-peptides, via immersion in a 0.1 mM solution of OEG$_6$-SH 10 for 2 min, was most effective. For the other experiments, the same procedure was repeated including only the blocking step 3 and various controls were performed (Au, Au + CB[7], Au + CB[7] + 4 or 5, Au + 5, Au + CB[7] + Fc-PEG).
Figure 2.5. 24 h incubation time. Scale bar = 200 μm. All the surfaces were blocked at the last step (blocking 3) for 2 minutes with OEG₆-SH.

Figure 2.6. 48 h incubation time. Scale bar = 200 μm. All the surfaces were blocked at the last step (blocking 3) for 2 minutes with OEG₆-SH.
Figure 2.7. Number of cells on substrate covered with 8 or 9 on CB[7]-coated gold plates, normalized to the number of cells on substrate coated with 8 at each timepoint. Statistical significance level $p < 0.001 (***)$.

Supramolecular coating with the Fc-cRGD 9 resulted in efficient adhesion and growth of hUVECs to the supramolecular surfaces as evidenced by the increase in surface coverage and cell density after 24 h. In contrast, surfaces pre-incubated with the Fc-cRAD 8 showed less spreading of cells and a significantly reduced number (3-fold) of adhered cells. Similarly, reference surfaces using bare gold, CB[7]-only, CB[7] with a ferrocenyl-PEG$_3$ linker or only 9, showed only minor cell adhesion and a more round cell morphology. After 48 h, a closed monolayer of cells was formed on the surfaces with CB[7] and Fc-cRGD 9, whereas this state could not be reached in any of the control experiments including surfaces with CB[7] and Fc-cRAD 8. This indicates that the cells do not recognize the RAD peptide, nor do they trigger certain mechanisms to proliferate as seen on the RGD coated surfaces. A live-dead assay confirmed that the hUVECs remain viable on CB[7]-SAMs incubated with the Fc-cRGD 9 compared to control tissue culture plates (TCP) and fibronectin coated TCP (Figure 2.8).

A wound assay on surfaces with CB[7] and Fc-cRGD 9 showed a full recovery of the cell monolayer within 8 h, indicating effective direction of cell growth by the supramolecularly immobilized peptide epitopes (Figure 2.9 left). To achieve patterned adhesion of hUVECs, glass substrates patterned with gold arrays were used. Prior to the functionalization of the gold arrays with CB[7], Fc-cRGD 9 and OEG$_6$SH as described above, the remaining glass areas were covalently blocked with polyethyleneglycol. After seeding hUVECs for 4 h on these arrays the bright field images show that cells adhere specifically to the functionalized gold by having multiple contact points and mostly spreading over multiple gold stripes (Figure 2.9 right) whereas on the bare gold, the cells tended to align along single gold stripes. Cells are able to sense the RGD sequence over the PEG-silane and to spread as they would do on monolayers.
Figure 2.8. Live-dead assay on Tissue Culture Plate (TCP, left), fibronectin coated TCP (middle) and CB[7] + Fc-cRGD 9 coated gold substrate (right) after 96 h proliferation. Scale bar: 200 μm.

Figure 2.9. (left) Wound assay on a confluent monolayer of HUVECs on CB[7] and 9: micrographs recorded every 2h from i) t=0 to v) t=8h). (right) Scale bar: 200 μm. hUVECs seeded on a gold array functionalized with CB[7], Fc-RGD 9 and OEG6-SH 10, after 4h proliferation, inset shows magnification.
3 Conclusion

In conclusion, the ferrocene-CB[7] based host-guest system allows for supramolecular control over cell adhesion. Ferrocene conjugated to bioactive relevant RGD peptide was synthesized and immobilized on a CB[7] monolayer on gold. A blocking step was found to decrease unspecific adhesion of cells on gold. The first example of spatial resolution of supramolecular cell adhesion using this system was demonstrated on a gold array. Combined with investigations into different surface types and supramolecular approaches, such systems could lead to beneficial switching properties, building further on contemporary covalent methods by introducing reversibility and adaptability to surface immobilization through the chemical fine-tuning of the host-guest chemistry.

Improvement of the blocking step could be performed by testing different PEG-thiol length and try to reduce even further the adhesion on RAD-coated substrates. Furthermore, the wound assay shows intense cell activity and migration within hours, it might be interesting to study cell adhesion at earlier time point than 24 h. Other factors such as the linker length, the multivalency may also play an important role in the cell adhesion qualities of the resulting supramolecular surface and will be studied in chapter 3. Finally, combination of the RGD sequence with the synergistic site PHSRN also located on fibronectin could enhance cell adhesion, surfaces displaying both sequences can easily be prepared and studied by mixing in different ratios both ferrocene-peptide conjugates.

4 Experimental

We thank Dr. S. Sahebali for help with statistical analysis, L. Olijve for help with NMR measurement, J. van Dongen for high resolution mass spectrometry measurements, M.Sc. S. Krabbenborg for the electrode surfaces and G. Kip for XPS measurements. Live/dead cell assay was performed by J. Brinkmann.

4.1. Synthesis

4.1.1. General

Reactions were performed at room temperature unless otherwise stated. MeOH, HPLC grade trifluoroacetic acid (TFA), HPLC grade formic acid (FA) and acetonitrile (MeCN) were purchased from Biosolve. All organic solvents were used as purchased. H$_2$O refers to Millipore grade distilled water. Reverse-phase liquid chromatography-mass spectrometry (LC-MS) analysis: LCQ Fleet from Thermo Scientific on a C18 column, Surveyor AS and PDA. Eluent conditions (CH$_3$CN/H$_2$O/0.1% FA), 5 minutes run: 0-0.3 min, isocratic, 2 % CH$_3$CN; 0.3-3.3 min, linear gradient, 2 - 70 %; 3.3-4.3 min, isocratic, 70 %; 4.3-4.7 min, linear gradient, 70 – 2 %; 4.7-5 min, isocratic, 2 % CH$_3$CN. 15 minutes run: 0-1 min, isocratic, 5 % CH$_3$CN; 1-10 min, linear gradient, 5 – 100 %; 10-11 min, isocratic, 100 %; 11-12 min, linear gradient, 100 -
Chapter 2

5 \%; 12-15 min, isocratic, 5 \% CH₃CN. \(^1\)H-NMR and \(^{13}\)C-NMR spectra were recorded using a 400 MHz or 500 MHz NMR (Varian Mercury). Proton chemical shifts are reported in ppm and calibrated with residual H₂O (4.79 ppm) as the internal standard. The splitting patterns are designated as: m, multiplet.

Purification of products by RP-HPLC was performed in a Shimadzu HPLC on a C18 preparative column from Phenomenex (21.20 x 150 mm) and a flow rate of 15 mL/min. The products were eluted by using different solvent gradients of solvents A and B (solvent A = 0.1% FA/H₂O; solvent B = 0.1% FA/CH₃CN). UV signal at 210 nm was used for detection.

4.1.2. Synthesis of ferrocenylamine-PEG-azide

![Figure 2.10. Synthesis of ferrocenylamine PEG azide 5.](image)

Ferrocenecarboxaldehyde, A (922 mg, 4.31 mmol) and azide amino, B (1.1 g, 5.04 mmol, 1.2 eq.) were dissolved in dry MeOH (18 mL) at rt and stirred under a positive argon pressure for 3 h. After complete conversion to the imine, C (judged by \(^1\)H-NMR of an aliquot measured in CD₃OD), the reaction was carefully treated portion-wise with NaBH₄ (277 mg, 7.32 mmol, 1.7 eq.) and stirring continued until no further gas evolution was observed. Then, the reaction was immediately quenched with 10 mL of 1 M aqueous NaOH and diluted with 50 mL CH₂Cl₂. The aqueous phase was then extracted 2 x 30 mL CH₂Cl₂ and the recombinant organic phase washed with 40 mL brine, dried (MgSO₄) and filtered. Evaporation of the organic solvents in vacuo afforded a dark orange-red crude oil, which was purified by silica gel column chromatography (column dimensions: 10.5 cm h x 3 cm w; pack column with CH₂Cl₂, then step-wise gradient of 5% and 10% MeOH until product exits the column) to afford the target compound, 1, as a dark orange-red oil (1.32 g, 3.19 mmol, 74%). Rf = 0.50 (10% MeOH / 1% NH₄OH in CH₂Cl₂); LC-MS (C18, see general methods, FA): LC: Rt = 4.61 min; HRMS (ESI+): calc. for C₁₉H₂₉FeN₄O₃ [M+H]⁺: 417.1589, found 417.1597; \(^1\)H-NMR (400 MHz, CDCl₃): \(\delta = 4.20\) (t, J = 1.6 Hz, 2H), 4.13 (s, 5H), 4.09 (t, J = 1.6 Hz, 2H), 3.66-3.59 (m, 12H), 3.51 (s, 2H), 3.36 (d, J = 5.2 Hz, 2H), 2.84 (d, J = 5.2 Hz, 2H), 2.01 (broad s, 1H) ppm; \(^{13}\)C-NMR (100 MHz, CDCl₃): \(\delta = 86.9, 70.7, 70.6, 70.3, 70.0, 68.4, 67.7, 50.6, 49.0, 48.8\) ppm; IR (neat): \(\tilde{\nu} = 3332, 3091, 2865, 2099, 1641, 1449, 1347, 1284, 1249, 1104, 1037, 1023, 1000, 923, 818\) cm\(^{-1}\).
4.1.3. Synthesis of cyclic RXDfK: X = A (6) or G (7)

All peptides were synthesized on solid-phase using a 2-Cl-Trt resin preloaded with Gly or Ala. Peptide elongation involved treating the resin with 20% piperidine in NMP to cleave the Fmoc-protecting group, followed by coupling of the next amino acid with DIPEA and HBTU. Before cleavage, the resin was rinsed seven times with diethyl ether and CH₂Cl₂. A mixture of TFE, acetic acid and DCM (1:1:3 v/v/v) was used to cleave the linear peptide from the resin without removing the side chain-protecting groups. The solution containing the cleaved peptide and the resin were separated by filtrations, solvents removed in vacuo, and the resulting solid was redissolved in H₂O/acetonitrile and lyophillized. The peptide chain was then cyclized using PyBOP as coupling agent. The peptide was dissolved in dry DMF (5 mL) and added dropwise with a syringe at 0.033 mL/min in a glass round-bottomed flask containing DIPEA (9 eq.), PyBOP (2.2 eq.) and dry DMF (10 mL) in dry CH₂Cl₂ (14 mL). The reaction mixture was allowed to react overnight under stirring at room temperature under a positive argon pressure. Solvents were then removed in vacuo. The cyclized peptide was then deprotected using a mixture of TFA/H₂O/TIS 95:2.5:2.5 v/v/v for 3 h. Solvents were removed in vacuo and the crude was redissolved in H₂O/acetonitrile and lyophillized. The peptide was finally coupled to NHS-activated pentynoic acid (synthesized according to previous literature)³¹ (1 eq.) in dry DMF (1.3 mL) in the presence of DIPEA (10 eq) for 1 h. Solvents were removed in vacuo. Purification was performed using preparative-HPLC with a gradient of 12-20% B. Yields for 2 and 3 were respectively 38 and 45%.

6: **LC-MS** (C18, see general methods, FA): **LC**: Rₜ = 2.57 min; **HRMS** (ESI+): m/z [M+H]+ calcd for C₃₃H₴₇N₉O₈: 698.3626; found: 698.3622; **¹H-NMR** (400 MHz, D₂O): δ = 0.90 (m, 2H, γCH₂ Lys), 1.32-1.70 (m, 10H, 2βCH₂ Lys, 2δCH₂ Lys, 2γCH₂ Arg, 1βCH₂ Arg, 3βCH₂ Ala), 1.87 (m, 1H, βCH₂ Arg), 2.39 (t, 1H, alkyneH), 2.43-2.54 (m, 5H, βCH₂ Asp and 2CH₂ pentynoic acid), 2.63 (dd, 3J = 7.6 Hz, 2J = 15.2 Hz, 1H, βCH₂ Asp), 2.74 (m, 1H, NH), 2.98 (dd, 1H, βCH₂ Phe), 3.11 (m, 3H, 2εCH₂ Lys, βCH₂ Phe), 3.22 (m, 2H, δCH₂ Arg), 3.92 (m, 1H, αCH Lys), 4.15 (m, 2J = 7.2 Hz, 1H, αCH₂ Ala), 4.45 (m, 1H, αCH Arg), 4.52 (dd, 3J = 5.2 Hz, 2J = 10.8Hz, 1H, αCH₂ Phe), 4.68 (m, 1H, αCH Asp), 7.26-7.41 (m, 5H, ArH) ppm. Not all NH signals were detected in the spectrum. Peaks assigned by analogy with ³².
7: LC-MS (C18, see general methods, FA): LC: Rt = 2.59 min; HRMS (ESI+): m/z [M+H]+ calcd for C_{32}H_{45}N_{9}O_{8}: 684.3469; found: 684.3468; ¹H-NMR (400 MHz, D_{2}O): δ = 0.91 (m, 2H, γCH_{2} Lys), 1.32-1.70 (m, 7H, 2 βCH_{2} Lys, 2 δCH_{2} Lys, 2 γCH_{2} Arg, 1 βCH_{2} Arg), 1.87 (m, 1H, βCH_{2} Arg), 2.40 (t, 1H, alkyneH), 2.44-2.61 (m, 5H, βCH_{2} Asp and 2CH_{2} pentynoic acid), 2.73 (dd, ²J = 8 Hz, ³J = 16 Hz, 1H, βCH_{2} Asp), 2.97 (dd, 1H, βCH_{2} Phe), 3.11 (m, 3H, 2 εCH_{2} Lys, βCH_{2} Phe), 3.21 (m, 2H, δCH_{2} Arg), 3.49 (d, 1H, αCH_{2} Gly), 3.86 (m, 1H, αCH_{2} Lys), 4.23 (d, ²J = 14.8 Hz, 1H, αCH_{2} Phe), 4.41 (m, 1H, αCH_{2} Asp), 4.59 (dd, ²J = 5.2 Hz, ³J = 10.8Hz, 1H, αCH_{2} Phe), 4.72 (m, 1H, αCH_{2} Asp), 7.26-7.41 (m, 5H, ArH) ppm. Signals NH were not detected in the spectrum. Peaks assigned by analogy with 32.

4.1.4. General protocol for the synthesis and purification of ferrocene-peptide conjugates

Prior to reaction, azide 5 (1 eq.) was weighed into a glass round bottom flask fitted with a magnetic stirrer bar, degassed with flushing argon and stored under a positive argon pressure. The azide was then dissolved in a mixture of degassed DMF/milliH_{2}O (3/1 v/v) and alkyne 6 or 7 (1 eq.), CuSO_{4} (0.3 eq.) and sodium ascorbate (0.5 eq.) were next added, followed by a small piece of copper ribbon. The reaction flask was heated at 40 °C for 24 h under a positive argon pressure, after which the solvents were removed in vacuo and the crude redissolved in H_{2}O/MeCN in readiness for purification by RP-HPLC.

Purification was performed using a gradient of A from 10 to 30%. Final yield after purification ranged from 32 to 39%.

8: LC-MS (C18, see general methods, FA): LC: Rt = 2.80 min; HRMS (ESI+): m/z [M+H]+ calcd for C_{52}H_{75}FeN_{13}O_{11}: 1114.5138; found: 1114.5145; ¹H-NMR (500 MHz, D_{2}O): δ = 0.75 (m, 2H, γCH_{2} Lys), 1.22-1.97 (m, 11H, 2 βCH_{2} Lys, 2 δCH_{2} Lys, 2 γCH_{2} Arg, 2 γCH_{2} Arg), 2.55-2.68 (m, 4H, βCH_{2} Asp and 2CH_{2} PEG), 2.95-3.23 (m, 10H, 2 βCH_{2} Phe, 2CH_{2} pentynoic acid, 2εCH_{2} Lys, 2δCH_{2} Arg), 3.62 (m, 8H, CH_{2} PEG), 3.73 (t, 2H, CH_{2} PEG), 3.91 (m, 3H, αCH_{2} Lys, CH_{2} PEG), 4.14 (m, 3H, αCH_{2} Ala, CH_{2} PEG), 4.28 (broad s, 5H, Fe), 4.33 (broad s, 2H, Fe), 4.42 (broad s, 5H, Fe), 4.45-4.55 (m, 5H, αCH_{2} Arg, CH_{2} PEG), 4.68 (t, 1H, αCH_{2} Asp), 7.22-7.33 (m, 5H, ArH), 7.83 (s, 1H, C=CH) ppm. Peaks were assigned by comparison with the data of 2.

9: LC-MS (C18, see general methods, FA): LC: Rt = 2.68 min; HRMS (ESI+): m/z [M+H]+ calcd for C_{51}H_{73}FeN_{13}O_{11}: 1100.4982; found: 1100.4969; ¹H-NMR (500 MHz, D_{2}O): δ = 0.64 (m, 2H, γCH_{2} Lys), 1.09-1.52 (m, 7H, 2 βCH_{2} Lys, 2 δCH_{2} Lys, 2 γCH_{2} Arg, 1 βCH_{2} Arg), 1.72 (m, 1H, βCH_{2} Arg), 2.40 (t, 1H, alkyneH), 2.44-2.61 (m, 5H, βCH_{2} Asp and 2CH_{2} pentynoic acid), 2.73 (dd, ²J = 8 Hz, ³J = 16 Hz, 1H, βCH_{2} Asp), 2.97 (dd, 1H, βCH_{2} Phe), 3.11 (m, 3H, 2 εCH_{2} Lys, βCH_{2} Phe), 3.21 (m, 2H, δCH_{2} Arg), 3.49 (d, 1H, αCH_{2} Gly), 3.86 (m, 1H, αCH_{2} Lys), 4.23 (d, ²J = 14.8 Hz, 1H, αCH_{2} Phe), 4.41 (m, 1H, αCH_{2} Asp), 4.59 (dd, ²J = 5.2 Hz, ³J = 10.8Hz, 1H, αCH_{2} Phe), 4.72 (m, 1H, αCH_{2} Asp), 7.26-7.41 (m, 5H, ArH) ppm. Signals NH were not detected in the spectrum. Peaks assigned by analogy with 32.
Supramolecular control over cell adhesion via Fc-CB[7] host-guest binding on gold surfaces

2.37-2.58 (m, 4H, 2βCH₂ Asp and CH₂ PEG), 2.78-3.06 (m, 10H, βCH₂ Phe, 2 εCH₂ Lys, βCH₂ Phe, 2CH₂ pentyanoic acid, δCH₂ Arg), 3.34 (d, 1H, δ₂= 14.8 Hz, αCH₂ Gly), 3.45 (m, 8H, 4CH₂ PEG), 3.58 (t, 2H, CH₂ PEG), 3.68 (dd, 1H, βCH₂ Lys), 3.76 (t, 2H, CH₂ PEG), 3.96 (broad s, 2H, CH₂ PEG), 4.05 (d, δ₂= 14.8 Hz, 1H, αCH₂ Gly), 4.14 (broad s, 5H, Fe), 4.18 (broad s, 2H, Fe), 4.26 (m, 3H, αCH Arg, CH₂ PEG), 4.38 (t, 2H, CH₂ PEG), 4.43 (dd, δ₃= 5.2 Hz, δ₂= 10.8Hz, 1H, αCH Phe), 4.56 (m, 1H, αCH Asp), 7.07-7.18 (m, 5H, ArH), 7.67 (s, 1H, C=CH) ppm. Peaks were assigned by comparison with the data of 3.

Figure 2.11. ¹H-NMR spectra in D₂O of Fc-PEG₃-N₃ 5 (bottom), cRGDfK 7 (middle) and Fc-PEG₃-cRGDfK 9 (top).
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4.2. Preparation and characterization of surfaces

20 nm gold coated glass surfaces, 1 inch diameter cut in 2 equal pieces or 10*10 mm square were purchased from Ssens (Netherlands), and activated using a piranha solution (H₂SO₄/H₂O₂, 3:1, v/v) for 10 to 15 s. Surfaces were then extensively washed with H₂O and incubated for 4 h in a 0.1 mM solution of cucurbit[7]uril in H₂O. Surfaces were then washed with H₂O and incubated for 3 h in a 50 μM solution of Fc-cRGD (9) or Fc-cRAD (8). They were again washed with H₂O and finally incubated for 2 min in a 0.1 mM solution of OEG₆-SH 10. After final rinsing with H₂O, surfaces were dried under gentle N₂ flow and used directly for the cell experiments or surface characterization.

Gold electrodes (5 μm wide, 10μm spacing) were fabricated and prepared as previously reported in literature⁷. After a final washing, the electrodes were modified as described above (starting with incubation in CB[7]).

4.2.1. X-ray Photoelectron Spectroscopy (XPS)

XPS measurements were performed on a PHI Quantera SXM, using a monochromated Al K-α X-ray source with an energy of 1486.6 eV. The X-ray beam has a diameter of 100 μm and a power of 25 W. Survey scans (-5eV to 1345 eV) were collected at 45 deg. take-off angle and at a pass energy of 224 eV (0.4 eV step size). Samples were neutralized with low energy Ar⁺ ions and electrons. Atomic concentrations were calculated using Multipak 9.2.1.0 software from PHI.

Figure 2.12. LCMS spectrum of Fc-PEG3-cRGDfK 9.

Gold electrodes (5 μm wide, 10μm spacing) were fabricated and prepared as previously reported in literature⁷. After a final washing, the electrodes were modified as described above (starting with incubation in CB[7]).

4.2.1. X-ray Photoelectron Spectroscopy (XPS)

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Figure 2.15. Fe2p spectra, 4 scans averaged, Gaussian fit (CB[7]+Fc-cRGD SAM)

4.2.2. Water Contact Angle

Water Contact Angle measurements were performed on a Krüss G10 contact angle measuring instrument, equipped with a CCD camera. Images were analyzed using the software Drop Shape Analysis version 1.90.0.2 and an ImageJ plug-in (Water contact angle).

4.2.3. IR-RAS

Fourier Transform Infrared Reflection Absorption Spectroscopy (FT-IR-RAS) measurements utilized 200 nm gold Si wafers, 2x2 cm. Polarized FT-IR-RAS spectra of 1000 scans at 2 cm⁻¹ were obtained using Thermo Scientific TOM optical module.
4.3. Cell experiments

Human Umbilical Vein Endothelial Cells (hUVEC) at passage between 2 and 5 were used for the cell experiments. Confluent T25 or T75 flasks of hUVECs were trypsinized, centrifuged and redispersed in EGM-2 Lonza medium for surface incubation.

![Image of cell experiments with CB[7], cRGD, and Fc-PEG](image)

**Figure 2.16.** 38 h incubation time. Scale bar = 200μm. Top lines: substrate was incubated with CB[7], then 8 or 9, and blocked at different time points. Lines 3 and 4: controls with CB[7] only or with CB[7] and Fc-PEG. With the blocking 3 step, high cell adhesion on the substrate coated with Fc-cRGD 9 could be observed compared to the substrate coated with Fc-cRAD 8.

4.4. Live/dead assay

The LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells from Molecular Probes, Invitrogen was used, at a 1/1000 dilution factor in PBS for both components. Cells were incubated with the resulting solution 96 h after cell seeding and imaged 30 to 45 minutes later using a fluorescence microscope, using the following filters: Calcein AM (green): Ex 460-490 nm, Em 525 nm and Ethidium homodimer-1 (red): Ex 510-550 nm, Em 590nm, LP.
5 References

Chapter 3

Molecular insights into ferrocene-peptide derivatives on CB[7] surfaces

Following up on chapter 2, a library of ferrocene-peptide conjugates was explored. Peptide sequences promoting cell adhesion were conjugated to one or two ferrocene moieties via a flexible PEG linker of various lengths. The affinity of these different molecules for CB[7]-coated surfaces was determined using QCM-D, and the benefits of longer linker and of bivalent compounds could be shown to increase the affinity. Next, an electrochemistry study was conducted to see whether the CB[7] – ferrocene pair could be controlled electrochemically. Finally, three methods were employed to study the effect of these molecules over cellular adhesion. Monolayers coated with the supramolecular complex revealed that the background adhesion remained high on control surfaces compared to active surfaces. Therefore gold arrays were tested and it was possible to quantify the effect of the coating, by measuring the spreading abilities of the cells on different conditions. Finally, a microfluidic approach was described to determine the velocity of cells over the supramolecular substrates, depending on the peptide sequence present at the surface. Overall, RGD peptides showed better cell spreading and stronger interactions with cells compared to RAD peptides and controls. PHSRN peptides alone revealed a lower cell density and spreading compared to PHSRN in combination with RGD, validating a known synergistic effect for these two sequences.
1 Introduction

Multiple parameters can dictate the binding of two molecules, such as structural variations in the chemical structure that can impact their accessibility (e.g., linker length) and valency. As described extensively in chapter 1, an important peptide sequence for integrin binding, the RGD peptide sequence, was discovered in 1984.\(^1\) Ten years later, the PHSRN sequence – also located in fibronectin, but in the 9\(^{\text{th}}\) type III repeat –\(^2\) was found to exhibit a synergistic effect with RGD.\(^3\) The two sequences are located close by (3 to 4 nm distance \(^4\)) and bind to the integrin \(\alpha_5\beta_1\). Both sequences are located in a loop and display an arginine (positively charged amino-acid).\(^3\) Its function is not fully known, though it has been hypothesized that either the PHSRN helps to direct the integrin to bind to fibronectin and RGD\(^5\), or that PHSRN acts as a second recognition site for integrins, which increases the activity of the protein (synergistic hypothesis) (Figure 3.1).\(^5,6\) The second hypothesis is currently favored, also because PHSRN on its own shows little activity towards \(\alpha_5\beta_1\).\(^7,8\) However, more recent studies have also shown the intrinsic activity of PHSRN alone, but less efficiently than RGD and a synergistic effect when both sequences are present.\(^9\)

![Figure 3.1. Simulation of the 9\(^{\text{th}}\) and 10\(^{\text{th}}\) type III repeat from fibronectin, in the functionally decoupled state. Synergy site and RGD loop are depicted in yellow. Adapted with permission from \(^10\).](image)

In order to be fully efficient, the peptide sequence of interest must be accessible for binding. In this regards, the distance between a surface and a ligand can be of utmost importance regarding its accessibility and thus its activity:\(^11\) the peptide sequence must be accessible and recognizable by the integrin. Each system is different and an optimal linker length is not universal. RGD peptides with various polyglycine linker lengths have previously been immobilized on poliacrylonitrile beads and affinity for platelet binding was measured. In this case the shorter and longer linkers resulted in a lower affinity compared to the intermediate linkers (9 to 13 glycines).\(^12\) In some cases, the presence of a linker is critical to obtain at all an affinity.\(^13\) With respect to the sequence of interest to us, Kessler reported an optimal linker length of 35 Å for the attachment of cRGDFK on surfaces.\(^11\)

Depending on the type of supramolecular surfaces, a multivalent guest can be necessary: bivalent guests are known in some systems to increase binding affinity. For example, bivalent ferrocene exhibits an 100-fold higher affinity for \(\beta\)-cyclodextrin monolayers
compared to monovalent ferrocene.\textsuperscript{14} In a similar manner, the affinity of bivalent adamantine for β-cyclodextrin surfaces is significantly greater than monovalent adamantane.\textsuperscript{15} Multivalency can also give macroscopic properties to materials: in a recent example, CB[7]- and aminomethylferrocene–functionalized surfaces put in contact with one another were capable of supporting 2 kg with a supramolecular contact surface of just 1 cm\textsuperscript{2}.\textsuperscript{16}

Here we investigate the influence of accessibility and valency, both regarding the affinity of the ferrocene-peptide conjugate for CB[7] surfaces, and for their potential influence over cellular adhesion.

2 Results and discussion

2.1. Design and synthesis of the ferrocene-peptide conjugates library

The choice of linker is critical for the design of peptide conjugates that necessitate further binding to proteins, e.g. integrins present at the cell surface: a long and flexible linker ensures adequate accessibility of ligand for integrin binding. However, an overly flexible linker risks back-folding, while an overly rigid linker may prevent correct orientation of the ligand for optimal ligand-protein interaction. Amino acid linkers are used in protein engineering (GGS repeats) for their ease of implementation in a protein construct\textsuperscript{17} but can be difficult to obtain synthetically due to the low solubility of the glycine. We decided not to use alkane chains: although more flexible than amide backbones, they can also display issues of solubility in aqueous solutions for longer chains and tend to aggregate.

For these reasons the choice of an oligo(ethylene glycol) (PEG) linker was favored for this system. PEG is flexible, water soluble, and biocompatible.\textsuperscript{21} Different lengths of PEG linkers are commercially available and can be readily functionalized with different small molecules and peptides. The PEG 3 linker discussed in chapter 2 was chosen for this study due to its successful use by our group on a similar application.\textsuperscript{22} PEG 10 was easily accessible as a monodisperse molecule and is comparable in length to the optimal linker lengths from other systems (Figure 3.2).\textsuperscript{12} The use of longer PEG linkers would complicate the peptide coupling step: there would be a need for longer reaction time and increased temperatures with the risk of degrading the product.
Figure 3.2. Synthesis of ferrocene-PEG$_x$-N$_3$ linkers 12 and 13 ($x = 5, 10$).

The aminomethyl group substituent to ferrocene was conserved, as it has been shown to favor a stable complex formation with CB[7] compared to aminoethyl or amine groups (Figure 3.2).$^{18}$ Substitution groups of ferrocene have a strong influence over its binding affinity with CB[7]. The presence of a carboxylic acid group (Figure 3.3 2 and 3) directly attached to the ferrocene is too large and prevents binding and interactions with the oxygen rim: charge repulsion between the oxygens is taking place. Substitution with trimethylamine (Figure 3.3 4) is possible due to the positive charge of the amine, interacting with the oxygen rim via ion-dipole interactions. To reach an optimal binding mode, a methyl group acting as a spacer is necessary to correctly position the positively charged amine with respect to the oxygen rim (Figure 3.3 5).

Figure 3.3. Structures of various ferrocenes and their affinity $K_a$ for CB[7] in aqueous solution (compounds 1-3: pure water, compounds 4-6: 50 mM sodium acetate, pH 4.5): 1 hydroxymethylferrocene ($3.10^9$ M$^{-1}$),$^{19}$ 2 ferrocenecarboxylic acid (no interaction),$^{19}$ 3 ferrocenecarboxylate (very low interaction),$^{19}$ 4 ferrocenyltrimethylammonium ($3.6x10^{10}$ M$^{-1}$),$^{18}$ 5 ferrocenylmethytrimethylammonium ($3.3x10^{11}$ M$^{-1}$),$^{18,20}$ 6 ferrocenylethyltrimethylammonium ($7.3x10^{10}$ M$^{-1}$).$^{18}$
Both linear and cyclic RGD and PHSRN derivatives were synthesized via Solid Phase Peptide Synthesis to examine a potential synergistic effect of RGD and PHSRN on the supramolecular surfaces. The linear peptides were modified on the resin with pentynoic acid or with a bisalkyne derivative to facilitate purification. For the synthesis of cRGD and cRAD, a linear sequence bearing a D-phenylalanine and a lysine residue: the D-phenylalanine to ensure an adequate conformation upon cyclization and the lysine for further chemical modifications. After overnight intramolecular cyclization and TFA deprotection, the lysine was selectively modified with the alkyne moiety over the arginine residue by controlling and maintaining the pH of the solution at 7.

A library of ferrocene-peptide conjugates was synthesized using copper-catalyzed click chemistry (Table 3.1) as described in chapter 2. Reaction times and catalysts quantities had to be increased for reactions involving PEG10 linker 13. Thin linker molecule was not as soluble as PEG3 14 and PEG5 12 linkers and this might have decreased the reaction efficiency. Purification was carried out using mild conditions (formic acid based HPLC) to avoid cleavage of the acid-labile ferrocene moiety and afforded the compounds at milligram scale, sufficient for testing (Figure 3.5). Assuming that the peptide sequence would have no influence over the binding between CB[7] and ferrocene, our aim was to test the extent with which multivalency or linker length affected binding affinity.

![Figure 3.4](image)

*Figure 3.4. Representative structures of peptides and peptide-ferrocene conjugates. c(RGDFK)-bisalkyne 16 and PHSRN-alkyne 19 peptide building blocks obtained after solid phase peptide synthesis. Fc2-PEG3-cRGD 26 obtained after click reaction between cRGDFK-bisalkyne 16 and Fc-PEG3-N3 linker 14. The peptide sequence can vary (15-22) as well as the linker to which it is coupled (12-14), resulting in a library of ferrocene-peptide conjugates (23-39).*
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**Table 3.1.** Overview of the ferrocene-peptide conjugates synthesized (in bold, compound numbers), their retention time on LCMS (15 min run, 5 – 100% MeCN) and their calculated and found masses in g.mol<sup>−1</sup>.

Ferrocenylmethyltrimethylammonium 5 affinity for CB[7] has already been studied in solution and a high affinity was observed (K_d = 10^{-11} \text{ M}^{-1}).^{20} However, it might be that the immobilization of CB[7] on surfaces modifies slightly the binding of ferrocene in the cavity, thus modifying the binding affinity of ferrocene derivatives for CB[7]. Our conclusion from initial experimentation with Surface Plasmon Resonance (SPR) were that ferrocenylmethyltrimethylammonium 5 bound with gold-CB[7] monolayers with micromolar K_d, which was lower than reported in solution.^{20} A solution of FcPEG_3 linker at 1 \mu M flown over a CB[7] monolayer showed a weak binding signal. Subsequent flow of the strong binder FcNMe_3 5 revealed an increase in binding, indicating that FcPEG_3 derivative has a lower affinity for CB[7] compared to 5, and that even 5 bound weaker to CB[7] on surfaces than in solution. If the binding was as strong as in solution (in the picomolar range), a stronger signal would be observed by SPR.

Quartz Crystal Microbalance with dissipation (QCM-D) is a technique that allows the measurement of the binding of compounds to surfaces. In our case, gold crystals were coated with CB[7] and different concentrations of a range of different ferrocene-peptide conjugate flown over the surface. If the dissipation remained within 10% of the change in frequency value, the Sauerbrey model could be applied and binding would be directly proportional to the change in signal.^{23} The change in frequency could then be plotted versus the ligand concentration and fitted with a Langmuir model on Origin. To determine the binding affinity of our ferrocene-peptide conjugates with CB[7] monolayers, Fc-PEG3-HSPNR 40 was flown over at different concentrations in the micromolar range after buffer equilibration for 5 minutes to obtain a stable baseline. After 15 minutes flowing of 40, a buffer wash was carried out for 15 minutes to remove material non-specifically adhered at the surface.
Figure 3.6. SPR sensorgram depicting the binding of 1 μM Fc-PEG$_3$-N$_3$ followed by 1 μM FcNMe$_3$I to CB[7] monolayer on gold in 20 mM NaPi buffer.

The $K_d$ of Fc-PEG$_3$-HSPNR for the CB[7] monolayer was determined to be 293 μM +/- 58 μM (Figure 3.8). This value is higher than expected, given the high affinity Kd value reported for ferrocenylmethyltrimethylammonium for CB[7] in solution. This could be due to the fact that one side of the cavity is not accessible at the surface, and thus that ferrocene is not able to protrude on the opposite side of the oxygen rim of CB[7] for an optimal binding. Although crystal structures were only obtained for CB[7] complexed with ferrocene without any substituent groups, modeling of ferrocene derivatives in CB[7] show that minor modifications to bare ferrocene result in the ferrocene moiety protruding out of the CB[7] cavity, and that depending on the size of the substituent, ferrocene can be pushed out further from the cavity. Besides the hydrophobic interaction between the ferrocene moiety and the inner cavity of CB[7], dipole-dipole interactions between the oxygen from the CB rim and the NH from the ferrocene derivative also play a large stabilizing role, as described in similar systems (CB[7] and 1,1'-bis(trimethylammoniomethyl)ferrocene and CB[8] and Phe-Gly-Gly$^{25}$).
Figure 3.7. Energy-minimized structures computed for CB[7] complexed with (left) ferrocenyltrimethylammonium 4, (middle) ferrocenylmethyltrimethylammonium 5 and (right) ferrocenylethyltrimethylammonium 6. In the case of βCD coated surfaces, binding of a bivalent ferrocene shows a significant improvement in binding affinity (approximately 100-fold increase) and we tested the same hypothesis with the CB[7] monolayer. The affinity of Fc₂-PEG₃-HSPNR for CB[7] monolayers was measured at 156 μM +/- 37 μM (Figure 3.9), which corresponds with a two-fold improvement in affinity. CB[7] monolayers are not as densely packed as βCD monolayers, and the PEG₃ linker may be too short for two Fc moieties to bind to two CB[7] cavities simultaneously, resulting in a small improvement in binding affinity.

As evidence for this, the affinity of Fc₂-PEG₁₀-HSPNR, featuring two longer PEG spacers, was tested for CB[7] and found to be 3.5 μM +/- 1.6 μM (Figure 3.10) (50 fold improvement compared to Fc-PEG₃-HSPNR). Longer linkers may now allow the two ferrocenes to bind two CB[7], thus increasing the affinity. It appears that because the CB[7] monolayer is not densely packed, the distance between two CB7 necessitates a longer linker length than the originally designed.

Figure 3.8: QCM-D data for Fc-PEG₃-HSPNR on CB[7] coated crystals. (left) Fifth resonance frequency overtone for various concentrations of Fc-PEG₃-HSPNR (10, 50, 100, 200, 250, 500 μM). (right) Change in frequency of the fifth overtone versus Fc-PEG₃-HSPNR concentration. Fit was performed using Origin, Langmuir fit. $K_d = 293 \mu M +/- 58 \mu M.$
Figure 3.9: QCM-D data for Fc$_2$-PEG$_3$-HSPNR on CB[7] coated crystals. (left) Fifth resonance frequency overtone for various concentrations of Fc$_2$-PEG$_3$-HSPNR (10, 25, 100, 500 µM). (right) Change in frequency of the fifth overtone versus Fc$_2$-PEG$_3$-HSPNR concentration. Fit was performed using Origin, Langmuir fit. $K_d = 156$ µM +/- 37 µM.

Figure 3.10: QCM-D data for Fc$_2$-PEG$_{10}$-HSPNR on CB[7] coated crystals. (left) Fifth resonance frequency overtone for various concentrations of Fc$_2$-PEG$_{10}$-HSPNR (0.5, 2, 5, 10, 20, 40, 50 µM). (right) Change in frequency of the fifth overtone versus Fc$_2$-PEG$_{10}$-HSPNR concentration. Fit was performed using Origin, Langmuir fit. $K_d = 3.5$ µM +/- 1.6 µM.

2.3. Electrochemical behavior of CB[7] surfaces

Aminomethylferrocene is known to form a reversible complex with β-cyclodextrin upon electrochemical stimuli, also when ferrocene is connected to proteins or other small molecules.$^{14,26}$ However in the case of CB[7], few studies have been performed in this direction. Kaifer’s group studied the influence of the pH on the formation of CB[7] and ferrocene derivatives.$^{27,28}$ upon increasing pH, the oxidation peak potential of aminomethylferrocene increased and shifted to lower potential. Simultaneously, the amine group NH captures a proton to become NH$_2^+$, thus increasing its affinity for CB[7]. However, these aminomethylferrocene derivatives usually bear a chemical group sensitive to pH and
minimal modifications (only modified with a methyl group). Salt concentration\textsuperscript{29} and solvents\textsuperscript{30} can also decrease the affinity of ferrocene for CB[7]. Here we wanted to study whether such reversible behavior could be detected with larger modification on the ferrocene group (addition of a PEG linker and peptide) and applicable as a potential controlled system for cell release.

After incubation of the gold substrates with CB[7], a cyclic voltametry scan between -0.2 V and 0.5 V was performed, and an oxidation peak could be detected at 0.15 V (Figure 3.11, black curve). However, after scanning a surface displaying CB[7] and Fc-cRGD under the same conditions, no such peak could be detected (Figure 3.11, red curve). This peak for CB[7] and peak absence for CB[7] + Fc-RGD are difficult to explain, and would contradict the results from Kaifer’s group who were able to detect a ferrocene signal when complexed with CB[7].

![Cyclic voltametry scan of gold substrate coated with (black) CB[7] and (red) CB[7] + Fc-cRGD. Range: -0.2 V to 0.5 V, 25 mV/s, third scan, 1 mV step.](image)

It is not the first time that puzzling electrochemical data have been reported for CB[7]-based systems.\textsuperscript{31} Our unexpected result could be explained by the slow scan rate, or alternatively because the addition of CB[7] has already been reported to cause a significant decrease in the intensity of the oxidation peak in the case of neutral ferrocene derivatives.\textsuperscript{27} Finally, it should be mentioned that these measurements were performed on surfaces and not in solution as reported in the literature; the amounts detectable are thus much lower and complicate the characterization. Therefore we are not yet at the stage to electrochemically control the reversibility of the ferrocene-CB[7] complex on gold surfaces.
2.4. Cellular evaluation

Following on from the cellular studies of chapter 2, the library of compounds with various linker lengths, peptide sequences and monovalent and bivalent ferrocene was then studied using three different cellular approaches to evaluate the library of ferrocene-peptide conjugates regarding cell adhesion promotion.

2.4.1. Cell experiments on monolayer

To gain more insights into the behavior of HUVEC cells on the supramolecular surfaces, the supramolecular surfaces incubated with endothelial cells were monitored overtime and the number of cells adhering on them was counted after 2, 3.5, 6, 24 and 48 h. The objective here was to determine which compound was more effective in cell adhesion, and whether differences could be noticed in early stages of adhesion and which were persistent overtime. Monitoring was performed with bright field microscopy to avoid fixing samples and to allow for overtime observation of the same substrates. Figure 3.12 depicts a typical set of images obtained over the course of this experiment. After 2 h, cells have started to adhere to the surface and to spread. Depending on the condition (RAD or RGD coated surface for example), the number of round cells may vary. After 3 h 30 min and 6 h, cells spread more, and 24 and 48 h time points show cell proliferation. Due to the gold layer, a poor contrast is obtained for images and counting can’t be streamlined. Non-adherent cells display a round morphology, while spread cells are more elongated and flat.

![Figure 3.12. Brightfield images of Fc-PEG3-cRGD 23 surface taken after a) 2 h, b) 3 h 30 min, c) 6 h, d) 24 h, e) 48 h.](image)

The linker length seems to have an effect on cell adhesion: overall, the longer the linker, the less the cells adhere at the surface. PEG is known as an anti-fouling agent and a longer linker might display that characteristic. Moreover, a longer linker means increased probability to fold back thereby reducing the accessibility of RGD to bind to integrins. It
Molecular insights into ferrocene-peptide derivatives on CB[7] surfaces

seems that the initially designed compound Fc-PEG<sub>3</sub>-cRGD 27 displays the highest affinity for HUVECs. Multivalent compounds don’t show improved activity compared to monovalent, and are even less efficient in the case of Fc<sub>2</sub>-PEG<sub>3</sub> compared to Fc-PEG<sub>3</sub>. As already observed by QCM-D, no significant improvement in binding was observed between the mono and bivalent systems displaying a short PEG<sub>3</sub> linker, which means that the bivalent system does not more strongly anchor the cells to the surface. An improvement in cell adhesion might be expected therefore for Fc<sub>2</sub>-PEG<sub>10</sub>, but again the increased PEG content might also lead to an increased antifouling effect, which would result in comparable cell density on Fc<sub>2</sub>-RGD surfaces compared to Fc-RAD surfaces (Figure 3.13). The potential antifouling properties could be tested by synthesizing a Fc-PEG-methyl derivative that would bind to the CB[7]-modified surface and not promote cell adhesion (lacking a positive charge and a specific peptide sequence). The HUVEC cells were seeded in normal EGM-2 Lonza medium containing 2% serum, which could explain why cells were also observed to adhere on control surfaces. Personal observation and comparison between these cellular experiments and others have shown that HUVEC cells do not differentiate strongly between the different conditions, and that other cell types such as C2C12 do. Moreover, residual adhesion was also observed on bare gold and gold-CB[7] surfaces (Figure 3.13), even though these were also blocked with EG6SH as the other surfaces, thus an optimization of the blocking step may be required.

![Graph showing cell adhesion](image)

**Figure 3.13.** Number of HUVEC cells adhering on supramolecularly coated gold surfaces, functionalized with CB[7], a ferrocene-peptide conjugate (if applicable) and blocked with EG6-SH, after 3 h 30 and 24 h adhesion time in 2% serum EGM-2 Lonza medium. Percentages are relative to the number of cells on cRGD-PEG<sub>3</sub>-Fc surfaces. Three positions on each surface at each time point were recorded and analyzed. Various linker lengths and RAD vs. RGD surfaces are compared here, together with control surfaces.
A synergistic effect was observed between PHSRN and RGD: cell adhesion reduced by a third for surfaces displaying only PHSRN compared to only RGD. When RGD and PHSRN were mixed, cell adhesion increased compared to RGD alone. The most active ratios seemed to be RGD 3:1 PHSRN in the early stage and RGD 1:1 PHSRN for longer time points (24 and 48 h) (Figure 3.14).

![Graph showing the percentage of cells relative to RGD surface over time](image)

**Figure 3.14.** Number of HUVEC cells adhering on supramolecularly coated gold surfaces, functionalized with CB[7], a ferrocene-peptide conjugate (if applicable) and blocked with EG₆-SH, after 3 h 30 and 24 h adhesion time in 2% serum EGM-2 Lonza medium. Percentages are relative to the number of cells on cRGD-PEG3-Fc surfaces. Three positions on each surface at each time point were recorded and analyzed. Various ratios of RGD and PHSRN ligands, at different time points are compared.

However, these results are only trends, since error bars are too large to draw solid conclusions. One reason for the large error bars could be the manner in which the surfaces are handled. It could also be because the cells, over the course of several hours secrete adhesion proteins themselves and that the supramolecular coating has therefore little influence on cell adhesion over such periods of time. Also, cells might still sense the gold and unspecific adhesion could remain to a certain level. Due to the limitations of the experimental setup, further studies should be performed at shorter time points.

### 2.4.2. Cell experiments on gold arrays

To obtain more quantitative data regarding the cell affinity for the supramolecular surfaces, cells were seeded for a shorter period of time (1 h), and fixed and stained to gain more insight into the structure of the cells (formation of focal adhesions, actin filaments structure). Also, by using electrodes, the surface available for cell adhesion is reduced and could in principle increase the differences between substrates.
Electrodes with 5 μm gold and 10 μm glass spacings were fabricated under clean room conditions. Cells were seeded under four different conditions, with the glass part of the electrodes previously functionalized with PEG silane to avoid adhesion on glass and direct cells to adhere specifically to the supramolecularly functionalized gold surface.

C2C12 cells were used as a model cell line in these experiments. On the control surfaces (gold, CB[7], CB[7] + Fc-RAD), cells remained elongated and mostly on one electrode (Figure 3.15). On the active RGD-coated surface, cells spread over multiple electrodes in a short period of time: they were able to sense and spread across the PEG-silane layer. Moreover, cells were forming focal adhesions and thus recognizing the integrins only on the active surface (Figure 3.15, green staining for phosphorylated Paxillin).

These qualitative results were confirmed by quantitative analysis. The number of electrodes over which a cell spreads was analyzed for each substrate and clear differences could be observed (Figure 3.16). While cells spread on average over 1.4 electrodes on the control surfaces, they are able to reach for 3, 4 and even 5 electrodes on the CB[7] + Fc-PEG₃-cRGD surface. Large error bars on the control surfaces were due to one set of repetition resulting in a particularly clear behavior, with cells spreading only on one electrode for the control, while they displayed cells spreading on one or two electrodes for the two other repetitions. This method seems appropriate to test the library of compounds.

![Figure 3.15](image)

*Figure 3.15. Gold electrodes, 10 μm glass blocked with PEG-silane, 5 μm gold coated with gold, CB[7], CB[7] + Fc-PEG₃-cRAD 29, CB[7] + Fc-PEG₃-cRGD 23 and blocked with EG₂SH. Staining for nuclei (DAPI, blue), actin (phalloidin, red) and focal adhesion (paxillin, green).*
Figure 3.16. Percentage and average of electrodes over which a cell spreads, depending on the coating applied on the gold (CB[7], Fc-PEG$_{3}$-cRAD 29 or Fc-PEG$_{3}$-cRGD 23, all blocked with EG$_{6}$SH).

We used this method to test several compounds from the library and see whether the results obtained on the monolayer were similar to the ones obtained on electrodes (Figure 3.17). The increased linker length (PEG3 vs PEG10) resulted in lower spreading of cells, as observed with the monolayer: it was probably due to the increase in PEG content and its antifouling properties. Going from monovalent to bivalent ferrocene peptide conjugates also decreased the cell spreading for the same reason.

Finally, the presence of the PHSRN sequence alone showed great differences compared to RGD, as expected from the literature 7,8 and as observed in the monolayer experiments. However, the combination of RGD and PHSRN did not improve compare to RGD alone. It should be noted that these observations were different from the monolayer experiments, where we looked at initial spreading instead of number of cells adhered and while the time scale was also different (2 h to 48 h for the monolayer experiments, compared to only 1 h adhesion time here). A synergistic effect has also been described in the literature,3 it could be that the ratio between RGD and PHSRN was not optimal here to obtain that effect.
Figure 3.17. Percentage and average of electrodes over which a cell spreads, depending on the coating applied on the gold (Fc-PEG$_3$-cRGD 23, Fc$_2$-PEG$_3$-cRGD 26, Fc-PEG$_{10}$-cRGD 25, Fc$_2$-PEG$_{10}$-cRGD 28, Fc-PEG$_3$-PHSRN 34 and Fc-PEG$_3$-cRGD 23:1 Fc-PEG$_3$-PHSRN 34 (RGD 1:1 PHSRN), all blocked with EG$_6$SH). Experiment was performed in duplicate.

2.4.3. Microfluidics

Analysis of monolayer and electrodes can display important information about cellular affinity under static conditions. However, applications such as stent coating would put the system into flow conditions. Moreover, specific cells in the blood, Endothelial Progenitor Cells (EPCs), are capable of differentiating into endothelial cells. Therefore, it would be interesting to study the impact of flow on cellular binding to the monolayer reminiscent of conditions in the blood stream to try to sequester them from the blood stream. For this purpose a microfluidic approach would be highly suited.

As suggested by the name, microfluidic devices have dimensions on the micrometer scale. Due to the reduced dimensions of the experimental setup, a reduced amount of cells, medium, and surface area are needed, which can be of great benefit for the screening of compounds available only minutes quantities (as in our case).  

The approach followed here involves the use of supramolecularly coated gold substrates as described above and PDMS comprising a single 200 µm wide channel (Figure 3.18 a). PDMS could not be covalently bonded to the gold surface, otherwise resulting in a peeling off of the gold layer. By simply placing the two components in formal contact —i.e. by layering one
on top of the other and applying force-, a microfluidic device could be created held together by hydrophobic interactions.\textsuperscript{34}

![Diagram of PDMS device with cells and PDMS surfaces](image)

**Figure 3.18.** (left) Scheme of the PDMS device. Recording is performed under the microscope, from the top of the device. (right) Cell velocity on CB[7] + Fc-PEG\textsubscript{3}-cRGD and on CB[7] + Fc-PEG\textsubscript{3}-cRAD coated surfaces, flow speed 0.5 \( \mu \)L/min. P-values < 0.05 is considered significant.

Freshly trypsinized HUVECs at high concentration were flown over at a low flow rate (0.5 \( \mu \)L / min) using a syringe pump. Under these conditions cells are either at the center of the channel and go straight to the outlet, or at a side (be it the PDMS surface or the supramolecular surface) and roll on it. It is possible to distinguish on the video in which direction the cells are rolling and thus to segregate cells rolling on PDMS (not relevant for us) from cells rolling on the coated surface (of interest for us). Cells that interact more with the surface exhibit a slower rolling speed than cells interacting less. By measuring the speed of cells flowing at the supramolecularly coated surface, it is possible to discriminate between different types of surfaces (Figure 3.18 b). A two-fold decrease in rolling speed could be observed in the rolling speed of cells on RGD coated surfaces compared to RAD coated surfaces. This experiment could be repeated with the different compounds of the library to assess them. HUVEC cells interact more with RGD-coated surfaces than with the control surface RAD. This result also indicates that the ligand is accessible to cells in suspension and could potentially allow for recruitment of cells with specific peptides recognition sequences.

Also, for potential application in stent coating, by using a specific recognition sequence for EPC, the ability to capture those could be tested. Further tests involving flowing blood could
also be envisioned to assess and improve the specificity of the surface towards endothelial cell migration and EPC recruitment.

3 Conclusion

As shown previously in literature, parameters such as multivalency, linker length and synergistic effects can influence cell adhesion. Here, we aimed at finding an optimal ferrocene-peptide conjugate by testing compounds with various linker lengths, combining RGD with PHSRN to study their binding to integrins of endothelial cells and testing bivalent ferrocene compounds. A library of compounds could be synthesized by reacting various ferrocene-PEG-azides with peptide-alkynes using Copper-catalyzed click chemistry and purification via preparative HPLC. Their affinity towards CB[7] surfaces was tested and found to be lower than expected (300 μM on surfaces vs. picomolar range in solution). According to these results, and for an improved coverage of the monolayers, the concentrations of ferrocene-peptide conjugates necessary for the surfaces preparations should be increased. However, the use of a bivalent ferrocene with PEG10 linker length allowed for low μM affinity and a 100-fold improvement compared to Fc-PEG3, probably because the low density of CB[7] molecules at the gold surface necessitate long linkers for two ferrocene moieties to be able to reach for two CB[7] cavities. Finally, cellular adhesion was evaluated using three different methods. Monitoring the number of surface adherent cells over time proved to be unreliable because of non-homogeneous cell distribution at the surface, and because cells were probably secreting ECM proteins, resulting in high error bars. Seeding cells on electrodes led to a higher differentiation between the different compounds. Although the method requires accuracy in surface preparation to properly block the glass, it showed significant results and the rest of the library could be tested in the same way. Variations in cell spreading depending on linker length or multivalency are not too pronounced, but a clear difference was observable for PHSRN coating: in the absence of RGD, cells did not spread, in the presence of RGD, they did. Finally, microfluidics allows for an evaluation under flow conditions, which is relevant for potential biomedical application of such coatings. Although the setup still requires some optimization, it is also a promising approach to test the remainder of the ferrocene-peptide conjugates presented in this chapter, as well as a general method to determine cell affinity for coated substrates.

4 Experimental

NH₂-PEG₃-N₃ linker was kindly provided by Dr. Freek Hoeben (SyMO-Chem). Maarten Bakker performed synthesis of the linkers 1 and 2. Peptide synthesis was performed together with Maarten Bakker, Rick Huisman, Paul van Bussel and Stefan van Uden. MSc. Jenny Brinkmann fabricated the electrodes in the clean room and carried out one repetition of the electrode
experiment. Microfluidics experiments were done by and together with Dr. Sheen Sahebali. Dr. Sheen Sahebali did analysis.

4.1. Synthesis of ferrocene-azole linkers

n = 2: Ferrocenecarboxaldehyde, 7 (179 mg, 0.837 mmol) and azide amino, 8 (300 mg, 0.979 mmol, 1.17 eq.) were dissolved in dry MeOH (3.5 mL) at rt and stirred under a positive argon pressure for 3 h. After complete conversion to the imine, 10 (judged by $^1$H-NMR of an aliquot measured in CD$_2$OD), the reaction was carefully treated portion-wise with NaBH$_4$ (54 mg, 1.42 mmol, 1.7 eq.) and stirring continued until no further gas evolution was observed. Then, the reaction was immediately quenched with 2 mL of 1 M aqueous NaOH and diluted with 10 mL CH$_2$Cl$_2$. The aqueous phase was then extracted 3 x 10 mL CH$_2$Cl$_2$ and the recombinant organic phase washed with 10 mL brine, dried (MgSO$_4$) and filtered. Evaporation of the organic solvents in vacuo afforded a dark orange-red crude oil, which was purified by silica gel column chromatography with a step-wise gradient of 5% and 10% MeOH until product exits the column, to afford the target compound, 12, as a dark orange-red oil (89%). **LC-MS** (C18, see general methods, FA): **LC**: $R_t = 4.99$ min; **HRMS** (ESI+): calc. for C$_{23}$H$_{37}$FeN$_4$O$_5$ [M+H]$^+$: 505.20, found 505.17; $^1$H-NMR (400 MHz, CDCl$_3$): $\delta = 4.20$ (t, J = 1.6 Hz, 2H), 4.13 (s, 5H), 4.10 (t, J = 1.6 Hz, 2H), 3.66-3.59 (m, 20H), 3.51 (s, 2H), 3.39 (d, J = 5.2 Hz, 2H), 2.83 (d, J = 5.2 Hz, 2H), 1.93 (broad s, 1H) ppm.

n = 7: Ferrocenecarboxaldehyde, 7 (52.17 mg, 0.244 mmol) and azide amino, 9 (150 mg, 0.285 mmol, 1.17 eq.) were dissolved in dry MeOH (1.1 mL) at rt and stirred under a positive argon pressure for 24 h. After complete conversion to the imine, 11 (judged by $^1$H-NMR of an aliquot measured in CD$_2$OD), the reaction was carefully treated portion-wise with NaBH$_4$ (15.7 mg, 0.413 mmol, 1.7 eq.) and stirring continued until no further gas evolution was observed. Then, the reaction was immediately quenched with 0.85 mL of 1 M aqueous NaOH and diluted with 10 mL CH$_2$Cl$_2$. The aqueous phase was then extracted 3 x 10 mL CH$_2$Cl$_2$ and the recombinant organic phase washed with 15 mL brine, dried (MgSO$_4$) and filtered. Evaporation of the organic solvents in vacuo afforded a dark orange-red crude oil, which was purified by silica gel column chromatography with a step-wise gradient of 5% and 10% MeOH until product exits the column, to afford the target compound, 13, as a dark orange-red oil (91%). **LC-MS** (C18, see general methods, FA): **LC**: $R_t = 3.21$ min; **HRMS** (ESI+): calc. for C$_{33}$H$_{57}$FeN$_4$O$_{10}$ [M+H]$^+$: 725.33, found 725.33; $^1$H-NMR (400 MHz, CDCl$_3$): $\delta = 4.20$ (t, J = 1.6 Hz, 2H), 4.13 (s, 5H), 4.10 (t, J = 1.6 Hz, 2H), 3.66-3.59 (m, 20H), 3.52 (s, 2H), 3.36 (d, J = 5.2 Hz, 2H), 2.84 (d, J = 5.2 Hz, 2H), 1.65 (broad s, 1H) ppm.

4.2. Peptide synthesis

cRGDFK and cRADfK were synthesized as described in chapter 2. Addition of the alkyne functionality was performed by coupling the peptide either to NHS-pentynoic acid or to a NHS-3,5-bis(prop-2-yn-1-ylxy)benzoic acid.

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PHSRN and HSPNR peptides were synthesized on solid-phase at a 200 μmol scale using a Wang resin preloaded with Asn or Arg. Peptide elongation involved treating the resin with 20% piperidine in NMP to cleave the Fmoc-protecting group, followed by coupling of the next amino acid (4 eq.) with DIPEA (16 eq.) and HBTU (3.9 eq). The peptide was finally coupled to pentynoic acid or to 3,5-bis(prop-2-yn-1-yl)oxy)benzoic acid in the same way as a normal amino-acid coupling. Before cleavage, the resin was rinsed seven times with diethyl ether and CH$_2$Cl$_2$. A mixture of TFA, water and TIS (93:2:5 v/v/v) was used to cleave the linear peptide from the resin and simultaneously remove the side chain-protecting groups. The solution containing the cleaved peptide and the resin were separated by filtrations, solvents removed in vacuo, and the resulting solid was redissolved in H$_2$O/acetonitrile and lyophilized. Yields for 19, 20, 21 and 22 were typically around 20 %.

4.3. Synthesis of ferrocene conjugates

The general protocol used to couple the peptides to the ferrocene linker is described in chapter 2. In the case of coupling of a ferrocene linker to a bivalent alkyne peptide, 2.1 eq of Fc-linker was added and amount of catalysts (CuSO$_4$ and NaAsc) were doubled. In the case of coupling to PEG$_{10}$ linker, a longer reaction time was needed (48h), the amount of catalyst was doubled and a second addition of catalyst was done after 24h.


CB[7] surfaces (monolayers, QCM-D crystals and electrodes) were prepared as described in chapter 2.

4.5. QCM-D experiments

Prior to use in QCM-D experiments, resonators were activated using a piranha solution (H$_2$SO$_4$/H$_2$O$_2$, 3:1, v/v) for 15 s. Surfaces were then extensively washed with H$_2$O and then incubated in a 0.1 mM solution of CB7 in milliQ H$_2$O for 4 h. They were then rinsed with H$_2$O and dried under N$_2$ flow.

QCM-D data were measured using a Q-Sense E1 with a peristaltic pump, Ismatec Reglo Digital M2-2/12. Gold-coated QCM-D resonators QSX 301 with a resonance frequency of 4.95 MHz +/- 0.05 MHz were purchased from LOT-QuantumDesign. All solutions were prepared with PBS buffer. Measurements were performed at 20 °C, with a flow of 50 μL/min. Prior to the binding of the ferrocene-peptide XX, YY or ZZ, surfaces were equilibrated by flowing over PBS buffer until a stable baseline was obtained.

4.6. Electrochemistry

Measurements were performed using the Autolab PGSTAT10. 200 nm thick gold disc were used and coated with CB7 as described in chapter 2. The electrolyte was a 0.1 M K$_2$SO$_4$ solution.
4.7. Gold electrodes preparation

Gold electrodes (5 μm wide, 10 μm spacing) were fabricated and prepared as previously reported in literature\textsuperscript{35}. The substrate was activated for 10 sec in piranha solution (H\textsubscript{2}SO\textsubscript{4}/H\textsubscript{2}O\textsubscript{2} 3/1), rinsed with copious amount of milliQ H\textsubscript{2}O and blocked with PEG-silane AB111226 by incubation at 60 °C for 2h in anhydrous toluene. Solution was discarded and substrates were then placed at 120 °C for 2h. Cleaning of the gold was performed by sonication (10 min in EtOH, rincing EtOH, then 5 min milliQ H\textsubscript{2}O, rincing H\textsubscript{2}O, drying under N\textsubscript{2} flow). After a final washing, and when applicable, the electrodes were modified first by incubation for 4 h in 0.1 mM aqueous solution of CB7 and rincing with H\textsubscript{2}O. Surfaces were then washed with H\textsubscript{2}O and incubated when applicable for 3 h in a 50 μM solution of Fc-cRGD (27) or Fc-cRAD (33). They were again washed with H\textsubscript{2}O and all surfaces (gold, CB7, Fc-cRGD and Fc-cRAD) finally incubated for 2 min in a 0.1 mM solution of OEG\textsubscript{6}-SH 10. After final rinsing with H\textsubscript{2}O, surfaces were dried under gentle N\textsubscript{2} flow and used directly for the cell experiments or surface characterization.

4.8. Preparation of microfluidics set-up

Master preparation: A silicon wafer was spin coated with 4 mL SU-8 50 at 500 rpm for 15 sec (acc. 110 rpm / sec) and at 1000 rpm for 60 sec (acc. 330 rpm / sec). The wafer was then placed on a bench heater at 65 °C for 10 min, and then at 95 °C for 30 min. Exposure to UV light with the mask of interest was performed for 25 sec. For post exposure bake, the wafer was heated for 1 min at 65 °C and for 10 min at 95 °C. Finally, the wafer was placed in SU-8 developer for 10 min under constant shaking, washed with isopropanol, dried and stored.

PDMS preparation: PDMS was prepared by mixing 184 silicon elastomer and 184 curing agent (10:1), put under vacuum to remove air bubbles and pour on the mask. After heating at 65 °C for 1 to 2 h, the mask was left to cool down until solidification of PDMS. Device was cut out and place in formal contact with the previously supramolecularly coated gold surface.

Analysis was performed by measuring the speed of cells rolling specifically on the supramolecular surface at the center of the microfluidic channel. 5 cells per condition were analyzed.

4.9. Cell culture

For the monolayer and the microfluidics experiments, hUVECs were treated as described in chapter 2.

For the electrodes experiments, C2C12 cells, from a mouse myoblast cell line, were used at passage between 15 and 20 for the cell experiments. 80% confluent T25 or T75 flasks of C2C12 were trypsinized, centrifuged and redispersed in DMEM medium supplemented with
penicillin/strep, NEAA, as well as 10% FBS for culturing and 0% FBS for surface incubation experiments.

4.10. Cellular evaluation

Substrates coated with CB7 and Fc-peptide were briefly dipped into 70% EtOH and rinsed twice with PBS.

Monolayer: HUVECs were seeded at a density of 60 000 to 80 000 cells/mL, 3 mL per well in 6-well plate at 37 °C, 5% CO2. After 3 h, the medium was removed, each well was washed once with 2 mL PBS buffer and transferred into a new well with fresh medium. 3 images per substrate were recorded at different time points (1.5, 3, 6, 24, 48h).

Electrodes: C2C12 cells in suspension in 0% FBS supplemented DMEM media were seeded on the substrates (20 000 cells/mL, 3mL/well) and left to adhere for 1 h at 37 °C and 5% CO2. Surfaces were then gently washed twice with PBS, cells were fixed for 10 min with 10% Formalin and then rinsed three times with PBS.

Staining: Surfaces were incubated with blocking solution (0.1% Triton, 0.5% w/w BSA in PBS pH 7.4) for 1 h at room temperature or overnight at 4 °C. Surfaces were then incubated Paxillin 1:500 in blocking buffer for 1 h, washed 3 times for 10 min with blocking buffer, and incubated for 1 h with secondary antibody-Alexa 488 (1:500) and phalloidin-Alexa 546 (1:500) in blocking buffer. Finally the surfaces were washed once for 10 min with blocking buffer and twice with PBS, incubated for 10 min with DAPI in PBS (1:1000), rinsed with PBS three times, and then stored at 4 °C. Experiments were performed in triplicate. Imaging was performed using an Olympus IX71 fluorescence microscope, at 40x magnification.

Microfluidics: HUVECs were trypsinized, centrifuged and resuspended in Lonza EGM-2 medium at a concentration of 500 000 cells/mL. Cells were flown at 0.5 μL/min in a 1 cm long channel (200 μm wide).

5 References

Chapter 4

Studies into beta-cyclodextrin-ferroocene interactions at the surface: controlled cell adhesion and release

Following on from the studies performed in chapters 2 and 3, the immobilization of ferroocene-peptides was studied on β-cyclodextrin-coated gold and glass surfaces. Infrared, water contact angle, electrochemistry and quartz crystal microbalance were used to determine the molecular assembly and affinity of the host-guest system. The surfaces were tested for cell adhesion as well as for controlled-cell release upon electrochemical signaling. Bivalent ferroocene-peptide conjugates bind to β-cyclodextrin with high affinity and could be electrochemically released from the surface. Differences between mono- and bivalent ferroocene compounds could be shown in cellular studies, and promising results regarding the controlled release of cells established.
1 Introduction

Immobilization of biomolecules such as proteins and peptides is of importance in biomedical coatings or in high-throughput screening.\(^1\) Biomolecules can be attached directly to gold surfaces via gold-thiol bonding or to other types of surfaces such as glass via polymers. The latter method requires mild conditions to preserve the integrity and the activity of the biomolecules. Proteins are particularly sensitive and mild conditions, often incompatible with polymer solubility, are required. Therefore, the functionalization of a polymer coating with proteins is usually performed using a post-modification strategy, which often results in non-selective attachment to the surface and a consequential loss in activity due to misorientation of the protein and obstructed access to the protein’s active site. In contrast to covalent-based methods, which lock’s in functionality at the material’s surface, supramolecular complexes are dynamic and exhibit a strong potential for the controlled release of biomolecules. Fluorescent proteins as a model system have been chemically modified with a supramolecular tag (ferrocene, naphtol) and immobilized on surfaces coated with cucurbit[7]uril \(^2\) or via a ternary complex methylviologen – cucurbit[8]uril – naphtol.\(^3\) The functionalization of guest molecules with short peptide sequence has been reported, for example the integrin binding RGD peptide, which enabled cell attachment on supramolecular surfaces.\(^4\)\(^-\)\(^6\) This property has been demonstrated recently with the use of electrochemistry as a trigger for disrupting the host-guest complex resulting in the release of cells from a supramolecular functionalized surface (Figure 4.1).\(^5\)\(^,\)\(^7\) Cells in their early attachment stage (5 to 10 min) could be released from surfaces along with the supramolecular surfaces on cue in response to an electrochemical signal.

Among known supramolecular hosts, beta-cyclodextrin (βCD) is a cyclic oligosaccharide composed of seven units, with a hydrophobic cavity and a hydrophilic exterior. CDs are therefore water soluble and able to solubilize hydrophobic guests in aqueous solution. This property makes them valuable excipients in industry, functioning as odor masking agents,\(^8\) food preservatives,\(^9\) drug delivery,\(^10\) or fiber agents.\(^11\) In particular, CDs can bind to fatty acids such as cholesterol and lithocholic acid, which has been applied to the production of cholesterol-free food.\(^12\) The inner and outer rim of CDs are rich in hydroxyl groups, who allow for their chemical modification with one or several groups, such as amines \(^13\) or sulfides.\(^14\)\(^,\)\(^15\) As a result, binding affinities to guest molecules can be enhanced\(^16\) and the attachment to surfaces made possible.\(^17\)\(^,\)\(^18\) Cyclodextrins can accommodate a variety of guests depending on the size of the CD cavity, sometimes in a reversible manner. Common guests for αCD are aminobenzoic acids,\(^19\) which are small guests, while γCD can form a complex with larger guests such as coumarin\(^20\)\(^,\)\(^21\) and adamantane\(^22\) derivatives.
Figure 4.1. Ternary complex (methylviologen – CB[8] – tryptophan) functionalized with RGD peptide, allowing cells to adhere specifically and to be released upon electrochemical stimuli. Adapted with permission from reference 5.

βCD guests include azobenzene and ferrocene, both of which form low affinity complexes with βCD (Kₐ = 2.4·10³ M⁻¹ for azobenzene ²³ and Kₐ = 1.2·10³ M⁻¹ for ferrocene ²⁴) and thus necessitate multivalent binding for efficient surface immobilization.²⁵ However, this low binding affinity of guest molecules can also be an advantage in cases where reversibility becomes desirable. As such host-guest complexes can be disrupted physically, for example by UV light ²⁶,²⁷ or electrochemistry ⁷, or chemically, by addition of a competing/competitor host ²⁸ or guest ² to displace the equilibrium towards release of proteins, cells or bacteria from a supramolecular surface. As described in chapter 1, the short peptide sequence RGD is a well-known cell adhesion promoter and therefore was an ideal choice for planned studies into supramolecular controlled cell adhesion.

In this chapter we investigate complex formation between ferrocene-peptide conjugates and βCD surfaces using IR-RAS, WCA and electrochemistry, as well as QCM-D. Furthermore, the binding of cells on RGD supramolecular surfaces are studied, as well as the potential for supramolecular-mediated release of cells.
2 Results and discussion

2.1. Design and synthesis

To construct the envisioned bioactive supramolecular surfaces, molecules displaying both a supramolecular tag and a bioactive moiety are needed. The ferrocene moiety was chosen as guest molecule for the βCD surfaces, as it is known to form a reversible complex with βCD upon electrochemical reduction of the ferrocene. Furthermore, to ensure a strong anchoring at the surface, a bivalent ferrocene was synthesized in addition to the monovalent ferrocene.

The specific immobilization of proteins favoring cell adhesion, such as fibronectin, is complicated by the sheer size of such proteins (around 450 kDa for the active dimeric form of fibronectin). Conventional approaches for protein modification, such as NHS ester or maleimide, result in non-specific labelling of the protein and therefore in random immobilization on surfaces, which does not guarantee a proper exposure and orientation of the ligand at the surface and thus availability for binding to integrins at the cell surface. Therefore, the cRGD motif was selected as peptide sequence for surface functionalization, as it is derived from fibronectin and has a high binding affinity for α5β3 integrin present at the surface of C2C12 cells used here as a model system.29,30 Specific attachment at the surface with high exposition to cell binding can then be achieved. The negative control cRAD was synthesized as well, to demonstrate the specificity of the cell attachment.

To benefit from the increased binding affinity of a bivalent ferrocene, the two ferrocene moieties must be able to reach and bind two βCD cavities simultaneously. Therefore a flexible poly(ethylene glycol) (PEG) linker was introduced between the two ferrocene groups, with the specificity of cell binding towards cRGD ensured by PEG’s biocompatibility and antifouling properties (Figure 4.2).

Finally, the chemical conjugation reaction to attach the peptide to the supramolecular unit needed to be performed under mild conditions to avoid peptide degradation, as well as to preserve the ferrocene moiety, which is acid-labile, and after peptide deprotection, since TFA would cleave off the ferrocene. Therefore, copper-catalyzed click reaction was chosen as it is orthogonal to the peptide side-chains and can be conducted in a peptide-friendly solvent mixture (DMF/H2O) on a fully unprotected peptide. Two surfaces were used for the studies: gold surfaces coated with βCD-7S were used for surface characterization, binding affinity experiments and reversibility (the gold surface acting as an electrode for the electrochemical activation of the surface), while glass-coated surfaces were preferred for the cell experiments for their ease of imaging with the fluorescent staining.

The detailed synthesis and purification of the compounds used in this chapter are described in chapters 2 and 3. For binding studies, a different peptide conjugate (HSPNR in place of
cRGD) was used as it was available in larger quantities and displaying similar charges as cRGD (Figure 4.2).

Figure 4.2. Compounds used in this chapter: Fc-PEG₃-cRGD 1, Fc-PEG₃-cRAD 2, Fc₂-PEG₃-cRGD 3, Fc₂-PEG₃-HSPNR 4, βCD-7S 5 and βCD-7NH₂ 6. See Chapters 2 and 3 for synthesis details.
2.2. Characterization of the surface

Gold surfaces functionalized with βCD-7S 5, which form stable and densely packed βCD monolayers, have been extensively used in the past by Huskens and coworkers. A. Three techniques were used to characterize the monolayer and complex formation at the surface, namely water contact angle (WCA), infrared-reflection absorbance spectroscopy (IR-RAS) and electrochemistry. Substrates were functionalized overnight with βCD-7S 5 and later incubated with either Fc-PEG3-cRGD 1 or Fc2-PEG3-cRGD 3. In general water contact angle is an indirect measure of the hydrophilicity of the surface. Functionalization of gold surfaces with βCD-7S 5 resulted in a decrease in WCA from 92 to 46.8 ° corresponding with a strong increase in hydrophilicity. Further complex formation with Fc-PEG3-cRGD 1 or Fc2-PEG3-cRGD 3 did not significantly modify the hydrophilicity of the surface, as cyclodextrin and peptide displaying similar chemical functions: alcohols, amines and acids are polar groups, which will increase the hydrophilicity of the monolayer. This result shows that βCD-7S is present at the surface, and is indicative of the presence of Fc2-PEG3-cRGD at the surface as well.

![Table 4.1. Water contact angle values (n=3).](image)

IR-RAS provides information at the molecular level about which groups are displayed on the gold surface. Though complex, the IR spectrum for βCD in solution can be used to characterize immobilized on the gold surfaces. For example, peaks at 1053, 1088, 1157, 1201, 1241 and 1273 cm⁻¹ are characteristic for the different stretching (CO and CC) and bending modes (COH, OCH and CCH). The sharp peaks observed at 1660 cm⁻¹ (βCD-7S 5, βCD-7S 5 + Fc-PEG3-cRGD 1 and βCD-7S 5 + Fc2-PEG3-cRGD 3) correspond to the C=O stretch of amides present both in the bCD-7S structure, 5, as well as in the RGD peptide backbone, while those at 1430, 1540 and 1577 cm⁻¹ can be attributed to the amino acid side chains. Furthermore, the intense broad peak at 3340 cm⁻¹ is indicative of the presence of the cyclodextrin’s secondary OH groups. Some contamination of the gold disc is visible on the IR spectrum between 2600 and 3000 cm⁻¹. These results confirm the presence of βCD-7S at the surface and are a second indication that ferrocene-peptide is immobilized at the surface.
Electrochemistry is the study of the redox properties of chemical species at an electrode interface. Here, βCD-7S is subject to charge transfer via the sulfur atom, as well as ferrocene which can be reduced to Fc⁺, thereby reducing its binding affinity to βCD significantly and causing its release from the surface. Given the redox activity of both Fc and βCD, care should be taken to selectively address the ferrocene over the βCD during electrochemistry studies. Scanning between -1 and 0.6 V shows release of the thioether peak at -0.5 V (Figure 4.4 a), therefore a scan between 0 and 0.6 V will only address ferrocene and not βCD. A linear sweep voltametry scan between 0 and 0.6 V showed a flat curve for βCD-7S 5 on gold, whereas a peak at 0.25 V could be detected for βCD-7S 5 + Fc₂-PEG₃-cRGD 3 functionalized surfaces (Figure 4.4 b). This indicates the presence of ferrocene and is concordant with separate electrochemical studies, which reported a ferrocene peak at 0.2 to 0.3 V depending on the solvent and on the reference electrode (Ag/AgCl or SCE).³²,³³

After multiple CV scans in the same area, the intensity of the ferrocene peak decreased, presumably due to release of Fc₂-PEG₃-cRGD 3 from the surface to solution (Figure 4.4 c). Finally, for the cell experiments, the signal leading to appropriate release of Fc₂-PEG₃-cRGD 3 from the surface was determined. A chronoamperometry signal of 900 sec at 0.3 V was found to release Fc₂-PEG₃-cRGD 3 from the gold surface (Figure 4.4. d), as indicated by the disappearance of the peak at 0.2 V.
Figure 4.4. Electrolyte: 0.1 M K₂SO₄. Reference electrode: Ag/AgCl, working electrode: gold surface, counter electrode: Platinum. a) 1ˢᵗ (black) and 2ⁿᵈ (red) CV scans on 200 nm thick gold discs coated with βCD-7S 5 (starting potential: 0 V, scan from -1 to 0.6 V, 0.1 V/sec, 0.001 V step potential). b) Linear sweep voltammetry of 200 nm thick gold discs coated with βCD-7S 5 (black, bottom) or βCD-7S 5 + Fc₂-PEG₃-cRGD 3 (red, top) (0 to 0.6 V, 0.1 V/sec, 0.001 V step potential). Peak at 0.2 V is a ferrocene peak. c) 1ˢᵗ (black) and 1⁵ᵗʰ (red) Cyclic voltammetry scans on 200 nm thick gold discs coated with βCD-7S 5 + Fc₂-PEG₃-cRGD 3 (0 and 0.6 V, 0.1 V/sec, 0.001 V step potential). d) Cyclic voltammetry scans on 200 nm thick gold discs coated with βCD-7S 5 + Fc₂-PEG₃-RGD 3 between 0 and 0.6V before (black) and after (red) Chronoamperometry (CA) (0.2 V step, 1000 sec, 0.1 sec interval time).

2.3. QCM-D measurements

The binding affinity between βCD and ferrocene on surfaces has been studied previously. However, even slight modifications to the chemical structures of the host-guest molecules might affect their binding properties. There a detailed study was undertaken to determine the binding affinity of the ferrocene-peptide derivative on the βCD monolayers. Quartz Crystal Microbalance with dissipation (QCM-D) is a technique that permits a quantitative measure of surface binding by monitoring changes in a crystal’s resonance frequency upon analyte-surface binding. QCM-D measurements were thus performed to evaluate the binding of ferrocene-peptide conjugates to βCD-surfaces. Gold crystals were coated overnight with βCD-7S 5, rinsed with milliQ, dried and then mounted in the QCM-D. After
equilibration with PBS until a stable baseline was reached, various concentrations of ligands Fc-PEG₃-HSPNR or Fc₂-PEG₃-HSPNR 4 were flown over for 15 min, and finally PBS was flown over again for 15 min (Figure 4.5, left). The difference in frequency between the maximum frequency reached and the equilibrated surface was plotted versus the ligand concentration (Figure 4.5, right). Since the dissipation remained within 10% of the change in frequency value, a rigid film is assumed to have formed at the resonator surface and that therefore the Sauerbrey model can be applied. The curve could be fitted using a Langmuir fit and a Kₐ value of 30 μM was obtained for Fc₂-PEG₃-HSPNR 4, which compares well with similar systems previously studied. In that case, two fluorescent proteins tagged with one ferrocene moiety each and covalently locked via disulfide bridge – resulting in a bivalent ferrocene construct - were studied by SPR and a binding affinity of 25 μM was measured/calculated using the same fitting method. With Fc-PEG₃-HSPNR, concentrations up to 1 mM were not sufficient to reach saturation and thus a reliable fit could not be obtained, as previously described.

![Figure 4.5. QCM-D data for βCD-7S 5 coated crystals. a) Fifth resonance frequency overtone for a range of concentrations of Fc₂-PEG₃-HSPNR 4 (10, 25, 50, 100, 500 μM). b) Change in frequency (Hz) of the fifth overtone versus Fc₂-PEG₃-HSPNR 4 concentration (μM). Spikes in the 500 μM curve are due to air bubbles. Fit was performed using Origin, Langmuir fit, resulting in a Kₐ value of 30 μM.](image)

### 2.4. Cellular evaluation of the surfaces

Cellular studies were performed to assess the cell adhesion properties of the glass surfaces coated with βCD-7NH₂ 6 and Fc₂-PEG₃-cRGD 3. C2C12 cells were first cultured according to standard protocol. Glass coverslips were functionalized with βCD-7NH₂ 6 and with either 1, 2 or 3, C2C12 seeded and cell adhesion monitored by confocal microscopy after 1 h adhesion (Figure 4.6). The differences observed in this study concern the cell morphology and not the number of adherent cells. Indeed, cyclodextrins are composed of sugars and can if anything favor cell adhesion and spreading. Cells seeded on the control surfaces (βCD-
7NH₂ 6 and βCD-7NH₂ 6 + Fc-PEG₃-cRAD 2) displayed a round morphology, indicative of adhesion in the absence of any specific interaction. In contrast, cells on RGD-functionalized surfaces (βCD-7NH₂ 6 + Fc-PEG₃-cRGD 1 and βCD-7NH₂ 6 + Fc₂-PEG₃-cRGD 3) appeared more elongated and formed focal adhesion (Figure 4.7) indicating binding of cRGD to integrins. Immunofluorescence staining with an antibody against phosphorylated Paxillin, a protein present in focal adhesion complexes, confirms this observation (Figure 4.7, green staining). The actin skeleton appeared more organized and connected with the focal adhesion in the case of an RGD coated surface. On the control surfaces, the actin skeleton is not organized yet; there is a lack of any specific interaction between the cells and the surface.

![Figure 4.6](image_url)

**Figure 4.6.** Brightfield imaging of C2C12 cells after 60 min adhesion on glass coverslip coated with βCD-7NH₂ 6, βCD-7NH₂ 6 + Fc-PEG₃-cRAD 2, βCD-7NH₂ 6 + Fc-PEG₃-cRGD 1 and βCD-7NH₂ 6 + Fc₂-PEG₃-cRGD 3. Scale bar: 50 µm (top), 100 µm (bottom).

To study the adhesion of the cells in more detail, pictures were taken at 10 min intervals over the first hour of adhesion, starting after addition of the cell suspension. The area of 15 cells on three different substrates was measured and plotted vs. time. Cells on Fc₂-PEG₃-cRGD 3-coated surfaces were observed to adhere and spread faster than on the control surfaces (βCD-7NH₂ 6, βCD-7NH₂ 6 + Fc-PEG₃-cRAD 2 and βCD-7NH₂ 6 + Fc-PEG₃-cRGD 1). Over the entire experiment, the cell spreading was consistently larger on substrates coated with the bivalent ferrocene-RGD derivative, which indicates that cells sense the higher affinity of this bivalent compound over the monovalent controls. These considerations show that cells specifically adhere on surfaces displaying the RGD peptide in a supramolecular fashion on βCD monolayers and in a short time-frame (after 10 minutes for Fc₂-PEG₃-cRGD). Therefore this system is promising for reversibility studies.
Figure 4.7. Fluorescence images of C2C12 cells after 60 min adhesion on glass coverslip coated with βCD-7NH₂ 6, βCD-7NH₂ 6 + Fc-PEG₃-cRAD 2, βCD-7NH₂ 6 + Fc-PEG₃-cRGD 1 and βCD-7NH₂ 6 + Fc₂-PEG₃-cRGD 3. Staining: phosphorylated Paxillin (green), actin (red), nucleus (blue). Scale bar: 50 μm (top), 100 μm (bottom).

Figure 4.8. Measure of the cell area of C2C12 cells over time on glass coverslip coated with βCD-7NH₂ 6, βCD-7NH₂ 6 + Fc-PEG₃-cRAD 2, βCD-7NH₂ 6 + Fc-PEG₃-cRGD 1 and βCD-7NH₂ 6 + Fc₂-PEG₃-cRGD 3.
2.5. Reversibility

The specific release of Fc₂-PEG₃-cRGD 3 from βCD-coated gold surfaces has been demonstrated as well the specific adhesion of cells to surfaces functionalized with RGD peptide-conjugates. These important developments suggest that an electrochemical release of cells on supramolecular surfaces might also be possible. To this end, gold electrodes were fabricated in the clean room. The glass was blocked with PEG-silane to ensure specific binding of the cells on the coated gold electrodes. Then the gold part was coated with βCD-7S 5 overnight and after rinsing, was incubated if needed with Fc₂-PEG₃-cRGD 3, rinsed and dried for use in cellular studies.

C2C12 cells were seeded on the functionalized electrodes. After 30 minutes incubation to allow for cells to sink, adhere and spread over the electrodes, observation under the microscope showed that they were adhering specifically on the electrodes, with cells covering multiple electrodes (Figure 4.9 top). A chronoamperometry signal of 0.3 V was then applied on the electrodes for 5 min. Pictures taken after that revealed that cells had been released from the Fc₂-PEG₃-cRGD 3 coated surface, while cells present on the βCD-7S 5 control surface were not displaced (Figure 4.9 bottom). The electrochemical release of cells via disruption of the supramolecular complex was thus achieved.

![Figure 4.9. Gold electrodes, 10 μm glass blocked with PEG-silane, 5 μm gold coated with (left) βCD-7S 5 + Fc₂-PEG₃-cRGD 3 or (right) βCD-7S 5. Pictures were recored before (top) and after (bottom) electrodes were activated for 5 min at 0.3 V (vs. Ag/AgCl).](image-url)
This result is further corroborated by a study reported recently using βCD and a tetravalent ferrocene displaying a RGD peptide. The authors were able to electrochemically release HEK293 cells, which also express α5β3 integrins. A signal of 0.55 V vs Ag/AgCl was applied for 5 minutes and after rinsing in medium, cells were released from the surface. In our case, we waited until specific adhesion and spreading on the electrodes was detected, after 30 minutes incubation.

Another study performed with a ternary supramolecular complex (methylviologen – CB[8] – tryptophan) showed release of cells from surface upon electrochemical stimuli. In this case as well, a signal at -0.5 V vs Ag/AgCl (to reduce methylviologen) was applied for 200 sec resulting in over 90% removal of cells from the surface.

Further experiments are currently underway using confocal microscopy to study in real time the rearrangement of the actin skeleton and to monitor cell release from the surface. Although it is possible to monitor cell spreading at the surface and the specific attachment to the electrodes, initial results regarding the electrochemical release of cells in real time are not fully conclusive. Partial focal adhesion retraction is visible in some cases, but there is no clear release effect. This could be due to the fact that there is no medium flow in the experiment. Indeed, in our initial experiment and in the two other examples described above, a rinsing step was performed voluntarily or due to the experimental setup (taking the substrate out for the electrochemical stimuli before returning to the incubation well) that can help to remove cells loosely attached to the surface. Also, cells might secrete proteins within the spreading time and thus no longer specifically attach to the ferrocene-cRGD. Therefore the experiments must to be repeated, using shorter adhesion time and with the introduction of a gentle flow to help cells to detach.

3 Conclusion

βCD surfaces have been widely studied in the past from a chemical perspective. Here, we show that it is possible to functionalize ferrocene with a short functional peptide without loss of binding affinity for the host-guest complex and without modification of the electrochemical properties of the supramolecular complex on surfaces. Cells adhere specifically to cRGD-coated surfaces via focal adhesions and display clear morphological and structural differences compared to βCD- or RAD-coated control surfaces.

Finally, we could demonstrate that electrochemical activation of the supramolecular surface leads to release of cells via interaction with the RGD peptide sequence. In the future, the study of the rearrangement of the cell cytoskeleton through transfection of cells with a fluorescent actin marker could provide more detailed information about the mechanism of cell adhesion and release from the surface.
Other peptide sequences targeting specific integrins or receptors present at the cell surface could be targeted in the same way for cell immobilization purposes. In this way, for example in a microfluidic setup, it might be possible to selectively attract certain cell-types and subsequently release them. One application could be the separation of different types of stem-cells after differentiation. If the supramolecular bioconjugate is labeled, e.g. with a fluorescent dye, then tracking of the cells via FACS might also be possible. One could also think of reversible attachment of other markers, such as virus epitopes for use in biosensors.

4 Experimental

βCD-7S 5 and βCD-7NH₂ 6 were provided by Dr. Alejandro Méndez Ardoy (UTwente). Cellular evaluation of the surfaces was carried out by Dr. Wies van Roosmalen (UTwente). Gold electrodes were fabricated by MSc. Jenny Brinkmann (UTwente). Reversibility studies were performed together with MSc. Jenny Brinkmann (UTwente).

4.1. Synthesis

Synthesis and purification of the compounds was described in chapters 2 and 3.

4.2. Surface preparation for βCD monolayer on glass

Glass coverslips were sonicated for 10 min in Hellmanex, then twice for 5 min in H₂O, dried under N₂ flow and exposed to O₂ plasma for 30 s. Surfaces were thoroughly washed with H₂O, then with EtOH and dried under N₂ flow. Surfaces were placed in a vacuum dessicator overnight with (trimethoxysilyl) propyl-ethylenediamine (TPEDA). The next day, the surfaces were washed with EtOH, dipped in dry toluene and then dried. Surfaces were incubated in a 1 mM toluene solution of 1,4-phenylene diisothiocyanate at 50 °C for 2 h under N₂ atmosphere, washed with toluene, EtOH and water, and subsequently incubated for 2 h at 50 °C with a 1 mM solution of per-6-amino-b-cyclodextrin (βCD-7NH₂) 6 in H₂O. Finally, the surfaces were washed sequentially with H₂O, EtOH and then thoroughly dried under N₂ flow.

Where applicable, substrates were then incubated for 3 h with 75 µL of a 100 µM aqueous solution of the ferrocene-peptide conjugate, rinsed with H₂O and dried under N₂ flow.

4.3. Surface preparation for βCD monolayer on gold

Prior to use in QCM-D experiments, resonators were activated using a piranha solution (H₂SO₄/H₂O₂, 3:1, v/v) for 15 s. Surfaces were then extensively washed with H₂O, EtOH, and then incubated in a 1 mM solution of heptakis[6-deoxy-6-[12-(thiododecyl)undecanamido]]-b-cyclodextrin (βCD-7S 5)¹⁴,¹⁵ in CHCl₃/EtOH 2/1, heated at 60 °C for 1 h, then left at room
temperature overnight, under a N₂ atmosphere. They were then rinsed with EtOH and dried under N₂ flow.

The same protocol was followed for the preparation of substrates for IR-RAS analysis. Where applicable, the substrates were then incubated for 3 h with 75 µL of a 100 µM aqueous solution of the ferrocene-peptide conjugate 1, 2 or 3, rinsed with H₂O and dried under N₂ flow.

4.4. Characterization

IR-RAS and WCA were performed as described in chapter 2.

Electrochemistry measurements were performed using the Autolab PGSTAT10. 200 nm thick gold discs were used and coated with βCD-7S 5 as described above. All measurements were performed using Ag/AgCl electrode as reference electrode, gold surface as working electrode and platinum as counter electrode. Where applicable, the substrates were then incubated for 3 h with 75 µL of a 100 µM aqueous solution of the ferrocene-peptide conjugate, rinsed with H₂O and dried under N₂ flow. The electrolyte was a 0.1 M K₂SO₄ solution.

4.5. QCM-D measurements

QCM-D data were measured using a Q-Sense E1 with a peristaltic pump, Ismatec Reglo Digital M2-2/12. Gold-coated QCM-D resonators QSX 301 with a resonance frequency of 4.95 MHz +/- 0.05 MHz were purchased from LOT-QuantumDesign. All solutions were prepared with PBS buffer. Measurements were performed at 20 °C, with a flow of 50 µL/min. Prior to the binding of the Fc₂-PEG₃-CRGD 3, surfaces were equilibrated by flowing over PBS buffer until a stable baseline was obtained.

4.6. Cell culture

C2C12 cells, from a mouse myoblast cell line, were used at passage between 15 and 20 for the cell experiments. 80% confluent T25 or T75 flasks of C2C12 were trypsinized, centrifuged and redispersed in DMEM medium supplemented with penicillin/strep, NEAA, as well as 10% FBS for culturing and 0% FBS for reversibility experiments.

4.7. Cellular evaluation on glass

Glass coverslips coated with βCD SAM and Fc-peptide 2, 3 or 4 were briefly dipped in 70% EtOH and rinsed twice with PBS. C2C12 cells in DMEM without addition of FBS were seeded on the substrates (125 000 cells/well in 2,5 mL) and left to adhere for 1 h at 37 °C and 5% CO₂. Cells were rinsed twice with PBS, fixed for 10 min with 10% Formalin and then rinsed 3 times with PBS.

Surfaces were then incubated with blocking buffer (0.1% Triton, 0.5% w/w BSA in PBS pH 7.4) for 1 h at room temperature or overnight at 4 °C. Surfaces were subsequently incubated
with an antibody against phosphorylated paxillin Y118, diluted 1:500 in blocking buffer for 1 h, washed 3 times for 10 min with blocking buffer, and incubated for 1 h with secondary antibody-Alexa 488 (1:1000) and phalloidin-Alexa 546 (1:500) in blocking buffer. Finally the surfaces were washed once for 10 min with blocking buffer and twice with PBS, incubated for 10 min with DAPI in PBS (1:1000) and rinsed with PBS 3 times. Coverslips were mounted using Aqua Polymount and stored at -20 °C.

Imaging was performed using a Nikon Eclipse Ti confocal laser scanning microscope fitted with a Plan Apo VC 20x (NA 0.75, WD 1.0) and Apo TIRF 100x oil (NA 1.49, WD 0.12) objective and 405/488/561 lasers.

4.8. Cellular evaluation on gold (reversibility experiments)

Gold substrates coated with βCD and ferrocene-peptide were briefly dipped into 70% EtOH and rinsed twice with PBS. C2C12 cells in suspension in 0% FBS supplemented DMEM media were seeded on the substrates (60 000 cells/well in 3 mL) and left to adhere for 30 min at 37 °C and 5% CO₂. The substrate was imaged at various locations, dipped in a becher containing 10 mL of DMEM without addition of FBS together with a Ag/AgCl electrode as reference electrode and a platinum as counter electrode and a potential of 0.3 V was applied for 5 min. Afterwards, the substrate was imaged again at the same locations using brightfield microscopy.

5 References

Chapter 5

Carborane as supramolecular connector for bioactive architectures

Supramolecular chemistry is an attractive means to generate dynamic and well-controlled bioactive surfaces. Novel host-guest systems are urgently needed to provide a broader affinity and applicability portfolio. The interaction between carborane and cyclodextrin was first studied in solution via FRET experiments, but the affinity was found to be too high to be reliably studied via this system. Therefore a synthetic strategy to carborane-peptide bioconjugates was developed to provide an entry to monovalent supramolecular functionalization of β-cyclodextrin coated surfaces. The β-cyclodextrin•carborane-cRGD surfaces are formed efficiently and with high affinity as demonstrated by IR-RAS, WCA, and QCM-D, compare favourable to existing bio-active host-guest surface assemblies, and display an efficient bioactivity, as illustrated by a strong functional effect of the supramolecular system on the cell adhesion and spreading properties. Cells seeded on the supramolecular surface displaying bioactive peptide epitopes exhibited a more elongated morphology, focal adhesions, and stronger cell adhesion compared to control surfaces. This highlights the macroscopic functionality of the novel monovalent supramolecular immobilization strategy on β-cyclodextrin surfaces.

Chapter 5

1 Introduction

Using supramolecular host-guest chemistry, biomolecules can be practically and reversibly immobilized on surfaces with retention of activity. For example, functional proteins and peptide epitopes modified with a ferrocene moiety have been immobilized on cucurbit[7]uril (CB7) surfaces for the preparation of protein arrays.1–3 Similarly, beta-cyclodextrin (βCD) monolayers have also been widely studied for the immobilization of ferrocene-labeled proteins or peptides via ferrocene-βCD host-guest binding.4,5 However, the weak binding of βCD to ferrocene means that βCD monolayers necessitate a multivalent interaction to enable efficient surface immobilization.6,7 For monovalent surface immobilization on CD-functionalized surfaces, there is thus a need for new guest molecules with stronger binding properties to βCD.

Carboranes are icosahedral cluster compounds consisting of boron, carbon and hydrogen atoms. Their exceptional chemical stability, the result of pseudo-aromatic delocalization of electrons, as well as their high resistance to biological degradation predisposes carboranes to various biomedical applications. Furthermore, their high boron content render carboranes particularly useful for boron neutron capture therapy (BNCT),8 while their well-defined structures and distinctive hydrophobic properties makes them useful molecular scaffolds for drug development. For example, carboranes have shown promise as pharmacophores (for reviews see 9,10) whose geometry and peripheral substituent groups can be tuned for the construction of various tight-binding enzyme inhibitors (such as carbonic anhydrase 11 and HIV protease 12). The supramolecular field has shown renewed interest in carboranes 13–18 and metallacarboranes 19–23 due to their ability to form strong non-covalent complexes with cyclodextrins for example, for the chromatographic separation of carborane 24–27 and for the solubilization of carborane complexes containing platinum(II)-based DNA intercalators.28,29

Figure 5.1. Supramolecular monolayer formation, starting with βCD-7S coated gold, further incubation with carborane-RGD and subsequent cell immobilization at the surface.

Here we study 1,2-closo-carborane (Cb) as a monovalent supramolecular guest molecule for βCD in solution and on surfaces. The binding was studied in solution through ITC and FRET experiments, and the formation of a carborane - βCD complex on surfaces established by measuring the host-guest binding affinity using QCM-D and via the non-covalent
immobilization of biologically relevant molecules to βCD surfaces (Figure 5.1). The potential utility of the approach for tissue engineering applications was demonstrated by immobilizing integrin-binding RGD peptides to enhance adhesion of C2C12 cells to a βCD monolayer.

2 Results and discussion

2.1. Carborane-thiol derivative synthesis

Our aim was to develop conditions to couple carborane to biomolecules compatible with a broad range of molecules including peptides and proteins. For this reason we became interested in thiol-functionalized carboranes. In some examples the thiol group is directly attached to the Cb cage, though these Cbs are arguably too sterically hindered to be broadly applicable for the conjugation of biomolecules. More attractive for us were the isolated examples of water soluble Cb bearing a thiol group attached via a short linker. Therefore, to enable mild conjugation of Cb, the 1-aminomethyl-1,2-closo-carborane precursor was prepared in three steps starting from decaborane B_{10}H_{14} (Figure 5.2). We modified the previously published synthesis by implementing a recently described acetylene insertion methodology, which is performed in ionic liquid. The aminomethylene-carborane 1 was then coupled to the protected cysteine Boc-Cys(Trt)-OH via TBTU-activation and the desired product, 3, obtained upon treatment with TFA/H_{2}O/triisopropylsilane (95/2.5/2.5, %v/v).

![Figure 5.2. Synthesis of carborane-thiol 3. a) 2-(prop-2-yn-1-yl)isoindoline-1,3-dione, [BMIM]Cl, toluene, 110 °C; b) NaBH₄, i-PrOH/H₂O; c) AcOH/H₂O, HCl, 75 °C; d) Boc-Cys(Trt)-OH, TBTU, DIPEA, DMF; e) TFA/H₂O/TIS (95/2.5/2.5, %v/v). BMIM = 1-Butyl-3-methylimidazolium, TBTU = O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethylenuronium tetrafluoroborate, DIPEA = N,N-diisopropylethylamine, DMF = N,N-dimethylformamide, TIS = triisopropylethylsilane.](image-url)
2.2. βCD-Carborane interaction in solution

βCD and carborane have already been shown to adopt different binding modes in solution phase.\textsuperscript{14,17} NOE and NOESY studies, together with molecular modeling, indicated that 1-phenyl-1,2-dicarba-closo-dodecaborane (phenyl-‘o-carborane’) forms a 2:1 binding complex with βCD.\textsuperscript{14} Separate studies in borate buffer held at pH 10.5 revealed a dissociation constant $K_d$ of 11.4 µM for the binding of βCD and o-carboranyl propanol.\textsuperscript{17} We determined the binding affinity of βCD for our carborane-thiol derivative in solution using two different methods: Förster Resonance Energy Transfer (FRET) and IsoThermal Calorimetry (ITC).

For the FRET studies, the fluorescent proteins, Cyan Fluorescent Protein (CFP) and Yellow Fluorescent Protein (YFP), were chosen as FRET pairs.\textsuperscript{36,37} Two variants of each protein were studied, a monomeric and a dimeric variant: the monomeric variants have no intrinsic affinity for each other, thus binding will occur only if they are attached to two elements having some affinity for each other, and the dimeric variants favor the formation of a stable heterodimer under the influence of the supramolecular elements.

These two proteins were fused to an intein and a chitin binding domain to generate a C-terminal thioester after purification on a chitin beads column. After expression in BL21 cells and purification, the YFP variants were ligated to βCD-cysteine and CFP variants to the carborane-cysteine on overnight incubation under reducing conditions. Purification of the ligated proteins with Amicon centrifuge filters with a MWCO of 10 000 Da yielded CFP-Cb (CCb) and YFP-βCD (YCD). Successful ligation was confirmed by SDS-PAGE gel and by LCMS-QTOF in purity higher than 95% (Figure 5.3). The shift to higher masses is slightly visible on the gel due to the small change in mass (+276 g.mol\(^{-1}\) for the carborane-cysteine, and +1280 g.mol\(^{-1}\) for the βCD-cysteine addition). No trace of unligated protein could be detected by QTOF or on the SDS-PAGE gel.
Figure 5.3. (top) SDS-PAGE gel (4 to 15 %) of the four expressed proteins next to carborane-cysteine ligated to CFPs or to βCD-cysteine ligated to YFPs. (middle) QTof-LCMS of ligated protein mCFP to carborane-cysteine 3. (bottom) QTof-LCMS of ligated protein mYFP to βCD-cysteine, after purification. The main peak was deconvoluted used the MagTran program and the expected mass for the ligated product was obtained.

The two sets of monomeric and dimeric proteins were then mixed to determine the affinity of the host-guest system. First, mCCb and mYCD were mixed in a 1:1 ratio in reducing phosphate buffer. Initial experiments performed with 1 μM concentrations of proteins were too dilute to observe energy transfer, therefore concentration of proteins was increased to 5 μM for the remainder of the experiments. Controls, where one or both of the proteins are lacking a supramolecular tag, showed a small increase of intensity at 517 nm, corresponding to some energy transfer between CFP and YFP. Upon mixing of the two ligated proteins, the intensity of the peak at 517 nm (YFP excitation peak) increased slightly compared to the control experiments (Figure 5.4). This increase was also visible in the FRET ratio calculated as the intensity at 517 nm (YFP maximum) divided by the intensity at 435 nm (CFP maximum), which increased from 0.47 in the control experiments to 0.53 upon mixing of the two ligated proteins. The addition of 15 equivalents of βCD resulted in slight decrease in FRET ratio (to 0.51) indicating partial reversibility of host-guest complex formation.

The same FRET experiment was repeated with the dimeric FP variants. Here as well, control experiments on proteins lacking one or both of the supramolecular elements, resulted in a lower FRET ratio (0.51 – 0.59) compared to the solution phase experiment involving the two ligated proteins (0.86) (Figure 5.5). The intrinsic affinity of the two proteins for each other via their dimerization interface accounts for the increased ratios compared to the monomeric protein variants. Similarly, addition of 15 equivalents of βCD results in partial disruption of the complex, as attested to by the drop in FRET ratio to 0.78.
These experiments are an indication that carborane and βCD exhibit weak though measurable affinity for each other, and that they form a reversible complex, as attested to by the loss in FRET upon addition of βCD as a competitor. To determine the binding affinities of carborane for βCD, a titration of CCb to YCD was performed, using the ligated monomeric and dimeric proteins variants. The difference in intensity at 517 nm was plotted vs. the concentration of YCD for each step, and the resulting curve fitted using Origin software and the following formula:

\[
\Delta I = I_0 - I = \frac{\Delta I_{\text{max}} \cdot c(\text{acceptor})}{K_d + c(\text{acceptor})}
\]
In the case of the monomeric protein, a $K_d$ of 4.0 μM +/- 0.2 was obtained (Figure 5.6 left), compared to 2.1 μM +/- 0.3 for the dimeric protein (Figure 5.6 right). These values were higher than expected considering the protein concentrations used to measure FRET, and the increase in intensity difference is thus attributed to a homoFRET effect at high YFP concentration. Furthermore the difference in observed FRET between monomeric and dimeric variants was lower than anticipated. For example, a comparable system yielded a 10-fold decrease in affinity in the case of the dimeric variant compared to the monomeric, with the protein concentration 5 times lower than used in the experiments in this chapter.36

This solution-phase system is thus suboptimal for measuring the binding affinity of carborane to βCD given the high $K_d$ of the host-guest system and the high intrinsic FRET. Steric hindrance of the proteins attached to the supramolecular elements might also prevent the formation of stable complexes. Finally, the linker length between the carborane and the cysteine may not be optimal: a longer linker may enable the formation of a more stable complex, for example.

Another method was thus investigated to determine the binding affinity of carborane-thiol and βCD. ITC measurements provide an indication of the thermodynamics of binding between two components in solution, without the need to use fluorescent dyes or proteins as described above. The binding of the two molecules is therefore unlikely to be influenced by substituents such as the fluorescent proteins in the FRET experiments.

In a standard ITC experiment, 10 mM βCD was titrated in 1 mM carborane-thiol 3, both dissolved in phosphate buffer with 50 mM TCEP to avoid the formation of carborane dimer via a disulfide bridge. A $K_d$ value of 97 μM was obtained (Figure 5.7) which is higher than reported in the literature for the binding of βCD and o-carboranyl propanol, a molecule structurally close to the carborane-thiol studied here.17 It could be due to the difference in pH (we measured at 7.5 instead of 10.5 in the reference) and in buffer (phosphate buffer

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**Figure 5.6.** Titration of 1 μM mCCb into mYCD (left), and of 1 μM dCCb into dYCD (right) in phosphate buffer with 1 mM TCEP. Curves were fitted using Origin. $K_d, \text{monomeric} = 4.0 \text{μM +/- 0.2}$, $K_d, \text{dimeric} = 2.1 \text{μM +/- 0.3}$.
instead of borate buffer). Furthermore, the linker group between carborane and the thiol substituent might be too short to allow for optimal binding.

The binding ratio, N, was determined to be 1.78, which corresponds to 1.78 carborane bound to 1 βCD molecule. The cavity is too small to accommodate two whole carborane molecules, however one carborane could bind in the βCD entering via the larger ring, with a second interacting with the primary alcohols on the smaller ring of βCD. A 1:1 binding ratio was expected, and two different behaviors might be studied here with the ITC and with the FRET experiments. Due to steric hindrance, only a 1:1 binding ratio would be possible with the FRET, whereas the ITC only involves two small molecules without any steric hindrance, allowing the possibility for a 2:1 binding. More studies with carborane derivatives, such as NMR studies, should be performed to elucidate the exact structure of the complex in solution.

![Figure 5.7. ITC measurement of βCD (10 mM, 70 μL) in carborane-thiol 3 (1 mM, 300 μL) in phosphate buffer/50 mM TCEP. Binding ratio N = 1.78 (1 βCD for 1.78 carborane-thiol), Kd = 97 μM.](image)

2.3. βCD-Carborane interaction on surfaces

2.3.1. Synthesis

The affinity between βCD and carborane-thiol of Kd = 97 μM measured by ITC showed that there is binding of more than one carborane molecule to βCD. By studying the interaction on surfaces it is in principle possible to control the binding ratio of βCD:carborane to 1:1 by blocking one of the entrances of the BCD cavity accessible to the carborane. Our aim was to
develop conditions to couple carborane to biomolecules, which might be compatible with a broad range of molecules including peptides and proteins. RGD peptides functionalized with a maleimide group could react with the carborane-thiol 3, resulting in a carborane-RGD bioconjugate. For the introduction of the maleimide group on the cyclic RGD peptide, running the reaction at pH 7 favored selective coupling of the NHS-activated maleimide at the lysine. Reactions between the Cys-functionalized Cb and the maleimide-cRGD and maleimide-cRAD were performed in a 1:1 (v/v) mixture of DMF/PBS at pH 7-7.5 to afford 6 and 7 in yields of 63% and 5% (Figure 5.8).

![Figure 5.8. Structures of βCD-7NH₂ 8 and βCD-7S 9 and synthesis of peptide-carborane conjugates, 6 (cRGD) and 7 (cRAD). a) DMF/PBS 1/1 %(v/v), 1 h, rt.](image)
Figure 5.9. Water contact angle values for gold, βCD monolayer and βCD complexed with Cb-cRGD 6 (n=4), with a representative picture. A high WCA angle value indicates a hydrophobic surface.

To gain more insights into the formation of the βCD•Cb complex on gold substrates functionalized with βCD-7S and further incubated with Cb-cRGD 6 was studied by Infrared Reflection Absorption Spectroscopy (IR-RAS). The IR spectra of βCD\(^{41}\) in solution exhibits characteristic absorption peaks at 1053, 1088, 1157, 1204, 1241 and 1267 cm\(^{-1}\) – corresponding to different stretching (CO and CC), and bending modes (COH, OCH and CCH), which are also observed on the gold surface (Figure 5.10). Sharp peaks at 1654 cm\(^{-1}\) (βCD) and 1661 cm\(^{-1}\) (βCD + Cb-cRGD) were observed corresponding to the C=O stretch of amides present in βCD-7S structure as well as in the cRGD peptide conjugates, while the intense broad peak at 3345 cm\(^{-1}\) is characteristic for the presence of the secondary OH groups. A peak at 2582 cm\(^{-1}\) (Figure 5.10, arrow) is observed in the case of βCD + Cb-cRGD, which is indicative of complexation between βCD and Cb (B-H) and has also been observed for a similar system in solution.\(^{42,43}\)

Both surface analyses provide convincing evidence for the immobilization of carborane-peptide conjugates to the βCD-7S gold monolayers via βCD•carborane complexation.

Figure 5.10. FT-IR-RAS of βCD (top, grey) and βCD + Cb-cRGD, 6 (bottom, black) on gold, in two different regions (left: 3600 – 2000 cm\(^{-1}\), right: 2000 - 1000 cm\(^{-1}\)). The arrow shows a characteristic peak of a βCD•carborane complex.
2.3.3. Quartz Crystal Microbalance measurements

Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) measurements were performed to gain a more detailed and quantitative measurement of the affinity of Cb-cRGD, 6, with βCD monolayers. In general, a change at the surface of a quartz crystal sensor, for example binding of a compound to the surface, results in a measurable change in the vibration frequency of the sensor. Therefore, various concentrations of Cb-cRGD in PBS, ranging from 10 to 500 μM, were flown over gold crystals pre-functionalized with βCD-7S. Dissipation remained within 10% of the change in frequency value, indicating the formation of a rigid film at the resonator surface: the Sauerbrey model could then be applied.\textsuperscript{44} The change in frequency of the 5\textsuperscript{th} resonance was plotted versus the concentration of 6 and this could be fitted with a Langmuir model, resulting in a K\textsubscript{d} value of 178 μM +/- 39 μM (Figure 5.11).

![Figure 5.11. QCM-D data for βCD-7S coated quartz crystals. a) Fifth resonance frequency overtone (Δf\textsubscript{5}) for various concentrations of Cb-cRGD (10, 50, 100, 250, 500 μM). b) Change in frequency of the fifth overtone versus Cb-cRGD concentration. Fit was performed using Origin, Langmuir fit, resulting in a K\textsubscript{d} value of 178 μM +/- 39 μM.](image)

The affinity of carborane for βCD monolayer compares favorably with other known guests of βCD, such as ferrocene and adamantane. Carborane binds to βCD with 5-fold greater affinity than aminomethylferrocene derivatives and is therefore better suited for monovalent surface immobilization.\textsuperscript{4} Lithocholic acid binds to βCD with high binding affinity in solution (K\textsubscript{d} = 1.2*10\textsuperscript{-6} M), but shows limited potential for βCD surface interactions due to the guest protruding the βCD at the smaller ring.\textsuperscript{45} The affinities of carborane and adamantane for βCD monolayer are comparable.\textsuperscript{46} However, carborane introduces to the system a unique quality: high boron content, which in principle can be further utilized for quantification of conjugation yields using sensitive spectral methods such as inductively atomic emission spectrometry with inductively coupled plasma (ICP AES), as has been shown for boron-containing BODIPY dyes.\textsuperscript{47}
2.3.4. Cellular evaluation of the surfaces

Strong and directional supramolecular surface immobilization techniques/strategies provide substantial opportunities for biomedical applications. To explore the potential of the βCD-Cb complex in this respect, the ability of surface-immobilized Cb-cRGD conjugates to induce specific cell adhesion was studied using the C2C12 mouse myoblast cell line. C2C12 cells express various integrin receptors, including αVβ3, which is known to bind to cRGDfK.48,49 For this experiment, the cells were passaged at 80% confluence to avoid differentiation. The surface characterization was performed on gold surfaces coated with βCD, however glass surfaces are more suitable for fluorescence microscopy and were thus favored for the cell experiments. Surfaces featuring a βCD monolayer and a βCD monolayer complexed with bio-inactive conjugate Cb-cRAD 7 were used as reference surfaces. Cyclodextrins are composed of oligomerized glucose and therefore do not specifically discourage cell adhesion, but lack a specific molecular entity to enhance cell spreading, such as the bioactive epitope cRGD. Cells seeded on either the control βCD or βCD + Cb-cRAD substrates remained round and did not form proper focal adhesion (Figure 5.12). On the other hand, cells seeded on the βCD + Cb-cRGD surfaces became strongly anchored to the surface, evident already within 1 h of seeding, with pronounced stretching of actin filaments as a consequence of cell and focal adhesion. These results show that the cells specifically recognize the RGD sequence through binding to integrins, and that the differences in cell morphology observed between the βCD + Cb-cRGD and βCD + Cb-cRAD surfaces are a specific consequence of the difference in integrin binding affinities between cRGD and cRAD.50

Figure 5.12. Scale bar: 50 um. C2C12 seeded on glass surfaces coated with a) βCD, b) βCD and Cb-cRAD, c) βCD and Cb-cRGD, fixed after 1 h and stained for nucleus (DAPI) and actin (Phalloidin). Focal adhesions are indicated by the white arrows.

A more in-depth analysis of the cell adhesion was performed using CellProfiler 51 to obtain a quantitative difference in cell morphology under the different surface immobilization conditions. Similar studies have been performed to correlate qualitative and quantitative aspects of cell pictures.52,53 A workflow chart providing information about e.g. cell area, perimeter or eccentricity was run and data were analyzed using the software GraphPad Prism. A repeated-measures one-way analysis of variance (one-way ANOVA) test was applied on the normalized averages for each repetition and each condition. As already indicated by the simple visual aspect and observation of the focal adhesions, statistically noticeable differences in eccentricity, perimeter, form factor, compactness and ratio
between the major and minor axis lengths was only observed for the βCD monolayer complexed with Cb-cRGD compared to βCD; there is a significant difference (p < 0.05) between the control surface βCD and the active surface (βCD with Cb-cRGD). Some of these characteristic changes strongly correlate with one another.

Figure 5.13. Statistical analysis of the cell experiments with CellProfiler and GraphPad Prism. Data were normalized and averaged for each repetition towards the control βCD.

For example, the eccentricity describes the elliptical character of the cell morphology: an increase in eccentricity describes a shape that transitions from a circle, through an ellipse, to a line. Similarly, the ratio major-axis length divided by the minor-axis length will be higher in the case of an elongated cell compared to a cell displaying a more rounded morphology. This is what can be observed in Figure 5.13 a and b: the eccentricity increases from 1 for bCD to 1.12 for the Cb-cRGD surface. The ratio also increases, from 1 to 1.14.

The form factor is defined as $4\pi \times \text{area}/\text{perimeter}^2$: this value will be equal to 1 for a circle and will decrease as the perimeter of the cell increases. We observed here an increase in perimeter (from 1 to 1.24), which correlated with a decrease in the form factor (1 to 0.76)
(Figure 5.13 c and d). These results confirm the qualitative observation from the pictures and thus the functional effect of the supramolecular system on the cell adhesion and spreading properties: cells are more elongated on the $\beta$CD + Cb-cRGD surface than on the control surfaces.

3 Conclusion

Supramolecular systems offer great opportunities for the development of dynamic and well-controlled biocompatible coatings. We report the synthesis of a carborane derivative monofunctionalized with cysteine for conjugation to biologically relevant molecules such as fluorescent proteins bearing a C-terminus thioester or active peptides under mild conditions. We demonstrate the utility of the approach by conjugating the cysteine-carborane derivative to cRGD analogs via Michael 1,4-addition to a maleimide group under ambient conditions (room temperature, pH 7-7.5). The functionalization of whole (fluorescent) proteins with the cysteine-carborane derivative via expressed protein ligation or maleimide coupling is also possible. Studies of the interaction in solution via FRET were not possible due to a low binding affinity which was incompatible with the requirements for FRET measurements (low protein concentrations preferred). ITC measurements indicated the formation of a complex with 1.78 carborane units to 1 $\beta$CD molecule, meaning that one carborane was interacting at each rim of the $\beta$CD. Formation of the $\beta$CD•carborane-cRGD complex on surfaces was demonstrated by IR-RAS and WCA, and the binding affinity quantified by QCM-D. A monovalent carborane is sufficient for efficient surface immobilization and functionalization. Furthermore, cells seeded on $\beta$CD + Cb-cRGD substrates exhibited a more elongated morphology and stronger cell adhesion compared to control $\beta$CD and $\beta$CD + Cb-cRAD substrates. This opens new possibilities, in combination with other host-guest pairs, to generate innovative supramolecular surfaces of biomedical interest, displaying proteins or peptides of interest in mild conditions. For example, it could be possible to combine different specific biomolecules with various supramolecular tags and affinities and exploit the reversibility potential of e.g. ferrocene towards $\beta$CD monolayers.

4 Experimental

Plasmids of mCFP, mYFP, dCFP and dYFP and $\beta$CD-Cys were provided by Dr. Dana Uhlenheuer. Synthesis of carborane-cysteine 3 was performed by Jiri Schimer. Dr. Alejandro Mendez Ardoy is thanked for the synthesis of the $\beta$CD derivatives.

4.1. Materials

TBTU was supplied by Iris Biotech. The $\beta$CD derivatives were a kind gift from Dr. Alejandro Mendez Ardoy (University of Twente, The Netherlands). Amino acids were supplied by
Novabiochem. All other chemicals and solvents were purchased from Sigma-Aldrich. Carboranes 1 to 3 were purified using column chromatography on silica (Sigma, pore size 60 Å, 70-230 mesh, 63-200 μm). The peptide conjugates were purified using preparative scale RP-HPLC Waters Delta 600 (flow rate 7 mL/min, gradient shown for each compound - including Rt) with column Waters SunFire C18 OBD Prep Column, 5 μm, 19 x 150 mm. Compound purity was determined by analytical Jasco PU-1580 HPLC (flow rate 1 mL/min, invariable gradient 2-100 % MeCN in 30 minutes, Rt shown beside each compound) with column Watrex C18 Analytical Column, 5 μm, 250 x 5 mm. Compounds were characterized using HRMS at LTQ Orbitrap XL (Thermo Fisher Scientific) and NMR (Bruker Avance I™ 400 MHz). Products 4 to 7 were purified using RP-HPLC on a Shimadzu HPLC equipped with surveyor PDA (C18 preparative column from Phenomenex (21.20 x 150 mm), flow rate 15 mL/min). Analysis was performed using LCQ Fleet from Thermo Scientific on a C18 column equipped with surveyor AS and PDA. Eluent conditions (CH3CN/H2O/0.1% CH3CO2H) for 15 min run: 0-1 min, isocratic, 5 % CH3CN; 1-10 min, linear gradient, 5 – 100 %; 10-11 min, isocratic, 100 %; 11-12 min, linear gradient, 100 - 5 %; 12-15 min, isocratic, 5 % CH3CN, flow rate 0.1 mL/min.

4.2. Protein expression and purification

Plasmids were provided by Dr. Dana Uhlenheuer. (mCFP: pHT476 (30), mYFP: pHT486 (88), dCFP: pHT483 (57), dYFP: pHT483 (2))

Plasmids were transformed into BL21 cells and selected on ampicillin agar plates. Preculture was performed in LB media with ampicillin overnight at 37 °C, 250 rpm. The day after, 10 mL of preculture was added to 1 L of LB medium, ampicillin (100 mg.L⁻¹) was added and the culture was incubated at 37 °C for 3 h at 250 rpm, until the absorbance at 600 nm (OD600) reached 0.6-0.7. The culture was cooled down to 20 °C, 0.5 mM IPTG was added and the culture was incubated overnight at 18 °C, 250 rpm. Cells were harvested by centrifugation (5000g, 20 min, 4 °C) and the pellet was frozen at -80 °C. After defreezing, the pellet was redissolved in 25 mL BugBuster containing 25 μL benzonase, incubated for 1 h at 4 °C on the shaker and centrifuged at 10 000g for 30 min at 4 °C. The supernatant was loaded on a chitin beads column (V = 7.5 mL) equilibrated with 3V of washing buffer (25 mM NaPi, 500 mM NaCl, 0.5 mM EDTA, pH 7). The supernatant was loaded, run through, the column was washed with 10 V of washing buffer. Next, the column was washed with 3 V of cleavage buffer (25 mM NaPi, 500 mM NaCl, 0.5 mM EDTA, 500 mM MESNA, pH 7.5), placed in the incubator at 18 °C overnight. 3 V of cleavage buffer was run through and fractions were collected. The pooled fractions were concentrated and the buffer exchanged 3 times to storage buffer (25 mM NaPi, 50 mM NaCl, 0.1 mM EDTA, 20 mM MESNA, pH 7.5) to reach a final concentration of 100 μM. After aliquoting the proteins were frozen in liquid nitrogen and stored at -80 °C. Around 40 mg per liter culture of mCFP and dCFP were obtained, as well as 24 mg for mYFP and 6 mg for dYFP.
SDS-PAGE electrophoresis was carried out on a Mini-PROTEAN 3 electrophoresis system (Biorad, Hercules, California). Precasted gels (4-15%, 10 well, 30 μL) were purchased from Biorad. Running buffer composition: 25 mM Tris-HCl, 250 mM glycine, 0.1% (w/v) SDS in H₂O. The runs were performed at room temperature at 90 V for 20 min and then at 140 V for 60 min. The gel was stained with Coomassie Brilliant Blue.

LCMS-QTof:

mCFP calculated: 29954 Da [M+H⁺], found: 29953 Da [M+H⁺]
dCFP calculated: 29972 Da [M+H⁺], found: 29973 Da [M+H⁺]
mYFP calculated: 28595 Da [M+H⁺], found: 28595 Da [M+H⁺]
dYFP calculated: 28612 Da [M+H⁺], found: 28610 Da [M+H⁺]

4.3. Protein ligation

Ligation of CFP-thioester to carborane-Cys: An 8 mM solution of carborane-Cys was prepared in ligation buffer (30 mM NaPi, 50 mM NaCl, pH 7.5), as well as a 400 mM MPAA + 80 mM TCEP stock solution in milliQ, pH adjusted to 7. Protein concentration was typically 2-3 mg/mL. The carborane-cys solution and the protein solution were mixed in a 10:1 ratio, together with the MPAA-TCEP solution (1:1 v/v with the protein), pH was adjusted to 7 and the reaction proceeded overnight at room temperature on a rotating wheel.

Ligation of YFP-thioester to βCD-Cys: An 8 mM solution of βCD-Cys was prepared in ligation buffer (30 mM NaPi, 50 mM NaCl, pH 7.5), as well as a 400 mM MPAA + 80 mM TCEP stock solution in milliQ, pH adjusted to 7. Protein concentration was typically 2-3 mg/mL for mYFP and 0.6 mg/mL for dYFP. The βCD-Cys solution and the protein solution were mixed in a 10:1 ratio, together with the MPAA-TCEP solution (1:1 v/v with the protein), pH was adjusted to 7 and the reaction proceeded overnight at room temperature on a rotating wheel.

Purification was performed with Amicon 0.5 mL, (10 000 g.mol⁻¹ MWCO). After 2V wash with buffer (30 mM NaPi, 50 mM NaCl, pH 7.4) (5 min, 13 400 rpm), sample was loaded and washed 5 times with buffer (5 min, 13 400 rpm). Finally, the ligated protein was eluted (2 min, 10 000 rpm) and stored in 25 μL aliquots at –80 °C.

Purity of the ligated protein was assessed by SDS-PAGE gel and by QTof LCMS.

mCFP-Cb (mCCb) calculated: 30088 Da [M+H⁺], found: 30255 Da [M+MPAA+H⁺]
dCFP-Cb (dCCb) calculated: 30106 Da [M+H⁺], found: 30106 Da [M+H⁺]
mYFP-βCD (mYCD) calculated: 29734 Da [M+H⁺], found: 29900 Da [M+MPAA+H⁺]
dYFP-βCD (dYCD) calculated: 29751 Da [M+H⁺], found: 29749 Da [M+H⁺]
4.4. ITC

ITC measurements were performed on a ITC200 Microcal-GE. 300 µL of 1 mM carborane-cysteine was placed in the cell, while 70 µL of 11.28 mM βCD-cysteine was loaded in the seringue. Both solutions were prepared in reducing phosphate buffer (30 mM NaPi, 50 mM NaCl, 50 mM TCEP, pH 7.4). 32 injections steps of 1.2 µL for 2.4 sec with 150 sec spacing were performed (initial volume of 0.4 µL for 0.8 sec with 150 sec spacing), with an initial delay of 10 sec and a stirring speed of 1500 rpm. The temperature of the cell was 25 °C.

4.5. FRET

FRET measurements were performed on a Cary Eclipse fluorescence spectrometer (Varian) equipped with a Perkin-Elmer PTP-1 Peltier temperature control system. Measurements were carried out in quartz cuvettes (Hellma), 10 mm light path, 200 µL volume for 1:1 mixtures or 2 mL volume for titration, at 5 µM concentration in phosphate buffer (30 mM NaPi, 50 mM NaCl, pH 7.4) with 100 mM TCEP, at 20 °C. Protein concentration was determined with NanoDrop ND-1000 spectrophotometer by UV/Vis spectroscopy, at 435 nm absorbance, using ε_{435} = 32500 M^{-1}cm^{-1} for CFP and ε_{515} = 84000 M^{-1}cm^{-1} for YFP. Fluorescence emission spectra were recorded at an excitation wavelength of 410 nm. Experiments were performed in duplicate.

4.6. Synthesis of carborane-cRGD and carborane-cRAD conjugates

4.6.1 (aminoethyl)-o-carborane hydrochloride (1) and carborane-cysteine (3) syntheses

1 and 3 were synthesized as described in reference 54.

4.6.2. cRGD-maleimide 4 and cRAD-maleimide 5 synthesis

cRGD and cRAD were synthesized according to previous literature.² 20 mg of peptide was reacted with NHS-activated maleimide (synthesized according to previous literature)⁵⁵ (1.4 eq.) in dry DMF (1 mL) for 1 h at rt in the presence of DIPEA (4 eq.). The solvents were then removed in vacuo and the peptide-maleimide conjugates were purified by preparative-RP HPLC (gradient 10 - 25% MeCN, 0.1 % HCO₂H in 20 min) to afford cRGD-maleimide 4 and cRAD-maleimide 5 in yields of 25% and 28%, respectively, both as white powders.

4: Analytical HPLC Rₜ = 2.55 min. MS (ESI+): calculated for C_{34}H_{46}N_{10}O_{10} [MH]^+ 755.79 Found 755.67.

5: Analytical HPLC Rₜ = 2.55 min. MS (ESI+): calculated for C_{35}H_{49}N_{10}O_{10} [MH]^+ 769.79 Found 769.67.

4.6.3. Cb-cRGD 6 and Cb-cRAD 7 synthesis

5.8 mg of cRGD-maleimide (resp. 3 mg of cRAD-maleimide) was dissolved in PBS (30 mM sodium phosphate, 50 mM NaCl, pH 7.4) and added to 1 (1 eq.) dissolved in 1 mL DMF.
reaction was stirred at room temperature for 1 h and the solvents were removed in vacuo. Purification was performed using preparative RP-HPLC (gradient 20-50% MeCN, 0.1 % HCO₂H in 30 min). Yields for Cb-cRGD 6 and Cb-cRAD 7 were 63 and 5 %, respectively.


7: Analytical HPLC Rₜ = 4.49 min. MS (ESI+): calculated for C₄₁H₇₀B₁₀N₁₂O₁₁S [MH]+ 1047.25. Found 1046.75

4.7. Surface preparation for βCD immobilization on glass coverslips

Glass coverslips were sonicated for 10 min in Hellmanex, then twice for 5 min in H₂O, dried under N₂ flow and exposed to O₂ plasma for 30 s. Surfaces were thoroughly washed with H₂O, then with EtOH and dried under N₂ flow. Surfaces were placed in a vacuum dessicator overnight with (trimethoxysilyl) propyl-ethylenediamine (TPEDA). The next day, the surfaces were washed with EtOH, dipped in dry toluene and then dried. Surfaces were incubated in a 1 mM toluene solution of 1,4-phenylene diisothiocyanate at 50 °C for 2 h under N₂ atmosphere, washed with toluene, EtOH and water, and subsequently incubated for 2 h at 50 °C with a 1 mM solution of per-6-amino-b-cyclodextrin (βCD-7NH₂, 8, Figure 1) in H₂O. Finally, the surfaces were washed sequentially with H₂O, EtOH and then thoroughly dried under N₂ flow.

Where applicable, substrates were then incubated for 3 h with 75 µL of a 100 µM aqueous solution of the carborane-peptide conjugate, rinsed with H₂O and dried under N₂ flow.

4.8. Surface preparation for βCD on gold substrates

Prior to use in QCM-D experiments, resonators were activated for 15 s using a piranha solution (H₂SO₄/H₂O₂, 3:1, %v/v). Surfaces were then extensively washed with H₂O, EtOH, and then incubated in a 1 mM solution of heptakis[6-deoxy-6-[12-(thiododecyl)undecanamido]]-b-cyclodextrin (βCD-7S, 9, Figure 1)³⁹,⁴⁰ in CHCl₃/EtOH 2/1, heated at 60 °C for 1 h, then left at room temperature overnight, under a N₂ atmosphere. They were then rinsed with EtOH and dried under N₂ flow.

The same protocol was followed for the preparation of substrates for IR-RAS analysis. Where applicable, the substrates were then incubated for 3 h with 75 µL of a 100 µM aqueous solution of the carborane-peptide conjugate, rinsed with H₂O and dried under N₂ flow.

4.9. Characterization of βCD – carborane-peptide surfaces

Fourier Transform Infrared Reflection Absorption Spectroscopy (FT-IR-RAS) measurements utilized 200 nm gold Si wafers, 2x2 cm. Polarized FT-IR-RAS spectra of 1000 scans with a resolution of 2 cm⁻¹ were obtained using a Thermo Scientific TOM optical module.
Water contact angle measurements were performed on a Krüss G10 contact angle measuring instrument, equipped with a CCD camera. Images were analyzed using the software Drop Shape Analysis version 1.90.0.2 and the ImageJ Contact Angle plug-in.

### 4.10. QCM-D measurements

QCM-D data were measured using a Q-Sense E1 with a peristaltic pump, Ismatec Reglo Digital M2-2/12. Gold-coated QCM-D resonators QSX 301 with a resonance frequency of 4.95 MHz +/- 0.05 MHz were purchased from LOT-QuantumDesign. All solutions of Cb-cRGD were prepared using PBS buffer. Measurements were performed at 20 °C, with a flow of 50 μL/min. Prior to the binding of the Cb-RGD 6, surfaces were equilibrated by flowing over PBS buffer until a stable baseline was obtained.

### 4.11. Cell culture

C2C12 cells, from a mouse myoblast cell line, were used at passage between 15 and 20 for the cell experiments. 80% confluent T25 or T75 flasks of C2C12 were trypsinized, centrifuged and redispersed in DMEM medium supplemented with penicillin/strep, NEAA, as well as 10% FBS for culturing and 0% FBS for surface incubation experiments.

### 4.12. Cell adhesion

Glass substrates coated with βCD and carborane-peptide were dipped in and out into 70% EtOH and rinsed twice with PBS. Cells in suspension in 0% FBS supplemented DMEM media were seeded on the substrates (20 000 cells/mL, 3 mL/well) and left to adhere for 1 h at 37 °C and 5% CO₂. Surfaces were then gently washed twice with PBS, cells were fixed for 10 min with 10% formalin and then rinsed three times with PBS.

Cells were incubated with blocking solution (0.1% Triton, 0.5% w/w BSA in PBS pH 7.4) for 1 h at room temperature or overnight at 4 °C. Surfaces were then incubated with Paxillin 1:500 in blocking buffer for 1 h, washed 3 times for 10 min with blocking buffer, and incubated for 1 h with secondary antibody-Alexa 488 (1:500) and phalloidin-Alexa 546 (1:500) in blocking buffer. Finally the surfaces were washed once for 10 min with blocking buffer and twice with PBS, incubated for 10 min with DAPI in PBS (1:1000), rinsed with PBS three times, and then stored at 4 °C.

Imaging was performed using an Olympus IX71 fluorescence microscope, at 40x magnification.

All experiments were performed in triplicates.

### 4.13. Cell analysis

Five pictures per substrate were taken for each of the three repetitions and analysis of the cell adhesion was performed using CellProfiler. Cells that could not be recognized by the software or that did not fall completely in the field of view were discarded from the analysis.
On average, between 30 and 40 cells per substrate per set remained for analysis, corresponding to approximately 100 cells per condition. Results were normalized towards the average value obtained for each experiment set for the βCD control surface.

5 References

Chapter 6

Supramolecular immobilization of RGD-containing fluorescent proteins via ternary complex formation

Since their discovery 50 years ago, fluorescent proteins have been widely used to study biological phenomena such as protein-protein interactions or to help visualize the close proximity of molecules via FRET. Here, a yellow fluorescent protein derivative, mCitrine, was used as a scaffold for the supramolecular immobilization of the RGD peptide promoting cell adhesion. Thanks to the fluorescent properties of the protein, it will be visible at the surface, while a RGD peptide sequence can be engineered into one of the loops of the protein, together with a WGG sequence at its N-terminus. Via a supramolecular ternary complex methylviologen – cucurbit[8]uril – tryptophan, the protein could be immobilized on surfaces and tested for cell adhesion. The protein remains fluorescent and promotes cellular adhesion of C2C12 cells, and important progresses towards specific adhesion of the protein at the surface via supramolecular interactions were accomplished.
1 Introduction

Fluorescent proteins (FP) are a useful tool to study biological processes. The first fluorescent protein reported was the Green Fluorescent Protein (GFP) back in the 1960’s\textsuperscript{1}, which was isolated from the jellyfish \textit{Aequorea} and crystallized in 1974.\textsuperscript{2} This protein has been extensively studied and mutated to obtain a range of colors and excitation wavelengths. First, mutations in the beta-barrel around the chromophore (Ser65 – Tyr66 – Gly67) led to a simplification of the emission spectra and to an increase in brightness.\textsuperscript{3,4} The yellow variant YFP could be obtained by one single mutation (T203Y)\textsuperscript{5} and its stability towards chloride ions and acidic pH was improved by the mutation Q69M to obtain Citrine.\textsuperscript{6} Finally, to favor the monomeric form of the protein, another point mutation (A206K) \textsuperscript{5} was performed, yielding mCitrine.

A toolbox of fluorescent proteins exists to create sensors and help the understanding of various biological phenomena such as protein localization, protein-protein interaction, small molecule affinity or cellular mechanisms.\textsuperscript{7,8} Nowadays, a large variety of dyes is also available, but they require post-modification of proteins or molecules, whereas coupling to FPs can be engineered and performed in one step. Fluorescent proteins can be paired to measure Förster Resonance Energy Transfer (FRET): an energy transfer occurs from a donor protein to an acceptor protein when the two are held in close proximity. This method is used to measure affinity between small molecules ligated to FPs,\textsuperscript{9} or to visualize protein interaction and cellular processes.\textsuperscript{10} FPs can also be fused for example to viruses capsids\textsuperscript{11} or to transmembrane proteins\textsuperscript{12} to visualize the localization of specific molecules within cells or organisms.

Mutations in FPs are usually carried out to ameliorate its fluorescence characteristics and its stability. But these proteins can also be genetically modified to add specific sequences and functions at particular locations along the amino acid sequence of the protein. Modifications on the more rigid beta-barrel can significantly modify the behavior of the protein, such as its fluorescence properties and its dimerizing capabilities, and are therefore not recommended.\textsuperscript{3} Instead, modifications in more flexible loop regions, further away from the chromophore, are favored, which are also more accessible for ligand-protein and protein-protein interactions. Indeed, circular permutations within GFP are possible without loss of fluorescence (Figure 6.1.),\textsuperscript{13} allowing the insertion of calmodulin (Ca\textsuperscript{2+} binder) or of a zinc finger in EYFP at position Tyr145 which fluoresce upon metal binding. To the best of our knowledge, it is the only example of epitope engineering in fluorescent protein.
Figure 6.1. Topologies of GFP, cpGFP, and chimeras with other proteins. The other proteins are depicted schematically as spheres, when their sequences remain contiguous, or as paired hemispheres, when GFP or cpGFP is inserted within them. N and C denote amino and carboxyl termini of the full protein or chimera. Tandem fusions b and f arbitrarily show the carboxyl terminus of the GFP or cpGFP fused to the amino terminus of the other protein, but also are intended to encompass the opposite order. Adapted with permission from reference 13.

FPs have in the past been used in combination with supramolecular surfaces to model protein immobilization on surfaces.\textsuperscript{14-16} Using the supramolecular systems described in chapters 2, 3 and 4, our group was able to modify and immobilize ferrocene-labeled YFP on CB[7] or βCD-coated surfaces and to achieve reversible micrometer resolution printing.\textsuperscript{15,16} Their relatively high stability facilitates their chemical modification with supramolecular tags for visualization of host-guest complex formation on surfaces. In figure 6.2, YFP and CFP were modified with bisadamantane and patterns could be formed on βCD-monolayers.\textsuperscript{17} The ternary complex formed by methylviologen (MV), cucurbit[8]uril (CB[8]) and tryptophan (W) is also a suitable candidate for such surface immobilization. Short peptide sequence such as WGG or FGG can be engineered into the N-terminus of proteins for further immobilization.\textsuperscript{18} Since it is a heterocomplex, formation of the complex is better controlled than the Phe - CB[8] - Phe interaction.\textsuperscript{19} Immobilization of a short peptide bearing a N-terminus WGG tag has already been successfully achieved to study the formation of the ternary complex MV - CB[8] - W.\textsuperscript{20} This complex also has the potential to undergo electrochemical reversibility, which could serve as a powerful means to manipulate the immobilization of biologically relevant compounds, such as peptides, proteins or cells on surfaces.
Here we make use of a scaffold possessing three distinct functions: a supramolecular tag for surface immobilization, fluorescence for the ease of detection and functional peptide sequence for biological activity. The fluorescent protein acts as a scaffold to accommodate the RGD and the WGG peptides and should allow for a specific supramolecular immobilization of the protein on surfaces, as well as cell adhesion activity towards endothelial cells. Once these properties are established, electrochemical activation of the surface should enable the release of the protein together with the cells.

2 Results and discussion

2.1. WGG-mCitrine-RGD design and cloning

The RGD peptide sequence was discovered 30 years ago from fibronectin, an extra-cellular matrix protein, and its cell adhesion properties have been widely applied since then (see Chapter 1 for more details). In previous research, Dr. Michael Sonntag and M.Sc. Jurgen Schill in our group cloned mCitrine to add a GRGDS sequence to it, either by insertion, or by replacement of existing amino acids. This work was performed at 10 different positions of the protein, mostly in loops, in order to minimize the impact on protein stability and its fluorescence. The activity of the different proteins was tested using four integrins known to bind to RGD (α6β3, α5β1 or α5β3) or serving as a negative control (α4β1).21 Two insertion positions, between amino acids 140 and 141, and between 194 and 195, were discovered to display the highest activity towards integrins α6β3 and α5β3. Surprisingly, both proteins exhibited low activity towards integrin α5β1. Although RGD is a known ligand for
this integrin, it could be that the geometry of the RGD in the loop at position 140/141 is not optimal, which could result in selectivity towards certain types of RGD binding integrins.\textsuperscript{23} The protein construct with the insertion at position 194/195 couldn’t be cloned successfully, and insertion of RGD at position 140/141 led to a stronger emission of the protein at 525 nm compared to insertion at position 194/195, or a double insertion: insertion at position 140/141 was thus the ideal candidate for further immobilization experiments.

Methyliviologen-CB8-tryptophan are known to form a stable complex on surfaces, with high affinity ($K_{\text{ter}} = 4.10^9 \text{ M}^{-1}$).\textsuperscript{19,20} In contrast to the complex with F – CB[8] – F, two MV or two W are incapable of simultaneously fitting into the same CB[8] cavity, which therefore the formation of the heteroternary complex. In this way the presence of all three components (MV, CB[8] and the protein) is assured and the FP can be anchored at the surface. Therefore the WGG sequence was engineered into the N-terminus of the FPs to facilitate studies into protein surface immobilization. An important design feature is that the WGG and RGD sequences are located at remote positions from one another on the fluorescent protein (Figure 6.3.), to ensure accessibility to cell integrins and thus facilitate surface immobilization via ternary complex formation.

![Supramolecular immobilization of RGD-containing fluorescent proteins via ternary complex formation](image)

**Figure 6.3.** (left) Structure of WGG-mCitrine-GRGDS140/141-Strep (crystal structure PDB: 3DQ3). The GRGDS sequence is inserted between the positions 140 and 141 of the protein (red loop) and WGG was added at the N-terminus for supramolecular immobilization. (right) Surface preparation: gold surfaces functionalized with 1% maleimide are incubated with the preformed MV – CB[8] – protein complex, resulting in supramolecular protein immobilization at the surface.

Cloning was performed in two steps. First, the pH293 vector of His-mCitrine-GRGDS 140/141-Strep was amplified via PCR with a WGG-SapI primer to introduce the WGG sequence at the N-terminus of the protein, and subsequently sub-cloned into TOPO-TA vector. After colony picking and sequencing, the construct was cloned in a PTWIN1 vector.
2.2. WGG-mCitrine-RGD expression and purification

The protein was successfully expressed in BL21 and purified by Ni-NTA column, followed by PD10 column to remove imidazole and chitin column to remove CBD and CBD-Citrine. After these three purification steps, the protein purity was assessed by SDS-PAGE gel (Figure 6.4.) and LCMS-QTOF (Figure 6.5.) to be >95%. The SDS-PAGE gel shows that the protein is still fluorescent and runs to the expected height, at circa 29 kDa. Both the flow through and the wash fractions were of similar purities, although some bands are visible corresponding to the CBD-Intein-WGG-His-mCitrine-RGD-Strep as well as for CBD-Intein. LCMS-QTOF confirmed that the target protein had the desired mass ($M_w = 29896 \text{ g.mol}^{-1}$). Finally, the fluorescence spectrum of WGGmCitRGD was recorded. Upon excitation at 515 nm, a single emission peak was detected at 525 nm, which corresponded to values previously reported in literature thus confirming the protein’s integrity (Figure 6.7.). From here on, the protein will be referred to as WGGmCitRGD.

![Figure 6.4. SDS-PAGE of WGG-mCitrine-140/141-Strep flowthrough (1) and wash (2). Left: UV illumination (350 nm), right: Coomassie blue staining.](image)

![Figure 6.5. Q-Tof LCMS of WGG-mCitrine-RGD (wash fraction). Calculated mass: 29896 g.mol$^{-1}$, found: 29896 g.mol$^{-1}$.](image)
Figure 6.6. Molecules used in this chapter.

Figure 6.7. Fluorescence emission spectrum of WGG-mCitrine-140/141-GRGDS-Strep, 1 μM, in phosphate buffer. Excitation: 515 nm.
2.3. Surface preparation

Immobilization of proteins on surface can be challenging given the relative instability of proteins. Here, the WGG tripeptide was conveniently engineered into the protein N-terminus to enable immobilization on surfaces that can be performed under conditions compatible with proteins (i.e. aqueous media, appropriate pH, short incubation times). To obtain patterns, a gold array (5 μm wide) on glass (10 μm wide) was fabricated in the clean room and functionalized. The glass array was blocked with PEG-silane to avoid unspecific binding of proteins and cells. The gold array was then further functionalized with disulfide-alkane-PEG 5 mixed with 1% of disulfide-alkane-PEG-maleimide 4 overnight (Figure 6.7). The alkane-PEG molecules create an anti-fouling layer to prevent unspecific adhesion of cells in the latter stage of the project. It also allows for a controlled density of maleimide functionality at the surface. The ternary complex preformed in PBS with methylviologen-thiol 1 (100 μM), CB[8] (100 μM) and WGGmCitRGD (70 μM) or with a control protein WGGCFP (20 μM) was incubated for 1 h with surfaces, dipped in PBS for rinsing and dried with N₂ flow.

Fluorescent patterns were observed on the arrays (Figure 6.8.), as seen with the profiles obtained with ImageJ. Furthermore, the presence of green dots on the fluorescence images suggested that protein aggregates were formed when WGGmCitRGD was used, which were not present in the case of WGGCFP. This could be due to the difference in protein concentration as well as in the treatment for protein expression (different expression media, different date of expression, different concentration for storage). Unspecific binding of protein on the glass could also be detected, as there is fluorescence on the glass, even though it has been coated with PEG-silane. The difference of intensity between gold and glass arrays is not very significant (Figure 6.8).

Upon addition of C2C12 onto those surfaces in serum-free medium, after 1 h incubation, fixation and staining, cells spread over multiple electrodes on the WGGmCitRGD substrate, whereas they remained on one electrode on the control surfaces (CFP) (Figure 6.8.). This indicates that the RGD sequence is active and responsible for specific cell spreading on the electrodes, and that the control protein CFP has no cell adhesion effect.

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1 The monolayer formation, as well as conditions for the functionalization of the ternary complex and its immobilization on gold surface have been studied and established by MSc. Jenny Brinkmann and will not be discussed here.
Supramolecular immobilization of RGD-containing fluorescent proteins via ternary complex formation

Figure 6.8. Scale bar 50 µm (top), 100 µm (bottom). a) MVSH 1 - CB[8] - WGGmCitRGD, b) MVSH 1 - CB[8] - WGGCFP, c) MVSH 1 - WGGCFP. (top) Fluorescence microscopy. (middle) ImageJ surface profiling over 5 electrodes. (bottom) cell experiments, C2C12 fixed after 1 h incubation time and stained for actin (Alexa 546-phalloidin, red) and nucleus (DAPI, blue).

We initially assumed that for proper complex formation at the surface, high concentrations of the three components of the ternary complex was needed (MVSH 1 = CB[8] = 100 µM, WGGmCitRGD = 70 µM). However, this high protein concentration might be the cause of the high unspecific binding observed in the first set of experiments (Figure 6.8.). Therefore, the concentration of the protein WGGmCitRGD was reduced to 4 µM. Addition of Tween 20 has also been shown to reduce unspecific adhesion of protein to surfaces and 0.1 % Tween 20 was added to the PBS buffer. Finally, the controls were performed with WGGmCitRGD protein instead of WGGCFP. As control experiments, the following setting were included: WGGmCitRGD and MVSH 1 without CB[8] to test for unspecific adhesion of the protein at the surface and to show the supramolecular character of the interaction, and WGGmCitRGD, CB[8] and MV lacking the thiol functionality, which should prevent the complex to be covalently attached to the surface and test for unspecific adhesion as well.

A reduction in protein concentration with simultaneous addition of Tween 20 dramatically improved the specificity of the complex formation: fluorescence could then only be detected on electrodes and no longer on the glass. However, aggregates were still present (Figure 6.9 top). An extra rinsing step of 15 minutes under gentle shaking in 0.1 % PBST was tested to see if protein aggregates would dissociate. This resulted in a complete loss of fluorescence on the surface displaying the supramolecular complex (Figure 6.9 a bottom) and in a slight loss of fluorescence on the control surface lacking the thiol anchor for MV
(Figure 6.9 c bottom), indicating unspecific adhesion of the complex at the surface and potential maleimide coupling to the protein. In the future, a surface without maleimide functionality would be an appropriate control to determine whether the protein can react with the maleimide.

![Image of fluorescence microscopy images](image)

*Figure 6.9. Scale bar 100 µm. Fluorescence microscopy images, 10x magnification. a) MVSH 1 - CB[8] - WGGmCitRGD, b) MVSH 1 - WGGmCitRGD, c) MV - CB[8] - WGGmCitRGD. (top) before rinsing. (bottom) after 15 min rinsing in 0.1% PBST.*

Upon incubation with cells for 1 h, fixing and staining for actin and nuclei on the surfaces described in figure 6.9, results showed that cells adhered and spread on the three conditions, including the control experiments without CB[8] or without thiol to anchor the methylviologen to the surface (Figure 6.10 top). The degree of cell spreading did not alter on surfaces rinsed for 15 minutes in PBS + 0.1 % Tween 20 (Figure 6.10 bottom). This suggests that there is a thin layer of protein WGGmCitRGD present on the glass, unspecifically adsorbed on PEG and not detectable under these conditions with fluorescence microscopy. Another hypothesis is that protein WGGmCitRGD could be present between the aggregates and the surface, which is difficult to detect by fluorescence microscopy, but nevertheless present. This layer might be detectable using a longer exposure time of the microscope’s shutter. But it might also be quenched by the gold array and thus be difficult, if not impossible to detect via conventional fluorescence microscopy. Alternative use of confocal microscopy might enable detection of protein at the surface.
Figure 6.10. Scale bar: 100 μm. Cell experiments, C2C12 fixed after 1 h incubation time and stained for actin (Alexa 546-phalloidin, red) and nucleus (DAPI, blue). a) MVSH-CB[8]-WGGmCitRGD, b) MVSH-WGGmCitRGD, c) MV-CB[8]-WGGmCitRGD. (top) before rinsing. (bottom) after 15 min rinsing in 0.1% PBST.

We also hypothesized that the high CB[8] concentration could induce protein complex formation via aromatic side chains complexation, such as Phe-CB[8]-Phe ternary complex formation.\(^{25,26}\) In a separate experiment, the CB[8] concentration was thus decreased from 100 to 10 μM while the concentrations of MVSH 1 and WGGmCitRGD protein were kept constant, but this resulted in an increased unspecific binding on the glass part of the arrays. To try to reduce the aggregate formation, protein sample was centrifuged at 13 000 rpm for 10 minutes before ternary complex formation, but aggregates could still be detected on surfaces.

3 Conclusion

Fluorescent proteins are a useful tool to study biological processes thanks to the ease of detection, their stability and the possibility to attach them to other proteins or molecules of interest. Here we successfully engineered a mCitrine fluorescent protein featuring a N-terminal WGG peptide for supramolecular immobilization, and an RGD peptide sequence in the loop between amino acids 140 and 141 to promote cell adhesion. This construct was cloned and expressed, as attested by SDS-PAGE gel and LCMS-QTOF analysis and is functional, both in terms of fluorescence and of integrin binding activity. Next, the protein was immobilized on gold arrays via the ternary complex MV - CB[8] - WGG and conditions for specific protein binding and cell activity were studied and improved. The RGD sequence inserted in the protein was active and promoted efficient cell adhesion.
Nevertheless, protein aggregates could still be detected and unspecific binding of the protein on gold occurred, as seen by the cell experiments. The expression of a fresh batch of protein for testing may reduce the formation of aggregates. Furthermore, the use of BSA instead of Tween 20 might lead to higher specificity. Indeed, Tween 20 at significant concentrations might perturb the ternary complex formation. Although BSA was first envisioned to prevent complex anchoring on the maleimide monolayer, it might be that low complex density does not prevent proper cell spreading. The platform described in this chapter is promising to study the attachment and the release of cells on a supramolecular surface. Fluorescence monitoring together with cell imaging could enable simultaneous visualization of the complex disruption upon electrochemical signal together with cell release. Virtually any protein of interest could be immobilized and controlled using this supramolecular system to form e.g. protein biochips or biosensors.

4 Experimental

Protein cloning, expression and purification were carried out by Dr. Michael Sonntag. MVSH synthesis was performed by MSc. Carmen Stoffelen (UTwente). Gold arrays were fabricated by MSc. Jenny Brinkmann. Characterization of the surfaces and cell experiments were performed at UTwente together with MSc. Jenny Brinkmann.

4.1. Protein cloning

WGG-mCitrineRGD was obtained via PCR amplification of the His-mCitrine-RGD from the pHTE293 constructs. Sub-cloning in TOPA-TA vector and subsequent cloning in pTWIN1 vectors were then performed.

4.2. Protein expression and purification

Preculture was performed in LB media with ampicillin overnight at 37 °C, 250 rpm. The day after, 20 mL of preculture was added to 1 L of Studier’s rich auto-induction medium (supplemented with 110 µg/mL Ampicillin and without a-lactose) and incubated at 37 °C for 3.5 h at 200 rpm, until the absorbance at 600 nm (OD600) reached 3.2. The culture was cooled down to 18 °C, 0.2 mM IPTG was added and the culture was incubated overnight at 18 °C, 200 rpm. Cells were harvested by centrifugation (10 000g, 10 min) and the pellet was frozen at -80 °C. 14 g of pellet was obtained per liter culture. To prepare samples for SDS-PAGE gel, 0.5 mL culture was centrifuged (13 000g, 10 min) and the pellet was dissolved in 500 µL BugBuster containing 0.5 µL benzonase. After incubating for 45 min at room temperature on a shaker, the solution was centrifuged at 13 000g for 10 min to separate the pellet from the supernatant. The pellet was dissolved in 200 µL of 6M urea and 5µL of pellet or supernatant was loaded on a SDS gel, revealing that most of the protein was in the pellet. To catch the protein from the soluble fraction, the pellet was dissolved in 30 mL BugBuster containing 30 µL benzonase, incubated for 1 h at room temperature on the shaker and
centrifuged at 10 000g for 40 min at 4 °C. The supernatant was loaded on a 3.5 L Ni-NTA column equilibrated with 20 mM TRIS, 150 mM NaCl at pH 7.9. The column was washed with 15 CV and eluted with 5mM, 20 mM and 300 mM imidazole (3CV). Protein was purified further on PD10 column and on chitin column.

4.3. SDS-PAGE

SDS-PAGE electrophoresis was carried out on a Mini-PROTEAN 3 electrophoresis system (Biorad, Hercules, California). Precasted gels (4-20%, 10 well, 30 µL) were purchased from Biorad. Running buffer composition: 25 mM Tris-HCl, 250 mM glycine, 0.1% (w/v) SDS in H2O. The runs were performed at room temperature at 80 V for 20 min and then at 140-160 V for 60 min. The gel was stained with Coomassie Brilliant Blue.

4.4. Fluorescence spectroscopy

The fluorescence spectrum of WGGmCitrineRGD was recorded on a Varian Cary Eclipse fluorescence spectrometer equipped with a Perkin-Elmer PTP-1 Peltier temperature control system. Measurement was carried out with quartz cuvette, 10 mm light path, 2 mL volume, at 1 µM concentration in phosphate buffer (30 mM NaPi, 50 mM NaCl, pH 7.4), at 20 °C.

4.5. Surface preparation

Gold arrays on glass were cleaned with piranha for 30s and rinsed thoroughly with milliQ and dried with N2 just prior to modification of the glass with polyethylene glycol (PEG). PEG functionalization was done as previously described. Briefly, substrates were immersed in 2% (v/v) 2-[Methoxy(polyethyleneoxy)propyl]trimethoxysilane in anhydrous toluene for 2h, rinsed in toluene and baked at 120 °C for 2h. Prior to further functionalization of the gold arrays, substrates were sonicated 5 min in toluene, rinsed in toluene, rinsed in ethanol, sonicated in ethanol 5 min, rinsed in ethanol and dried with N2 or directly incubated in mixed maleimide solution 4 + 5 as described above.

Substrates were immersed overnight in mixed 1 mM solution of 1 and 2 in a 99:1 (v/v) ratio in ethanol under N2 protection. Complex assembly of MVSH 1 - CB[8] - WGGmCitrineRGD was done by preassembly in 0.1x PBS in solution, followed by subsequent incubation on the mixed maleimide monolayers for 1 h. Final concentrations for incubation: MVSH 1= 100 µM, CB[8] = 10 or 100 µM, WGGmCitrineRGD = 4 or 70 µM. After incubation, substrates were rinsed in milliQ and dried with N2.

4.6. Cell culture

C2C12 cells were cultured as described in chapter 3.

4.7. Cell experiments

Cell adhesion experiments were performed as described in chapter 5.
5 References

Supramolecular surfaces for cell adhesion

There is an urgent need for smart biomaterials for diagnostics and biomedical applications, such as trigger-responsive hydrogels or antimicrobial coatings. In response to this demand, the development of new surfaces to stimulate cell adhesion and migration, using supramolecular host-guest complexes, is reported. Supramolecular chemistry makes use of strong non-covalent interactions between molecules to form a specific complex that can in some cases be disrupted upon external stimuli such as an electrochemical potential or light. The reversible character of the interaction makes this system a potential candidate for use in smart biomaterials. In this thesis, a set of different supramolecular systems for the immobilization of biomolecules on surfaces was explored. The supramolecular elements were functionalized with bioactive molecules such as the peptide sequence Arg-Gly-Asp (RGD), coming from the fibronectin protein present in the extra-cellular matrix, or genetically modified fluorescent proteins to enhance cell adhesion. The first chapter presents the use of such supramolecular systems in biodevices, for example as biological coatings for implants, as biosensors to help with the quantification and the detection of relevant biomarkers or as protein biochips. It sets the stage for the design and exploration of the novel supramolecular systems as reported in this thesis.

Ferrocene (Fc) can form a complex with beta-cyclodextrin (βCD) or with cucurbit[7]uril (CB[7]), both in solution and on surfaces. In chapter two, after synthesizing a ferrocene conjugated to a RGD peptide, the formation of CB[7] surface coated with the ferrocene-peptide conjugate was investigated, as well as the bioactivity of such supramolecular surfaces i.e. regarding cellular adhesion. We showed that the surface was biologically active and that a blocking step was necessary to avoid unspecific adhesion of cells on the gold surface.

With these testing conditions in hands, a library of Fc-peptide conjugates was synthesized to study the influence of various molecular parameters. In chapter three the formation of the complex on these surfaces depending on the linker length and of the multivalency of the interaction was investigated. The interaction between ferrocene and CB[7] is not as strong on surfaces as in solution. On CB[7] coated gold surfaces binding affinities of the monovalent constructs were in the micromolar range. A bivalent ferrocene-peptide conjugate improved the binding affinity 2-fold, and subsequent tripling the linker length of this bivalent ferrocene derivative improved the binding affinity 50-fold. Cellular evaluation of the library was performed on gold monolayers and arrays, under static and flow conditions with microfluidics. Different characteristics, such as the number of cells adhering to the surface overtime, the spreading of cells over arrays or their velocity in a microfluidics setup were measured and can be used as a general method to determine cell affinity for coated substrates.
Since ferrocene is also a known guest for βCD, the ferrocene-peptide library was studied on βCD monolayers. The bivalent ferrocene-peptide showed improvement for cell adhesion compared to the monovalent ferrocene-peptide: because of the low affinity of ferrocene for βCD, the density of RGD at the surface was probably too low, compared to the density reached with bivalent Fc-RGD. Moreover, the interaction between ferrocene and βCD can be disrupted upon electrochemical stimuli and induces a release of the Fc-RGD from the surface, yielding a reversible system. This phenomenon was also studied at the cellular level, to determine whether cells could be released from the surface upon disruption of the interaction. This host-guest system has a potential for the controlled release of cells from surfaces.

Chapter five focuses on carborane, a spherical structure composed of 12 boron and carbone atoms as a novel supramolecular immobilization platform that binds strongly to βCD. The complex formation was studied in solution and on surfaces. The carborane derivative was coupled to a cyan fluorescent protein and βCD to a yellow fluorescent protein to investigate the potential of the supramolecular for protein assembly in solution, via FRET studies. However, the two supramolecular elements do not assemble at the low concentrations typically required for protein-based FRET studies. An RGD peptide moiety was attached to a carborane moiety via maleimide-cysteine coupling and the novel bioconjugates were immobilized on βCD monolayers. Noteworthy, because the binding affinity between carborane and βCD was found to be higher than between ferrocene and βCD, a monovalent carborane was sufficient to display a high density of RGD at the surface and thus promote a specific adhesion of endothelial cells. On these surfaces, cells exhibited a more elongated morphology, focal adhesions, and stronger cell adhesion compared to control surfaces.

Finally, a supramolecular ternary system (methylviologen – CB[8] – tryptophan) was studied in chapter six. A fluorescent protein, mCitrine, containing engineered RGD sequences in one loop, and previously tested for activity towards αvβ3 and α5β1 integrins present at the surface of endothelial cells, was immobilized via its N-terminal tryptophan on a methylviologen-CB8 monoloyer. The activity of the protein immobilized on surfaces was studied and showed enhanced cell adhesion compared to protein not displaying the RGD sequence. Furthermore, conditions were optimized to obtain a specific interaction of proteins on the supramolecular monolayer.

Different sets of supramolecular host-guests systems have been studied in this thesis. Overall, these platforms are of great interest to immobilize micro-quantities of proteins and peptides on surfaces and can find applications in biosensors or regenerative medicine.
Throughout this thesis, several supramolecular host-guest systems have been explored as supramolecular biomaterials for cell adhesion and spreading at the molecular and cellular levels. CB[7] and βCD monolayers on gold and on glass have been functionalized with ferrocene or carborane labeled with RGD peptides. Furthermore, a ternary complex between methylviologen, CB[8] and tryptophan allowed for the supramolecular immobilization of mCitrine-RGD, a fluorescent protein labeled with an active RGD sequence. The cellular activity of the surfaces was assessed by looking at the number of cells adhered at different time points, their morphology and their spreading. How do these different supramolecular systems compare with each other? How could they be improved for further biomedical applications?

βCD monolayers are currently better defined than CB[7] monolayers. βCD-7NH₂ is covalently bound to the glass substrates and forms a densely packed monolayer. Similarly, βCD-7S also forms a strong interaction via its sulfur atoms with gold surfaces and well-defined monolayers can be obtained due to the ordering of the alkane chains. On the contrary, the formation of CB[7] monolayers relies solely on non-covalent polar interactions between its oxygen rim and the gold surface. The CB[7] packing has been studied by other groups and a coverage of about 50% of the surface was observed. The remaining 50% can lead to unspecific adhesion of proteins or cells on the surface and can potentially decrease the intended effect of the coating. From a molecular point of view, βCD monolayers thus seem most appropriate for further applications and studies towards biomaterials, especially when a precisely controlled surface is required.

However, cyclodextrins are composed of sugar units and therefore can result in unspecific cellular adhesion. CB[7], on the contrary, displays low unspecific cellular adhesion, as seen in chapters 2 and 3. A couple of groups have reported the synthesis of substituted CB[n]. This entry could be of great interest for surface immobilization, since it could increase the binding affinity of the guest (less steric hindrance since it would be further away from the surface) and permit a higher coverage of the surface with CB[n]. Subsequent improvements in CB[n] functionalization and their surface adhesion are thus expected to allow for a substantial potential for supramolecular cellular adhesion platforms.

In this thesis, the modification of the supramolecular elements with RGD peptides was described as bioactive unit to functionalize the surfaces. But it is also possible to immobilize proteins using supramolecular elements. Previous reports detail the site-specific labeling of fluorescent proteins with the same supramolecular ferrocene-tag and their subsequent surface immobilization. Many protein expressed in the laboratory could be engineered to display a C-terminus thioester, allowing for a practically and easy reaction with a cysteine-supramolecular tag. Similarly, antibody fragments could in principle also be modified. Point mutations to introduce a tryptophan residue at the N-terminus of a peptide or protein allow the formation of a ternary complex on surfaces with methylviologen and CB[8], as described in chapter 6, and do not require further ligations of proteins, which can be useful in the case
of unstable proteins. Unspecific labeling of proteins with a supramolecular tag has been shown in literature. Our attempts to do so with NHS-activated ferrocene yielded inconclusive results due to the fact that the ferrocene moiety is easily cleaved off during analysis. However, similar labeling using carborane derivatives, for immobilization on βCD surfaces, could in principle be more successfully detected and analyzed by using Inductively Coupled Plasma – Atomic Emission Spectroscopy (ICP-AES) or NMR.

To drive one of these supramolecular host-guest systems towards application for biomedical coatings, the next step would be the choice of the surface. βCD covalent attachment to polymer surfaces is possible and would allow for modulation of the intrinsic activity of the polymer, depending on the intended application. For example, hydrophilic polymers could be used for catheter coating, displaying an amine-reactive group to be able to attach βCD-7NH2 at the surface. In my opinion, only βCD-7NH2 or substituted CB[n] should be used for further application, to ensure a maximum of homogeneity of the surface, as well as of resistance towards treatment and manipulation of the biomedical device coated with this material.

Biomaterials often require a high specificity for the envisioned use. Throughout this thesis, the RGD peptide was used, as it is active towards a wide variety of cell lines and has been extensively studied. However, for most applications, such as biosensors, a higher specificity is required and this peptide sequence is therefore not appropriate. The design of the supramolecular conjugates offers a large flexibility in this respect. At the last coupling step, a pentynoic acid molecule is coupled to the active peptide via a traditional amide coupling to allow for the click reaction and conjugation with the supramolecular tag. Michael addition between a cysteine residue and a maleimide functionality is also possible depending on the system. If one considers the intended application of these supramolecular coatings, i.e. stent coatings promoting cellular adhesion and migration of endothelial cells to avoid restenosis, a peptide more specific towards endothelial cells than RGD should be chosen. Unspecific adhesion of blood elements could be studied in a microfluidics setup to obtain an appropriate coating, favoring the cell adhesion and migration, and preventing the adhesion of blood elements.

Finally, the ability of the βCD-Fc complex to be electrochemically controlled can be of great interest in the field of biosensors. In this way, specific proteins or cells could be pulled out from a solution e.g. in a microfluidic device displaying a ligand labeled with ferrocene, and subsequent electrochemical release would allow for a higher density and purity of the protein or cell in solution and facilitated analysis.

Overall, supramolecular host-guest systems are versatile and can be conjugated to a variety of proteins and peptides. These can then be immobilized on surfaces, and depending on the application, the affinity required, the necessity of a controlled release of elements or of conjugation calculation, cyclodextrins, cucurbiturils, ferrocene or carborane can be used.
List of publications


Curriculum Vitae

Pauline Neirynck was born on January 7th 1988 in Lausanne, Switzerland. After obtaining her Baccalauréat in 2005 and following two years of Classes Préparatoires in Physics and Chemistry at Lycée Janson de Sailly in Paris, France, she entered in 2007 the Ecole Nationale Supérieure de Chimie (ENSCM) in Montpellier, France studying Organic and Biochemistry. As part of her studies, she worked at the Salk Institute for Biological Studies in California and at Henkel in Germany. She also took part in the Erasmus program with the ETH Zurich, Switzerland. In November 2010 she graduated with an internship at DSM Biomedical Coatings on the synthesis and characterization of antimicrobial coatings comprising colorless silver nanoparticles. From January 2011 she started a PhD project at Eindhoven University of Technology in the Netherlands under the supervision of prof.dr.ir. Luc Brunsveld on supramolecular surfaces for cell adhesion. The most important results of this work are presented in this dissertation.
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