Cucurbit[8]uril ternary complexes for biomolecular assemblies in solution and at interfaces

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Emanuela Cavatorta

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To my family

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1. Cucurbit[n]uril assemblies for biomolecular applications

The dynamic and non-covalent nature of supramolecular assemblies makes them a perfect tool for mimicking and interacting with biological systems. Several types of supramolecular assemblies have recently been employed to engage with small and large biomolecules or even cells and bacteria and these have confirmed the enormous potential of adopting supramolecular chemistry for the next generation of biomedical devices. In this respect, the host-guest molecular recognition based on cucurbit[n]uril (CB[n]) has met several challenges, such as limited toxicity, high binding affinity and selectivity under physiological conditions and diluted environments typically also in the presence of several interfering components. In this chapter, the unique characteristics of molecular recognition using CB[n] are discussed and state-of-the-art biomolecular applications are given.

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1.1. Introduction

Cucurbit[n]urils (CB[n]) are a relatively young class of macrocycles that are able to form stable host-guest complexes with various guests. These guests range from small molecules such as drugs, dyes and amino acids, to polymers, peptides and even protein domains *via* their appended sidechains. This wide range of possible guests has stimulated an increasing number of fundamental molecular recognition studies that have found application in numerous fields, from catalysis to drug delivery and fabrication of (bio)materials.¹⁻⁵

The first synthetic report of cucurbituril originates from 1905 by Behrend and coworkers, but it was only in 1981 that Mock's group revealed the molecular structure of this "condensation product", giving it the name of CB[6].⁵ The name was chosen to highlight the symmetric shape of these compounds that resembles that of a pumpkin, botanically classified as a *Cucurbitacea*. In fact the CB[n] structure is given by the repetition of n glycoluril units connected by 2n methylene bridges, so that the hydrophobic cavity is decorated by two symmetric carbonyl-fringed portals (Fig. 1.1). Over the last 15 years, the conditions of the acid-catalyzed condensation of glycoluril and formaldehyde were varied leading to the successful purification of various homologues, including CB[5], CB[6], CB[7], CB[8], CB[10] and CB[14]. The latter has a folded structure and is so far the largest member of this family (Table 1.1).⁶⁻⁸ In the last two decades several groups⁸⁻¹⁴ succeeded to synthesize functionalized CB[6] and CB[7] enabling further chemistry and often better solubility in either water or organic solvents. Various derivatives of CB[n] have been discovered by now such as hemicucurbit[n]urils (one half of CB[n] cut along the molecular equator), inverted (containing one glycoluril unit directed toward the inside of the cavity) and nor-sec-CB[n] (missing one methylene bridge). For more information on the binding properties and applications of these CB[n] derivatives the reader is referred to other reviews.^{5, 15}

Fig. 1.1. (a) Molecular and schematic structure of cucurbit[n]uril macrocycles. (b) Calculated electrostatic potential (EP) maps for CB[5], CB[6], CB[7] and CB[8] as cross sectional and side views. Scale bar approx. 4 Å. Adapted with permission.¹⁶ Copyright (2012) American Chemical Society

Table 1.1 Structural parameters of CB[n] as graphically shown in Fig. 1.1a.

Host	Cavity diameter ^a (a)	Portal diameter ^a (b)	Height ^b (c)
	[Å]	[Å]	[Å]
CB[5]	4.4	2.4	9.1
CB[6]	5.8	3.9	9.1
CB[7]	7.3	5.4	9.1
CB[8]	8.8	6.9	9.1
CB[10]	11.7	10.0	9.1

Notes: (a) Data from ref. 5. (b) Data from ref. 17.

The CB[n] family is a powerful supramolecular platform for developing innovative applications mainly because of the following factors: (1) commercial availability in four different sizes; (2) high binding affinity and selectivity towards their guests; (3) accessibility of synthetic routes for several homologues and derivatives; (4) high stability; (5) solubility in both organic and aqueous solvents; (6) remarkably low or absence of toxicity *in vitro* and *in vivo*; (7) temporal and spatial control of the molecular recognition process, such as by chemical, electrochemical and photochemical triggers.^{1, 2}

In this chapter we first discuss the origins of the molecular recognition properties of the CB[n] family (Section 1.2) and methods to gain control over the binding affinity to CB[n] (Section 1.3). Then, we describe the amino acid based recognition of CB[n] (Section 1.4) and subsequently we review the literature on how these properties can be successfully employed to dynamically probe the properties and function of peptides, proteins and cells, which is important for bioanalytical and biomedical applications (Section 1.5).

1.2. Molecular recognition properties of CB[n]

1.2.1. Interactions with the carbonyl portals of CB[n]

In aqueous environment the molecular recognition properties of CB[n] originate from the unique combination of their structure and their cavity size. As mentioned before, the structure of the CB[n] family is highly symmetric, with a hydrophobic concave cavity laced on both sides by carbonyl rims (Fig. 1.1). The electrostatic potential map visualizes the high density of electrons localized uniformly at the portals of CB[n] and explains the affinity of cucurbiturils for positively polarized species through cation-dipole and hydrogen bonding interactions (Fig. 1.1).¹⁶ For example, optimal ion-dipole interactions and desolvated carbonyl portals were found in the crystal structure of the complex of CB[7] and the diamantane diammonium ion guest to form an ultra-strong complex with a binding constant of $K = 7.2 \times 10^{17} \text{ M}^{-1}$.¹⁸

However, these ion-dipole and hydrophobic interactions cannot be the main driving force for the complex formation. Installing one or even two ammonium groups on a ferrocene molecule led to more stable interactions as expected (1 and 2, Fig. 1.2), yet the overall enthalpy of binding with CB[7] was not increased when compared to a neutral ferrocene derivative (3, Fig. 1.2).¹⁹ The differences in overall binding energy in these three guests were ascribed solely to the contribution of the solvation entropy. Desolvation of the guest upon complex formation is more favorable for the charged species. While on the one hand the cationic derivatives can interact more favorably with the carbonyl portals in the complex, on the other hand they also interact stronger with the aqueous

environment prior to complex formation. The balance between favorable and unfavorable interactions is reversed for the complex between the neutral derivative **3** and CB[7], so that the overall enthalpies of the three complexes are similar.²

Fig. 1.2. Chemical structures of neutral and cationic ferrocene guests for CB[7] with the following thermodynamic parameters¹⁹ determined by calorimetry: K (**1**) = $3.2 \times 10^9 \text{ M}^{-1}$; K (**2**) = $4 \times 10^{12} \text{ M}^{-1}$; K (**3**) = $3 \times 10^{15} \text{ M}^{-1}$; ΔH° (**1-3**) = 90 kJ mol^{-1} and $T\Delta S^\circ$ (**1**) = -36 kJ mol^{-1} , $T\Delta S^\circ$ (**2**) = -18 kJ mol^{-1} , $T\Delta S^\circ$ (**3**) = -2 kJ mol^{-1} .

This concept was recently further studied in detail by Masson and coworkers.²⁰ In this study the binding affinities of CB[7] were measured for a series of homologues of *N*-benzyl-trimethylsilylmethylammonium cation guests in which only the para-substituent was varied systematically (Fig. 1.3). The binding affinities of substituted silanes **4** towards CB[7] relative to the unsubstituted silane (**4**, X = H) vary only weakly between 0.9 (**4**, X = CH₃) to 3.1 (**4**, X = SO₂CF₃) and correlate well with linearly combined Swain-Lupton field/induction and resonance parameters, which are derived from the Hammett parameters (Fig. 1.3).²⁰ In this way and supported by calculations, Masson and coworkers found that the differences in binding affinities throughout the series are due to changes in the solvation of the ammonium unit (for the free guests) by water and changes in the interaction between the ammonium unit and the CB[7] rim. Although each of these factors is strongly dependent on the resonance and field/inductive effects that the substituents exert on the charge of the ammonium unit, they are of opposite sign and in balance, yielding binding affinities that are barely affected by changes in substituents (Fig. 1.3). Important to realize is that the solvation of the entire host-guest complex is hardly affected by the substituent effect as the carbonyl rim of CB[7] cancels out most of the positive charge on the ammonium group and CB[7] shields the ammonium group from any water solvation.²⁰ This means that the contribution of the entropic desolvation of the free guest can be used to fine-tune

the binding affinities. The results also demonstrate that the binding properties of CB[n] in aqueous environment are only marginally affected by the coulombic interactions of the guest with the macrocycle portals, while the release of water molecules from the macrocycle upon guest binding is found to be driving the interactions (Section 1.2.2).

Fig. 1.3. Binding affinities for CB[7] of silanes 4 (K_X) relative to silane 4 with $X = H$ (K_H) as a function of a linear combination of Swain-Lupton field/inductive (F) and resonance (R) parameters that are derived from the Hammett parameters ($\sigma = 0.67F + 0.33R$). Adapted with permission - Published by The Royal Society of Chemistry.²⁰

1.2.2. Release of high energy water molecules from the CB[n] cavity

In contrast to other macrocycles such as calixarenes and cyclodextrins, which have an open or cone (vase-like) shape and are polarizable, the concave, rigid and weakly polarizable CB[n] cavity provides further understanding to the exceptionally strong binding of CB[n], also towards neutral guests (Fig. 1.1).

Due to the shape and structure of the CB[n] cavity, a limited number of water molecules occupies the cavity of CB[n] before complex formation, with weaker mutual interactions when compared to water molecules in the surrounding bulk (Fig. 1.4). The average number of hydrogen bonds per water molecule ranges from 2.55 for CB[8], which resembles the value of bulk water, to 0.99 for CB[5] (Table 1.2). Also, the dispersion interactions between the weakly polarizable water molecules and the cavity of CB[n] are weaker with respect to the interactions

between water molecules in bulk, and the water-water hydrogen bonds in the cavity are weaker when compared to bulk water due to the constrained space in the cavity for optimal hydrogen bond formation.¹⁷ The energy needed to remove the first water molecule from the cavity ($E_{\text{pot}}(\text{H}_2\text{O})$) was estimated by molecular dynamic simulations and varies from a maximum of 19.4 kcal/mol for CB[8] to a minimum of 15.1 kcal/mol for CB[5] (Table 1.2 and Fig. 1.4 c).¹⁷ Consequently, the molecules of water within the CB cavity are high-energy molecules that go to a lower energy level when being displaced from the cavity to the bulk water by encapsulation of the guest (Fig. 1.4).

Fig. 1.4. (a) Schematic representation of the release of high energy water molecules upon complex formation. Adapted with permission.³ Copyright (2012) American Chemical Society. Schematic representation of the effect of (b) the host shape and (c) size on the water molecules network. Adapted with permission.¹⁷

Biedermann and coworkers^{17, 21} demonstrated, using molecular dynamic simulations and isothermal titration calorimetry (ITC) experiments, that the release of these high-energy water molecules is *the* major driving force for the complex formation in CB[n], overcoming the direct host-guest interaction energy. The total energy needed for removing all internal water molecules ($\Delta E_{\text{pot}}(\text{all})$ in Table 1.2) perfectly explains the molecular recognition properties through the differently sized CB[n] homologue series (n=5-8). While in the case of the smallest cavities a higher energy gain exists for removing one water molecule ($E_{\text{pot}}(\text{H}_2\text{O})$), the larger cavities contain a higher number of water molecules inside leading to the highest total gain in energy in the case of CB[7] (Table 1.2 and Fig. 1.4c). These calculations are in agreement with the extremely high binding constants reported in the case of CB[7]. In addition, although the values of $E_{\text{pot}}(\text{H}_2\text{O})$ for CB[8] are similar to the ones for bulk water, the removal of all internal water molecules from the CB[8] cavity is energetically favored with respect to the bulk and even with respect to the smaller homologues CB[5] and CB[6] ($\Delta E_{\text{pot}}(\text{all})$, Table 1.2). According to simulations, the specific geometric constraints of the CB[8] cavity size create a void in the water network and favor the release of these high energy water molecules from the host upon guest complexation.^{17, 21}

Table 1.2. Calculated hydration properties of CB[n] with respect to bulk water.

	Cavity volume [Å ³]	Number cavity H ₂ O	Number H- bonds	$E_{\text{pot}}(\text{H}_2\text{O})^a$ [kcal/mol]	$\Delta E_{\text{pot}}(\text{all})^b$ [kcal/mol]
<i>Bulk water</i>			2.54	18.9	reference
<i>CB[5]</i>	45	2.0	0.99	15.1	-9.9
<i>CB[6]</i>	118	3.3	1.31	15.4	-12.2
<i>CB[7]</i>	214	7.9	2.01	17.8	-24.5
<i>B-CD</i>	262 ^c	4.4 ^d	2.96 ^{d, e}		
<i>CB[8]</i>	356	13.1	2.55	19.4	-15.8
<i>CB[8]-paraquat</i>	155	5.5	1.85		-27.0

Notes: (a) Energy required to remove a single water molecule from the cavity. (b) Energy required for removing all water molecules from the cavity and transfer them to the bulk water with respect to the bulk water itself taken as reference. Data from literature.^{17, 21} (c) Data from literature.²² (d) Data from literature.³ (e) This value is including H-bonds with the host.

1.2.3. Enthalpy-driven hydrophobic effect for CB[n]

The release of high energy water molecules is associated mostly to enthalpic contributions, as shown by systematic calorimetric studies of 1:1 complexes of CB[n] with neutral guests.¹⁷ In the case of CB[7] and CB[8] the binding with guests is usually entropically unfavorable and enthalpy driven. There is a perfect correlation between the trends in solvent effects (the release of high energy water molecules) and in enthalpy contributions. In particular according to the lock-and-key principle a tighter fit between the guest and the host provides the full release of water molecules from the cavity and therefore a larger enthalpic gain. A tighter fit corresponds also to a decrease in the degrees of freedom of the guest and consequently to a larger unfavorable entropy contribution. Moreover the entropic loss is in line with the model in which the water molecules are more mobile in the cavity due to a lower number of mutual hydrogen bonds formed with respect to the bulk. In the case of CB[6], due to the more restricted orientation of the water molecules, solvents effects for CB[6] could generate either enthalpic or entropic contributions and therefore the overall binding can be enthalpy or entropy driven.¹⁷

The enthalpy-driven hydrophobic effect for CB[7] seems to explain also its extremely high binding constants determined for some guests in comparison to the similarly sized β -CD (Fig. 1.4b). For instance neutral and cationic ferrocene derivatives bind to CB[7] in the range of 10^9 – 10^{10} M⁻¹ and 10^{12} – 10^{13} M⁻¹, resp., while they bind in the range of 10^3 – 10^4 M⁻¹ in the case of β -CD.²³ The CB[7] cavity, with its symmetric barrel structure, almost shields the encapsulated water molecules from interacting with the aqueous environment and forming hydrogen bonds with the bulk, thus creating high-energy water molecules and high binding enthalpies for the complex formation (Fig. 1.4b and Table 1.2).³ In contrast, the β -CD macrocycle contains fewer water molecules while its cone shape allows more water molecules to participate with hydrogen bonding to the bulk water and the cavity, which means that the water molecules inside the cavity are stabilized and therefore a lower enthalpic gain occurs upon guest complexation and water release (Fig. 1.4b and Table 1.2).³

1.2.4. Enthalpy-driven hydrophobic effect for CB[8] heteroternary complexes

The maximization of the release of energetically frustrated water molecules from the large cavity of CB[8] (Table 1.2) is achieved by the complexation of one or two guests to form a 1:1 or 1:2 complex, respectively. When the two guests in the ternary complex are the same, a so-called homoternary complex is formed, while otherwise a heteroternary complex is formed (Fig. 1.5). For example, CB[8] can bind to 1 equivalent of the electron-deficient paraquat and this 1:1 complex can then bind to 1 equivalent of the electron-rich 2,6-naphthol forming a heteroternary complex in aqueous solutions.⁹ As in this example, the first guest is typically electron-poor and the second guest electron-rich, generating a charge-transfer (CT) donor-acceptor complex.

Fig. 1.5. Stepwise formation of the heteroternary complex in CB[8] between an electron-poor guest G1 and an electron-rich guest G2 in aqueous solutions. Adapted with permission.¹⁶ Copyright (2012) American Chemical Society.

Biedermann and Scherman¹⁶ demonstrated in a systematic study that the CT interaction contributes to the stabilization of the ternary complex and, in contrast to earlier reports,^{9, 24} that the release of energetically frustrated water molecules is still energetically driving the complex formation (*vide infra* and Fig. 1.6). Employing UV-Vis spectroscopy, a range of donor-acceptor pairs (in absence of CB[8]) was investigated and the intensity and shift of the position of the absorbance bands of the CT complexes were monitored upon changing the polarity of the solvents (Fig. 1.6a). The higher the polarity of the solvent, the lower was the energy of the CT band, *i.e.* the CT absorbance band appeared red-shifted. Therefore, the authors concluded that polar solvents were able to stabilize the acceptor-donor pair in the absence of CB[8] and lower the charge repulsion. This indicates that the donor and the acceptor in the CT excited state are in their (mono)cationic forms (Fig. 1.6b).¹⁶ However, CB[8] can stabilize the CT complex much more strongly than any polar solvent used in the study, including water (Fig. 1.6a). In the presence of CB[8] the absorption maximum of the CT band of the paraquat/2,6-naphthol CT complex red-shifted to $\lambda = 560$ nm for the ternary complex, which is 94 nm more when compared to the case when the complex was dissolved in water in the absence of CB[8]. The authors attributed the findings to the large uniform negative electrostatic potential in the cavity of CB[8] (Fig. 1.1e), which lowers the energy band gap of the CT complex by (1) raising the energy of the HOMO of the electron-rich donor and (2) stabilizing both cationic excited states of the donor and the acceptor (Fig. 1.6b).¹⁶

The study of solvent effects for ternary complexes showed that after the complexation of the first guest, the residual water molecules in the cavity can form a much lower number of mutual hydrogen bonds and their energy is much

higher with respect to the bulk and to the free CB[8] cavity (Fig. 1.6c and Table 1.2).²¹ Even though the number of water molecules is reduced upon complexation of the first guest (from 13 H₂O for CB[8] to 5.5 H₂O for CB[8]·paraquat, Table 1.2), the overall energy for removing all residual encapsulated water molecules is twice as high when compared to free CB[8] (Table 1.2).²¹ After binding the first guest to CB[8] a smaller cavity volume remains and this leads to a distortion and a reduction of the number of the hydrogen bonds formed per water molecule. In addition due to the presence of the first guest only a part of the CB[8] portal is available for interactions between encapsulated and bulk water molecules.^{3, 21}

The second guest should not only be electrostatically compatible with the first guest but also sterically able to displace all residual water molecules.²¹ For example, while 1-naphthol is more electron-rich than the isomer 2-naphthol, the heteroternary complex of CB[8]·paraquat·1-naphthol has a less favorable enthalpy contribution than that of 2-naphthol ($\Delta H^0 = -9.2$ kcal/mol and -12.5 kcal/mol, respectively).²¹ Molecular dynamics simulations explained the differences in binding showing that the more electron-rich isomer is sterically unable to release all water molecule from the cavity.²¹ This comparison again demonstrates that the molecular recognition properties of CB[8] are more strongly driven by solvent effects than by CT interactions.

Fig. 1.6. (a) Absorbance maximum of the CT band of the 1:1 pair paraquat/2,6-naphthol in polar organic solvents as a function of their Taft π^* parameters. (b) Energy diagram for the charge transfer interaction between G1 and G2. (a-b) Adapted with permission.¹⁶ Copyright (2012) American Chemical Society. (c) Schematic representation of the solvent effects for ternary complex in CB[8] in aqueous environment. Adapted with permission.²¹ Copyright (2013) American Chemical Society.

1.3. Control over the binding affinity with CB[n]

The molecular recognition properties of CB[n] with diverse guests allow for control over their binding affinity through changes in the chemical, electrochemical, or photochemical conditions applied. The simplest condition that can be exploited for changing the affinity of the complex is pH, e.g. deprotonation of a guest generally decreases the complexation affinity with CB[n]. Typically this strategy is applied in molecular machines such as switchable (pseudo)rotaxanes.¹ Another way to change the affinity of the complex is by adding a competitively binding guest. In this case a competitor of similar binding affinity is added in excess or a competitor with a stronger binding affinity is added. For example, the formation of the stable 1:1 adamantylamine·CB[8] complex can trigger the dissociation of the heteroternary complex between a cationic derivative of pyrene, a functionalized methylviologen and CB[8].²⁵

Electro- and photochemical reactions can control the charges and the steric hindrance of the guests and consequently their affinity to the CB[n] host. The methylviologen dication can be reduced to the radical cation (Fig. 1.7a) that destabilizes the charge-transfer complex with e.g. azobenzene (Fig. 1.7b - c) and—in contrast to the oxidized form—is able to dimerize within the CB[8] (Fig. 1.7c).²⁶

Fig. 1.7. (a) Redox-controlled equilibrium between the dication paraquat MV^{2+} and the radical cation $MV^{+•}$, and photoisomerization of azobenzene derivatives AB. (b) Stepwise formation of CB[8]-mediated heteroternary complex with MV^{2+} as the first guest and *trans*-azobenzene as the second guest. (c) Orthogonal reversible photochemical and electrochemical control over the equilibrium of formation of the heteroternary complex $(MV^{2+} \cdot trans-AB) \subset CB[8]$. Adapted by permission from Macmillan Publishers Ltd: Nat. Commun.²⁷ copyright (2012).

Both photochemical and electrochemical control were reported for a CB[8] heteroternary complex with paraquat as the first guest ($K_1 = 8.5 \times 10^5 \text{ M}^{-1}$)²⁸ and the light-responsive *trans*-azobenzene as the second guest ($K_2 = 1.4 \times 10^4 \text{ M}^{-1}$) as depicted in Fig. 1.7.^{27, 29} Upon UV light irradiation the photostationary state of the azobenzene shifted towards to the *cis*-isomer (Fig. 1.7a). The *cis*-azobenzene is

too sterically hindered to participate in ternary complex formation, and its interactions with the cavity are too weak ($<10^3 \text{ M}^{-1}$) to compete with the paraquat guest.²⁷

In the next section further examples of these molecular switches are presented in the context of amino acid and peptide recognition. The possibility of controlling in time and space the assembly and disassembly of CB[n] complexes expands the toolbox for their applications.

1.4. CB[n] recognition of amino acids, peptides and proteins

Amino acid and peptide guests offer both biocompatibility and bioactivity and expand the applications of CB[n] complexes to *in vitro* and *in vivo* conditions. Urbach and Inoue have made major contributions in finding new complexes between amino acids or peptides with CB[n] in an aqueous, pH neutral, physiological environment.³⁰ Among the 20 genetically encoded amino acids, CB[6] forms complexes with aliphatic cationic residues such as lysine (K),³¹ while CB[7] and CB[8] bind with affinities higher than 10^3 M^{-1} selectively with the aromatic residues tryptophan (W), phenylalanine (F) and tyrosine (Y).³² Enthalpy is the driving force for binding aromatic amino acids with CB[7] (Table 1.3).

As derived from molecular dynamics simulations, the three aromatic amino acids are able to eliminate all water molecules from the CB[7] cavity.³³ Unfavorable free guest desolvation (for tyrosine) and restriction of motion (for tryptophan, see $-T\Delta S$ in Table 1.3) upon complexation are thought to be involved in the differences in binding affinity when compared to phenylalanine.³³ The presence of a positive charge, which is the driving force for the molecular recognition in vacuum,³³ has only an additional stabilizing role in solution and explains the selectivity of CB[7] toward *N*-terminal phenylalanine residues in the tripeptide FGG (G = glycine) with respect to a sequence with an internal, uncharged phenylalanine such as in GFG.³⁴

The *N*-terminal phenylalanine residue of human insulin was selectively recognized by CB[7] acting as an artificial receptor for the protein.³⁴ This concept was further explored in the development of a bioanalytical method for monitoring the protease activity of thermolysin on an unlabeled peptide.³⁵ This enzyme

recognizes an internal phenylalanine residue while its lysis product has an *N*-terminal phenylalanine. The assay was based on the 100-fold difference in affinities of CB[7] for these positional isomers and their different ability of competing with the fluorescent dye acridine orange to bind to the macrocycle. The enzymatic reaction was monitored by fluorescence spectroscopy following the increase of the emission of the dye as it was displaced from the CB[7] cavity by the *N*-terminal phenylalanine lysis products.³⁵

Table 1.3. Thermodynamic binding constants for CB[7] complexes with amino acids, peptides and proteins.^a

<i>Guest</i>	K^b [M ⁻¹]	ΔG^0^c [kcal mol ⁻¹]	ΔH^0^b [kcal mol ⁻¹]	$-T\Delta S^0^d$ [kcal mol ⁻¹]	<i>Ref.</i>
<i>F</i>	1.8 (±0.5)×10 ⁵	-7.2 (±0.9)	-7.3 (±0.7)	0.14 (±0.66)	33
<i>Y</i>	1.6 (±0.3)×10 ⁴	-5.7 (±0.6)	-6.6 (±0.4)	0.9 (±0.4)	33
<i>W</i>	1.2 (±0.1)×10 ³	-4.2 (±0.9)	-6.9 (±0.1)	2.7 (±0.1)	33
<u>F</u> GG	2.8 (±0.1)×10 ⁶	-8.9 (±0.1)	-17.5 (±0.1)	8.7 (±0.1)	34
<u>G</u> <u>F</u> G	2.2 (±0.1)×10 ⁴	-6.0 (±0.1)	-9.3 (±0.1)	3.3 (±0.1)	34
<u>G</u> <u>Y</u> G	2.7 (±0.1)×10 ³	-4.7 (±0.1)	-2.2 (±0.1)	-2.5 (±0.1)	34
<i>human insulin</i>	1.5 (±0.4)×10 ⁶	-8.5 (±0.1)	-10.8 (±0.5)	2.3 (±0.4)	34

Notes: (a) *N*-terminus is charged. Standard deviations are given in parentheses. For the peptides the residue in the cavity is underlined and the standard one letter code for amino acids is used. (b) Values measured by ITC in phosphate buffer at pH 7, see references for further details. (c) Gibbs free energy values calculated from *K* values. (d) Entropic contributions to ΔG^0 calculated from *K* and ΔH^0 values.

In contrast to CB[7], CB[8] can either host homodimers of two aromatic amino acid residues to form a homoternary complex, or host heterodimers of paraquat and an aromatic amino acidic residue to form a heteroternary complex. The crystal structure of the homoternary complex CB[8]·(FGG)₂ revealed the encapsulation of two aromatic residues into the cavity of the CB[8] in a face-to-face arrangement and additionally the coulombic interactions of the carbonyl fringed portals with the *N*-terminal phenylalanine residue and the amidic protons on the peptide backbone (Fig. 1.8).³⁶ As observed in the case of CB[7], the positive charge

adjacent to the aromatic amino acid residue drives the selectivity of CB[8] towards the *N*-terminal phenylalanine and tryptophan, $K_{\text{ter}} = 2.3 \times 10^{10} \text{ M}^{-2}$ and $3.6 \times 10^9 \text{ M}^{-2}$ (Table 1.4), respectively, over the internal or C-terminal positions (no binding detectable by ITC).³⁶

Fig. 1.8. (a) Formation of CB[8]-mediated homoternary complex with two molecules of peptide guest X. (b) Crystal structure of CB[8]·2(FGG), hydrogens and solvating water were removed; the dashed lines indicate key electrostatic interactions. Adapted with permission.³⁶ Copyright (2006) American Chemical Society.

Only few publications reported the use of a homoternary complex with CB[8] involved in controlled biomolecular assemblies. The binding of two equivalents of FGG with CB[8] enabled the specific formation of discrete assemblies of peptides (Fig. 1.8b and Table 1.4),^{36, 37} and protein dimers (Fig. 1.9)³⁸ and wires³⁹ in solution. The CB[8]-mediated dimerization of the monomeric caspase-9 tagged with a short *N*-terminal FGG motif provided a protein dimer with catalytic activity.⁴⁰ A strategy for the supramolecular control over the formation of dimers of peptides and proteins was achieved by reversing the homodimerization of FGG-tagged fluorescent proteins with the bivalent guest (FGGG)₂ (Table 1.4).⁴¹

Table 1.4. Equilibrium association constants for homoternary CB[8] complexes with amino acids and peptides.^a

<i>Guest</i>	K_1 [M ⁻¹]	K_2 [M ⁻¹]	K_{ter} [M ⁻²]	<i>Ref.</i>
<i>F</i> ^b	-	-	1.1 (±0.2)×10 ⁸	32
<i>Y</i> ^b	-	-	<10 ³	32
<i>W</i> ^b	-	-	6.9 (±1.3)×10 ⁷	32
<u>EGG</u>	-	-	1.5 (±0.2)×10 ¹¹	36
(<u>EGGG</u>) ₂ ^c	9.0×10 ⁶	-	-	41
<u>WGG</u>	1.3 (±0.2)×10 ⁵	2.8 (±0.3)×10 ⁴	3.6 (±0.2)×10 ⁹	36
<u>AEFRH</u>	7.6 (±1.2)×10 ⁵	4.9 (±0.2)×10 ⁴	3.7 (±0.7)×10 ¹⁰	37
<u>LVFIA</u>	1.3 (±0.2)×10 ⁵	6.4 (±1.1)×10 ⁴	7.7 (±2.3)×10 ⁹	37
<u>VIFAE</u>	3.7 (±4.6)×10 ⁷	4.2 (±0.4)×10 ⁵	1.6 (±0.3)×10 ¹³	37

Notes: (a) *N*-terminus is charged. Standard deviations are given in parentheses. Values measured by ITC in phosphate buffer at neutral pH, see references for further details. For the peptides the residue in the cavity is underlined and the standard one letter code for amino acids is used. The peptides YGG as well as all scrambled peptide sequences GxG, GGx with x = F, Y, or W did not show affinities measurable by ITC.³⁶ (b) Values of K_1 and K_2 are not reported in literature. (c) Binding affinity measured for the 1:1 complex (FGGG)₂-penta(ethylene glycol) with CB[8].

Heteroternary complexes offer easier applicability due to the fact that each guest is selectively recognized by the host in a binding process divided into two stages. In the most reported case the first guest is paraquat, which is able to bind to CB[8] only in a 1:1 stoichiometry due to the electrostatic repulsion exerted by its double positive charge in its oxidized form. The second guest sequentially binds to the CB[8]·paraquat complex.²⁸ Examples of amino acid-based heteroternary complexes are presented in Table 1.5.

Urbach and coworkers designed a polyvalent system where CB[8] mediated the “duplexing” of peptides containing one or more tryptophan residues with a series of synthetic analogues bearing one or more methylviologen side chains (Fig. 1.9).⁴² Recently, a recombinant immunoglobulin with a tryptophan tag was selectively conjugated *via* CB[8] with methylviologen derivatives of a bioactive peptide.⁴³

Fig. 1.9. Schematic representation of a homoternary and a heteroternary CB[8]-mediated assemblies. (a) Two monomeric yellow fluorescent proteins tagged with *N*-terminal FGG peptide motif dimerize in presence of CB[8]. Adapted with permission.³⁸ (b) A divalent scaffold displaying viologen groups (in red) forms with CB[8] a divalent receptor for a peptide with two tryptophan groups (in blue). Adapted with permission.⁴² Copyright (2009) American Chemical Society.

Table 1.5. Equilibrium association constants for heteroternary complexes of CB[8]·paraquat with amino acids and peptides.^a

Guest	K_2 [M ⁻¹]	K_{ter}^b [M ⁻²]
<i>F</i>	5.3 (±0.7)×10 ³	4.5 (±0.1)×10 ⁹
<i>Y</i>	2.2 (±0.1)×10 ³	1.9 (±0.1)×10 ⁹
<i>W</i>	4.3 (±0.3)×10 ⁴	3.6 (±0.1)×10 ¹⁰
<u>W</u> GG	1.3 (±0.3)×10 ⁵	1.1 (±0.2)×10 ¹¹
G <u>W</u> G	2.1 (±0.1)×10 ⁴	1.8 (±0.1)×10 ¹⁰
GG <u>W</u>	3.1 (±0.4)×10 ³	2.6 (±0.1)×10 ⁹
GG <u>W</u> GG	2.5 (±0.2)×10 ⁴	2.1 (±0.1)×10 ¹⁰

Notes: (a) *N*-terminus is charged. Standard deviations are given in parentheses. For the peptides the residue in the cavity is underlined and the standard one letter code for amino acids is used. Values measured by ITC in phosphate buffer at pH 7 from ref. 28. (b) K_{ter} is calculated considering the equilibrium association constant for the complex CB[8]·paraquat²⁸ equals to $K_1 = 8.5 (\pm 0.3) \times 10^5 \text{ M}^{-1}$.

Recently, also a 1:1 binding of CB[8] with the tripeptide YLA was found to feature a nanomolar binding affinity.⁴⁴ Both the *N*-terminal tyrosine and the neighboring leucine residues are inside the cavity while the alanine interacts with the portals (Fig. 1.10), which is promising for achieving more selective protein recognition.⁴⁴

Fig. 1.10. Peptide sequence of the peptide YLA and semi-empirical model of the 1:1 complex with CB[8]. Adapted with permission.⁴⁴ Copyright (2015) American Chemical Society.

1.5. CB[n] for bioanalytical and biomedical applications

In this section selected examples from the literature are shown in which CB[n] is considered for bioanalytical and biomedical applications. While the whole CB[n] family has powerful self-assembly characteristics such as a large range of binding affinities and stimuli responsiveness to create platforms for the investigation of biological systems, the ability of the CB[8] homologue of simultaneously complexing two guests is of particular interest and has initiated the development of many systems that exploit CB[8] to connect materials and surfaces ranging from polymers, hydrogels, and nanoparticles to planar surfaces.

1.5.1. CB[n]-mediated assembly of bioactive polymers and hydrogels

CB[8] is used as a supramolecular crosslinking unit to form biocompatible and dynamic hydrogels.⁴⁵ Self-assembled CB[8]-mediated hydrogels in water were formed by grafting F or W onto styrene copolymers. CB[n] have also been used to supramolecularly graft bioactive ligands onto linear co-polymers. Scherman and coworkers illustrated this concept using CB[8] to connect mannose-functionalized viologen to pendant naphthol moieties on a methacrylate polymer through ternary

complex formation.⁴⁶ A tetravalent lectin was used to crosslink the polymeric backbones by multivalent interactions with the mannose ligands.⁴⁶ Alternatively, simply mixing CB[6]-grafted hyaluronic acid (CB[6]-HA) with spermidine-functionalized bioactive peptides resulted in the formation of supramolecular host-guest systems that could be used for *in vitro* and *in vivo* studies as shown by Kim and coworkers (Fig. 1.11a).⁴⁷ For example, when the spermidine-functionalized formyl peptide receptor-like 1 (FPRL1) specific peptide WKYMV was used in this system, elevated Ca^{2+} and extracellular signal-regulated kinase (pERK) phosphorylation levels in FPRL1-expressing human breast adenocarcinoma cells were observed, in agreement with the therapeutic signal transduction of this specific peptide.⁴⁷ Another interesting hydrogel system was made by mixing CB[6]-HA with HA carrying 1,6-diaminohexane or spermine as pendant moieties (in their protonated forms) to make ultrastable host-guest complexes between the two HA-polymers (Fig. 1.11b).⁴⁸ This hydrogel was further modified modularly with, amongst others, a bioactive peptide-tagged CB[6], which was anchored to (residual) diaminohexane moieties in the hydrogel.⁴⁸ When an RGD-tagged CB[6] was incorporated into the hydrogel, human fibroblast cells were entrapped in the hydrogel and proliferated approximately 5-fold in 14 days. The cells showed a spread morphology, which matched characteristic cell adhesion and proliferation behavior in an RGD environment. In contrast, when hydrogels lacked the RGD-tagged CB[6], cell proliferation within the hydrogel network was relatively low and the cells retained a round morphology showing poor adhesion.⁴⁸

Another strategy to create supramolecular polymers decorated with bioactive ligands makes use of threading ligand-functionalized host molecules onto various polymers. For example Kim and coworkers threaded mannose-functionalized CB[6] onto the decamethylene segments of a linear chain of polyviologen.⁴⁹ The self-assembled mannose-pseudopolyrotaxanes not only induced bacterial aggregation effectively, but also exhibited high inhibitory activity against bacterial binding to host urinary epithelial cells (Fig. 1.11c). The most potent inhibitor was the mannose-pseudorotaxane threaded with only three mannose-CB[6] with, on average, 33 mannose units. Its inhibitory potency was 300 times higher than compared to free mannose and approximately 1.6 times higher with respect to the

compounds bearing 110 or 55 mannose units, indicating that the density of bioactive ligands along the rotaxane is of key importance for optimizing the interactions.⁴⁹

Fig. 1.11. (a) The structures of the CB[6]-grafted hyaluronic acid (CB[6]-HA) and of the spermidine-tag where the tags can be an imaging probe as FITC and a peptide as WKYMV are reported. (b) Hydrogel formation of CB[6]-HA and polyamine-conjugated hyaluronic acid (PA-HA, blue). Residual free guest sites are modularly modified with various tag-CB[6]s for cell probing. Adapted with permission.⁴⁸ Copyright (2012) American Chemical Society. (c) Mannose-pseudopolyrotaxanes, composed of various densities of CB[6]-based mannose wheels threaded on polyviologen, showed the highest recognition activity towards *E. coli* in the case of the lowest density of mannose-CB[6] relatively to monomeric mannose. Adapted with permission.⁴⁹

1.5.2. CB[n]-mediated assembly of bioactive nanoparticles

CB[n] host-guest recognition can create supramolecular nanoparticles (SNPs). Different multivalent polymeric building blocks can be held together by specific non-covalent interactions allowing control of their size as well as their assembly and disassembly.⁵⁰ Four structural molecular elements were used to form the SNPs: CB[8], a methylviologen-grafted polymer, and mono and multivalent azobenzene-functionalized molecules (Fig. 1.12a).⁵⁰ The higher the amount of the multivalent azobenzene component with respect to the monovalent one, the larger the size of the particles was, yielding SNPs with sizes ranging from 55 nm to 110 nm in diameter. Electrochemically and photo-responsive SNPs were formed in a one-pot mixing process through heteroternary complexation.⁵⁰ A mesoporous silica nanoparticle core with a layer-by-layer coating of CB[7] alternated with polymeric layers with bis-amines was designed as a stimuli-responsive drug delivery system.⁵¹ The recognition by the portals of CB[7] of two bis-amino functionalities on the polymer layers worked as a supramolecular glue and provided structural stability to the nanoparticle shell. The release of the cargo was achieved by controlling the dissociation of the complex, induced by adding amino-adamantane, or by acidifying the solution to endosomal pH in cancer cells. Cellular uptake and triggered cargo delivery was shown in several types of cancer cell lines and *in vivo* conditions using mice as a model system.⁵¹ The results indicated that CB[n] nanoparticles provide a method for manipulating cellular behavior.

The use of the smaller homologues of CB[n] to display ligands on a nanoparticle surface relies on their chemical functionalization. In a contribution from Kim and coworkers, a covalently polymerized network of side chain-functionalized CB[6] was used as a host template to form nanocapsules.^{52, 53} Through host-guest interactions of two spermidine conjugates, a typical polyamine guest of CB[6], bearing galactose⁵² or folate⁵³ as targeting ligands, was introduced to the spherical polymeric network of CBs for receptor-mediated endocytosis.

The examples discussed above involve the dynamic display of ligands on the surface of nanoparticles by host-guest chemistry: such assembly allows the pre-organization of the ligands in an array of multiple binding sites for cell receptors, which leads to a more favorable multivalent interaction. For therapeutic applications additional characteristics are relevant, especially when considering *in*

vivo applicability: (1) controlled cargo release, (2) persistence, clearance or accumulation in the body, and (3) enzyme degradability into non-toxic byproducts after the release of the load. Typically a decoration with hydrophilic polymers such as carboxybetaine analogues⁵⁵ or poly(ethylene glycol) improves the *in vitro* and *in vivo* stability of nanoparticles. Vesicles based on an amphiphilic CB[6] that was derivatized at its periphery with hexa(ethylene glycol) units containing disulfide bonds allowed for incorporation of spermine-modified cell targeting ligands and imaging probes through specific host-guest interactions between spermidine and the CB[6] host.⁵⁴ When these supramolecular vesicles were loaded with doxorubicin, both the internalization into cervical cancer (HeLa) cells by receptor-mediated endocytosis and the triggered release of entrapped drugs by the cytoplasmic reducing environment was demonstrated (Fig. 1.12b).⁵⁴

Instead of *displaying* ligands and stabilizing agents on the surface of nanoparticles, the high affinity and selectivity of the molecular recognition of CB[7] can *shield* cytotoxic moieties. A pyrene imidazolium-labeled hydrophilic peptide and a methylviologen-capped long alkyl chain were coupled by the CB[8] cavity to form a supramolecular peptide amphiphile (Fig. 1.13a).²⁵ In aqueous environment these amphiphiles can self-assemble further into nano-sized vesicles. After internalization in HeLa cells the vesicular structures triggered cell death by the addition of adamantylamine, which bound stronger to CB[8] and thus displaced both viologen and pyrene guests from the cavity. The disassembly of the complex and the particle was confirmed by the increase in fluorescence emission of the pyrene and concomitant cytotoxicity of the uncomplexed viologen in HeLa cells. In contrast, the addition of electron-rich naphthol - which replaced only pyrene in the CB[8] cavity yielding a ternary complex with viologen - caused an increased pyrene emission but no cytotoxicity (Fig. 1.13a).²⁵

A similar concept was applied to mediate the therapeutic activity of gold nanoparticles inside living cells (Fig. 1.13b).⁵⁶ When capped by CB[7], diaminoethane-terminated gold nanoparticles showed a reduced toxicity by the shielding of the positive charges in the macrocycle cavity and were internalized in human breast cancer cells MCF-7. The administration of the stronger binding guest adamantylamine triggered the intracellular activation of the *in-situ* cytotoxicity of

the polyamine moieties by dethreading of CB[7] from the nanoparticle surface (Fig. 1.13b).⁵⁶

Fig. 1.13. (a) Supramolecular peptide amphiphiles formed by CB[8] complexation of pyrene-labeled peptides and viologen lipid and self-assembled into vesicles with triggered fluorescence and cytotoxicity. Adapted with permission.²⁵ (b) Structure of an diamino-hexane-terminated gold nanoparticle (AuNP-NH₂) and the activation of the cytotoxicity of the nanoparticles capped with CB[7] (AuNP-NH₂-CB[7]) by reversing the complex by adamantylamine (ADA). Adapted by permission from Macmillan Publishers Ltd: Nat. Chem.⁵⁶ copyright (2010).

Another field in which the strong, selective and reversible complexes with CB[7] can find application is bioanalytical chemistry. Plasma membrane proteins were selectively captured, extracted and recovered by a synthetic receptor-ligand pair between CB[7] and 1-trimethylammoniummethylferrocene ($K \approx 10^{12} \text{ M}^{-1}$).⁵⁷ Proteins

of the plasma membrane of rat fibroblast Rat-1 cells were ferrocenylated on their amine residues by EDC/NHS chemistry, isolated from the cell lysate by CB[7]-immobilized sepharose beads and finally released, either by heat, or by treatment with a competitor (1,1-bis(trimethylammonium)methylferrocene, $K \approx 10^{15} \text{ M}^{-1}$).⁵⁷

1.5.3. CB[n]-mediated assembly on bioactive surfaces

Self-assembled CB[7] monolayers (CB[7] SAMs) with an approximate 40% surface coverage can be formed on gold surfaces simply by immersion of the substrate into a 0.1 mM aqueous solution of the macrocycle.⁵⁸ Binding studies with neutral adamantyl guests at a single molecule level by dynamic force spectroscopy showed that the binding affinity is similar to that in solution.⁵⁹ Stable and reversible immobilization of a fluorescent protein, which was selectively modified with a single ferrocene guest moiety, on CB[7] SAMs has been reported. This method allows the fabrication of stable protein monolayers with controlled protein orientation.⁶⁰ Similarly, synthetic integrin binding cyclic RGD peptides labeled with ferrocene were displayed on a CB[7] monolayer on gold.⁶¹ Endothelial HUVEC cells specifically adhered to these surfaces under supramolecular control (Fig. 1.14).⁶¹

Fig. 1.14. Supramolecularly controlled cell adhesion on a CB[7] monolayer on gold, pre-incubated with a ferrocene-modified cell adhesion ligand (Fc-cRGD) or the inactive compound Fc-cRAD. Adapted with permission - Published by The Royal Society of Chemistry.⁶¹

The fabrication of CB[8] SAMs takes advantage of the possibility of including two guests in its large cavity. A monolayer of either the first⁶² (Fig. 1.15 a), or the second guest²⁷ is assembled and subsequently the ternary complex can form on *e.g.* gold surfaces. Alternatively, an amino-terminated glass surface can be directly

coated with CB[8].⁶³ Supramolecular recognition of amino acid residues on native proteins, such as bovine hemoglobin and catalase, allowed the layer-by-layer assembly of CB[8]-protein stacks while maintaining the protein activity.⁶³ Another strategy to achieve stable CB[8]-modified surfaces was recently presented by Scherman and coworkers by preparing surface-mounted CB[8] rotaxanes to yield an interlocked architecture. This strategy prevents the dissociation of the first host-guest complex methylviologen-CB[8].⁶⁴

Modifying a gold substrate in a stepwise manner, first with a thiolated methylviologen and subsequently with CB[8], allowed the selective supramolecular immobilization of peptides bearing an *N*-terminal tryptophan.⁶² The electrochemical reduction of the methylviologen drove the release of the peptides.⁶² A similar strategy was developed for creating methylviologen-modified gold and SiO₂ surfaces as well as SiO₂ nanoparticles (Fig. 1.15b).⁶⁵ In this work the surface assembly of the supramolecular ternary complex between methylviologen, CB[8] and 2-naphthol was investigated by SPR. Three assembly routes were compared: (1) incubation of the gold sensor with a preassembled complex of methylviologen-CB[8], and then with a solution of naphthol; (2) stepwise incubation of a methylviologen monolayer with CB[8] followed by naphthol; (3) incubation of a methylviologen monolayer with a mixed solution of naphthol and CB[8]. (Fig. 1.15c) Whereas the first route led to a higher surface coverage when compared to the second, the simultaneous incubation of CB[8] and the second guest naphthol by the third route gave the highest coverage values, by favorably making use of the high ternary association constant.⁶⁵ The latter assembly protocol allowed the oriented immobilization and micro-scale patterning of fluorescent proteins on glass surfaces through the grafting of an amino-methylviologen on a reactive *N,N*-carbonyldiimidazole monolayer.⁶⁵

Fig. 1.15. Fabrication of CB[8] SAMs using the first guest to covalently anchor the ternary complex to the surface. (a) Reversible trap-and-release of peptides bearing an *N*-terminal tryptophan (in red) was achieved onto a gold substrate modified in a stepwise manner with viologen and CB[8]. Adapted with permission.⁶² Copyright (2011) American Chemical Society. (b) Oriented positioning of proteins on silica particles and on glass or gold surfaces is mediated by CB[8] *via* incubation of the substrates with a solution of CB[8] and a naphthol guest moiety chemoselectively ligated to yellow fluorescent protein (YFP). (c) SPR sensograms of the three fabrication routes (see text for explanation). Diamonds indicate switching of the flow back to PBS buffer. (b-c) Adapted with permission.⁶⁵ Copyright (2012) American Chemical Society.

In order to create a supramolecular system for the selective adhesion and electrochemically controlled release of cells, gold surfaces were coated with a cell-repellent monolayer, and a ternary complex was formed of thiolated methylviologen, CB[8] and tryptophan *N*-terminated peptide, bearing RGD ligands for integrin cell receptors (Fig. 1.16a).⁶⁶ A mixed monolayer of tri(ethylene glycol) and the labile 2-mercaptoethanol (99:1 v/v) was fabricated followed by the insertion of the preassembled ternary complex for dynamic display of the RGD ligands. Mouse myoblast C2C12 cells selectively adhered on these surfaces, and

the electrochemical reduction of the surface-bound methylviologen led to the release of the WGGRGDS peptide from the ternary complex and subsequently to cell detachment (Fig. 1.16a).⁶⁶

Fig. 1.16. (a) Strategy for cell-repellent gold surfaces for supramolecular CB[8]-mediated cell adhesion. In the mixed monolayer of thiols 2 and 3 (99:1 v/v) the short, labile 3 is replaced by the preformed ternary complex of the thiolated methylviologen, CB[8] and WGGRGDS. The RGD ligand on the peptide promotes cell adhesion, and the electrochemical reduction of the viologen leads to the release of cells. Adapted with permission.⁶⁶ (b) The peptide sequence GGWGG in one solvent-exposed loop of the mini-protein construct 1 is displayed on the outer membrane protein eCPX and binds CB[8] on a viologen-CB[8]-modified surface or promotes bacterial aggregation *via* multiple homoternary complexes. Adapted with permission.⁶⁷ Copyright (2015) American Chemical Society.

The same strategy was extended to the selective dynamic immobilization of living *E. coli* bacteria.⁶⁷ The supramolecular functionality GGWGG was incorporated on the bacterial surface by genetically modifying a transmembrane protein to display a cysteine-stabilized mini-protein (knottin) containing the GGWGG sequence in an accessible loop (Fig. 1.16b). The bacteria selectively

recognized the antifouling surfaces in the presence of methylviologen and CB[8] by heteroternary complexation. Moreover, the addition of CB[8] to a GGWGG-modified bacterial suspension caused aggregation by the formation of multiple supramolecular homoternary complexes between *E. coli* cells and CB[8].⁶⁷

When aiming for the supramolecular recognition of proteins, bacteria and cells at surfaces, the antifouling properties of the substrate need to be further optimized.⁶⁸ Mixed self-assembled monolayers were prepared incubating gold coated surfaces with the symmetric tetra(ethylene glycol)-terminated undecyl disulfide as the backfilling component and with the asymmetric maleimide and tri(ethylene glycol)-terminated undecyl disulfide as the reactive component.⁶⁹ A Michael addition was used to functionalize the maleimide groups at the surface covalently with a methylviologen thiol allowing for the specific supramolecular binding of a library of B-trypsin inhibitory knottins decorated with GGWGG guest tags for CB[8] either at the *N*-, or at the *C*-termini or at both ends.⁷⁰ As expected, the bivalent knottins showed a stronger binding affinity for CB[8] ($1.3 \times 10^6 \text{ M}^{-1}$) on the surface when compared to either of the two monovalent knottin analogues under the same conditions, or the bivalent one when measured in solution in the presence of an excess of viologen to avoid homoternary complex formation ($1.6 \times 10^5 \text{ M}^{-1}$). These knottins showed trypsin-inhibitory activity in solution and maintained it once supramolecularly immobilized on the surface.⁷⁰

Alternatively, supported lipid bilayers (SLBs) on glass can produce antifouling surfaces for CB[8]-mediated immobilization of proteins⁷¹ and bacteria²⁹ (Fig. 1.17). To achieve selective CB[8]-mediated ternary complex formation, the placement of viologen guest groups on the lipid bilayer was achieved by inserting a cholesterol-functionalized methylviologen into the bilayer as a 1:1 complex with CB[8], which enhances its incorporation into the bilayer (Fig. 1.17a).⁷¹ In this case, a fluorescent protein was immobilized on the viologen-CB[8] SLB *via* a tryptophan residue positioned at the *N*-terminus of the protein in the presence of CB[8] as witnessed by quartz crystal microbalance (QCM).⁷¹

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conjugated to a mannose moiety that can be recognized by the tetravalent protein ConA and by the FimH receptors of *E. coli*. The antifouling properties of the SLBs were investigated comparing ethylene glycol-based SAMs on gold, and gel and liquid-state SLBs for nonspecific bacterial adhesion. The liquid (L_α) phase is characteristic for unsaturated lipids (e.g. DOPC), with low melting temperature, generating SLBs with a disordered packing and a high lateral lipid mobility (on the order of $\mu\text{m}^2\text{s}^{-1}$). In contrast, saturated acyl chains (e.g. DPPC) provide a gel phase (L_β) bilayer with a tight packing, increased melting temperature and reduced lateral mobility of at least one order of magnitude.²⁹ The zwitterionic and hydration properties of both SLBs resulted in better antifouling performance compared to the SAM. However, the liquid-state bilayer gave the best results in agreement with models that predict an out-of-plane mobility for liquid state lipids resulting in an undulatory motion of the bilayer.²⁹ On the liquid-state bilayer, bacterial cells were captured by the ternary complex formation between *trans*-azobenzene-anchored mannose groups and CB[8], and finally released upon UV photoirradiation and concomitant isomerization to the *cis* isomer (Fig. 1.17b).²⁹

1.6. Scope and outline of the thesis

The work that is described in this thesis is based on the use of CB[8] ternary complexes for the formation of biomolecular assemblies in aqueous media. Various types of assemblies are explored either in solution or at the interfaces of nanoparticles and surfaces with living cells.

In Chapter 2, the homoternary complex between CB[8] and two peptides having an *N*-terminal phenylalanine residue is studied in detail. Their complexation is systematically investigated by calorimetric and ¹H-NMR titrations to evaluate the cooperativity of the binding of the second guest, which is not unequivocally known to date.

In Chapter 3, CB[8] mediates the formation of supramolecular vesicular nanoparticles of an amphiphilic ternary complex between a methylviologen guest and an azobenzene guest, which are functionalized with a hydrophobic and a hydrophilic tail, respectively. The photoisomerization of the azobenzene is studied to drive the assembly and disassembly of the particles. The nanoparticles' interface is used to multivalently display cell-adhesive ligands and promote targeted delivery of cargo proteins to cells.

In Chapters 4 and 5, CB[8] is tethered onto surface immobilized methylviologen as the first guest and used to display the second guest molecules that are functionalized with cell adhesive ligands. In Chapter 4, an azobenzene as second guest is studied for controlling cell adhesion in time and at the subcellular scale by employing the light-switchable nature of the ternary complex of azobenzene. In Chapter 5, a small library of miniproteins knottins having one to four tryptophan moieties is used as a second guest. An RGD motif situated on a solvent-exposed loop prompts cell adhesion. The differences in binding affinity among the knottins are evaluated.

In Chapter 6 a strategy for the CB[8]-mediated assembly of cells onto a supported lipid bilayer is developed. This strategy is independent from naturally occurring cell receptors, introducing multiple naphthol guests ligands on the glycocalyx of cells *via* metabolic cell labeling and subsequent strain-promoted

azide-alkyne cycloaddition. The naphthol ligands installed on the cells were recognized by the surface-bound methylviologen-CB[8] artificial receptors.

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2. Assessment of cooperativity in homoternary peptide-cucurbit[8]uril complexes

Evaluating the cooperativity in the interactions in cucurbit[8]uril (CB[8]) ternary complexes is required for the design of self-assembled systems based on such ternary interactions. Here the cooperativity of homoternary complexes between CB[8] and N-terminal phenylalanine peptides is assessed by means of concentration-dependent calorimetric and ^1H -NMR titrations. The sensitivity of the fitting of the cumulative heat of complexation of the calorimetric titrations is evaluated in terms of fitting error and enthalpy-entropy compensation. Within the acceptable range of degrees of cooperativity, the NMR spectroscopic analysis of the separate species determined non-cooperative binding to be the most probable scenario. The binding behavior of CB[8] homoternary complexes is similar to CB[8] heteroternary complexes, with an enthalpy-driven tight fit of the guests in the CB[8] cavity overcoming the entropic penalty. Also for these type of complexes a non-cooperative binding is the most probable.

2.1. Introduction

Supramolecular chemistry involves the non-covalent synthesis of artificial receptor-ligand complexes.¹ As non-covalent interactions in biological systems induce the formation of ordered, complex and dynamic structures, the specific molecular recognition properties between the ligand (guest) and the receptor (host) in supramolecular host-guest systems allow for the design of synthetic non-covalent, ordered structures.² Introducing host and guest moieties on various building blocks constitutes therefore a way to engineer the self-assembly properties of molecular systems with the ultimate goal of creating programmable and functional architectures.

The cucurbit[*n*]urils (CB[*n*]) form a new class of macrocyclic hosts that show remarkable molecular recognition properties in water.^{3, 4} Given by the repetition of *n* glycoluril units, CB[*n*]s are characterized by a rigid, symmetric structure. In addition to the direct interactions of a guest with the polar portals and hydrophobic cavity of the host, the highest affinities between CB[*n*]s and their guests occurs when high energy solvation water molecules are released from the cavity, which generates an enthalpic gain upon complexation.³

CB[8] is the first homologue large enough to promote the binding of two equivalents of an organic guest in a ternary complex.^{5, 6} This ternary complex forms a powerful paradigm for self-assembly. For example, a heteroternary complex, formed from the well-defined sequential binding of two different guests inside the CB[8] cavity, can drive the self-assembly of programmable structures such as polymers,^{7, 8} hydrogels⁹ and nanoparticles.¹⁰⁻¹² Also homoternary complexes can be used for this purpose, as witnessed, in particular, by the binding of *N*-terminal aromatic amino acidic residues such as tryptophan or phenylalanine (Phe).¹³ This type of CB[8]-peptide complexes extends the application of CB[8] assemblies into biological systems.¹⁴⁻¹⁷

A ternary complex offers the opportunity of tuning the assembly properties by cooperativity. Cooperativity describes the relationship between the affinities of binding of the first and second equivalent of guest by the host.¹⁸ In comparison to the affinity of the first guest molecule, the binding of the second guest can be

either favored (positive cooperativity), unfavored (negative cooperativity), or unaffected (independent/non-cooperative). The principle of cooperative interactions is common in living systems and modulates the function of a receptor by the concentration of the ligands. For example, the binding of oxygen to the four pockets of hemoglobin is a positive cooperative process resulting in an increase of the binding affinity of hemoglobin for the substrate oxygen upon each molecule of oxygen bound.¹⁹

Proper design of the stability and dynamics of self-assembled systems based on ternary interactions requires a thorough understanding of the, possibly cooperative, binding behavior of the ternary complex interaction motif. In a systematic study of the sequence-specific recognition of peptides by CB[8], the homoternary complex between PheGly₂ and CB[8] was proposed as a synthetic, positively cooperative receptor-ligand interaction.¹³ An overall ternary binding constant K_{ter} of $1.5 \times 10^{11} \text{ M}^{-2}$ was reported for the complex $\text{CB[8]} \cdot (\text{PheGly}_2)_2$.¹³ The positively cooperative nature of this complex was suggested on the basis of ¹H-NMR experiments, but the extent of cooperativity was not quantified.¹³

The aim of this study is to assess the degree of cooperativity for the ternary complexes between CB[8] and two peptides both with an *N*-terminal phenylalanine, followed by either two (PheGly₂) or six glycine (PheGly₆) residues. To this end we used isothermal titration calorimetry (ITC) and ¹H-NMR titrations to study the dependence of the affinity of CB[8] on the concentration of the guest. A key issue is to dissect the contributions of the bindings of the first and second guest molecules to the overall ternary complex formation. This is addressed by performing concentration-dependent titrations, an evaluation of the error sensitivity in the ITC experiments, and by a spectroscopic analysis of the separate species by ¹H-NMR.

2.2. Results and discussion

Fig. 2.1 graphically shows the first and the second binding events between the host CB[8] (H) and the peptide guest (G), leading to the formation of the 1:1 complex HG and the homoternary complex HG₂, respectively. The first equilibrium

binding constant K_1 arises from the interaction of a single guest G with the host H. For the second binding step, the dissociation of a guest is associated with a pre-factor 2 ($2 \cdot k_{d,2}$) to account for the presence of two identical guest molecules in the cavity. Overall, the degree of cooperativity, defined by the ratio K_1/K_2 , governs which of the three scenarios, positive, negative and non-cooperativity, applies, depending on whether K_2 is larger than, smaller than or equal to $\frac{1}{2}K_1$, respectively.

Fig. 2.1. A homoternary complex is formed in the cavity of CB[8] (host, H) binding two units of the peptide PheGly_n (guest, G). The stepwise equilibrium binding constants for the formation of the 1:1 complex HG and the 1:2 complex HG₂ between the guest G and the host H are indicated as K_1 and K_2 , respectively. The molecular structures of the molecules used in this study are shown at the bottom.

An important aspect for the assessment of the degree of cooperativity is to work in an as wide as possible range of concentrations of H and G in order to make use of the different concentration dependencies of the binding constants for the formation of HG and HG₂. For a given overall binding constant K_{ter} , different degrees of cooperativity are expected to give different species distributions. This means that the distributions of the concentrations of H, HG and HG₂ - while keeping the initial concentrations of host and guest constant - correspond to unique scenarios of K_1/K_2 . To be able to accurately determine the ratio K_1/K_2 , different distributions of H, HG and HG₂ can be measured starting from different initial concentrations of host and guest.

To find a suitable working range of concentrations, two main conditions were taken into account. Firstly, the solubility of CB[8] in aqueous media, around 100 μM ,²⁰ sets the upper limit of the working range. Secondly, the concentrations must be high enough to provide complexation, which defines the lower limit. Considering that in the course of a titration all species must be present at about equal concentrations, say x , it follows that:

$$K_{ter} = \frac{[HG_2]}{[H][G]^2}$$

$$[H] \cong [G] \cong [HG_2] = x$$

Thus:

$$K_{ter} = \frac{1}{x^2}$$

For an expected¹³ overall ternary binding constant K_{ter} on the order of 10^{11} M^{-2} , this yields approximately $x = 3 \mu\text{M}$. Hence the proper working range of concentrations was set between 1 and 50 μM .

ITC studies were performed to determine the ratio between K_1 and K_2 for the ternary complexes of CB[8] with the peptides PheGly₂ and PheGly₆. The simultaneous fitting of the ITC data sets measured at three different concentrations provided a restricted range of physically acceptable K_1/K_2 ratios. Specifically, consistent with the optimal range of concentrations discussed above, CB[8] was loaded in the cell at concentrations between 10 and 50 μM and titrated with a solution of the peptide guest. All solutions were prepared in PBS (10 mM phosphate buffer, 2.7 mM potassium chloride and 137 mM sodium chloride, pH 7.4). The enthalpograms obtained for each host-guest complex are given in Fig. 2.2a/e. A mathematical model was used to fit the experimental heats with a least-squares minimization routine (see Experimental section). The heat of complex formation was expressed as a function of the species concentrations, and the thermodynamic parameters K_1 , K_2 , ΔH^0_1 , and ΔH^0_2 were used as fit parameters, as described in the Experimental section. Heats of dilution for each set of initial concentrations were also included in the model, and calculated values were confirmed by reference experiments (see Experimental section). The best fits (Fig. 2.2a/e) provided the optimal four parameters ΔH^0_1 , ΔH^0_2 , K_1 and K_2 , and thus the

optimal K_1/K_2 ratio for each peptide guest. K_1/K_2 values of around 2 were found for both peptides ($K_1/K_2 = 2.1 \pm 0.8$ for PheGly₂ and 1.8 ± 0.4 for PheGly₆, Table 2.1), which agrees with a non-cooperative binding scenario.

Fig. 2.2. ITC study for the binding of CB[8] with PheGly₂ (a-d) and PheGly₆ (e-h). For each ITC experiment (a and e), the experimental data (data points) for three different initial CB[8] concentrations were fitted simultaneously to a model with K_1 , K_2 , ΔH°_1 , and ΔH°_2 as fit parameters (solid lines). Representative plots of the normalized least-squares fit error, ΔH° and $-T\Delta S^{\circ}$ calculated at fixed values of the K_1/K_2 ratio for PheGly₂ (b-d) and for PheGly₆ (f-h). The non-cooperative case ($K_1/K_2 = 2$) is indicated by the red line and the areas highlighted in green represent the acceptable ranges of K_1/K_2 in terms of both fit error (within 20% of the minimum error) and of enthalpy-entropy compensation (see text).

To evaluate how sensitive the fit error is to variations of the K_1/K_2 ratio, the least-squares error was calculated for different degrees of cooperativity. Thus the parameters ΔH°_1 , ΔH°_2 , and K_2 (correlated to K_1) were optimized for chosen values of K_1/K_2 . Fig. 2.2 shows the dependence of the fit error (Fig. 2.2b/f) on the ratio K_1/K_2 , and the correlated enthalpies (Fig. 2.2c/g) and entropies (Fig. 2.2d/h). An evaluation of these parameters allowed to determine a range of possible degrees of cooperativity (indicated in green in Fig. 2.2).

Values of the fit error within 20% from the minimum error were defined as acceptable. This 20% cut-off value was selected based on the variability of the minimum error observed in triplicate calorimetric experiments. Therefore, the upper boundary of the range of acceptable degrees of cooperativity was set at values of K_1/K_2 equal to 6 for PheGly₂ and to 3.5 for PheGly₆. For higher values of K_1/K_2 (strongly negative cooperativity) the fit errors became quickly unacceptably high (Fig. 2.2b/f). Regarding the thermodynamic parameters, such high K_1/K_2

ratios gave more exothermic enthalpies and less favorable entropies for the second step (Fig. 2.2).

The lower limit of the range was determined considering that, even though the fit errors did not rise as quickly as at the upper limit, the binding enthalpies and entropies for the first and second binding events diverged more and more for values of K_1/K_2 lower than 0.5. Specifically, an inversion of the signs and order of ΔH^0_1 and ΔH^0_2 , as well as of $T\Delta S^0_1$ and $T\Delta S^0_2$, was observed for values of K_1/K_2 below 0.2 for PheGly₂ and below 0.1 for PheGly₆ (Fig. 2.2). Under these conditions, the second binding event became less enthalpically favored (and more entropically favored) than the first step. Both steps would be thus associated with large enthalpy-entropy compensation effects and opposite driving forces, *i.e.*, strongly enthalpy-driven for the first step and strongly entropy-driven for the second. In particular, the unfavorable positive enthalpy contribution (Fig. 2.2c/g) and the highly favorable entropy (Fig. 2.2d/h) for the second step are not realistic considering that CB[8] complexation is known to be enthalpically driven and entropically unfavorable.^{3, 21} Overall, the considerations made in terms of fit error and of enthalpy-entropy compensation determined a range of acceptable K_1/K_2 ratios between 0.2 and 6 for PheGly₂ and between 0.1 and 3.5 for PheGly₆, which are highlighted in green in Fig. 2.2. For both peptides these ranges indicate either a non-cooperative or a weakly, negative or positive, cooperative system.

For both PheGly₂ and PheGly₆ the second binding event has a larger enthalpic gain than the first, as well as a larger entropy loss (Table 2.1). This indicates a tighter fit for the second guest in the CB[8] cavity, which is logical as it involves interaction with an already partially filled cavity. It is also in agreement with studies performed by Biedermann and coworkers²¹ that show that in the case of heteroternary complexes, a more favorable enthalpy for the second aromatic guest correlates with a less favorable entropy contribution. Similar to what was shown for the heteroternary complexes, this can be expected also in the case of the homoternary complexes studied here, as the first guest reduces the cavity volume of CB[8] in such a way that the potential energy of the residual cavity water molecules is increased, thus leading to a stronger enthalpic response upon release of these water molecules upon the binding of the second guest. Oppositely, the

tightly packed ternary complex reduces the degrees of freedom of both guests and therefore brings an additional unfavorable entropy contribution.²¹

Table 2.1. Thermodynamic binding constants for the complexation of PheGly_n peptides with CB[8].

	ITC			¹ H-NMR ^c	
	PheGly ₂ ^a	PheGly ₂ ^b	PheGly ₆ ^a	PheGly ₂	PheGly ₆
K_1/K_2	2.1 (0.8)	-	1.8 (0.4)	0.5	1.2
$K_1 [M^{-1}]$	$2.2 (1.1) \times 10^5$	-	$8.7 (0.6) \times 10^4$	3.8×10^5	9.2×10^4
$K_2 [M^{-1}]$	$1.0 (0.2) \times 10^5$	-	$5.1 (1.3) \times 10^4$	7.8×10^5	7.7×10^4
$K_{ter} [M^{-2}]$	$2.3 (1.4) \times 10^{10}$	$1.5 (0.2) \times 10^{11}$	$4.4 (1.1) \times 10^9$	3.0×10^{11}	7.1×10^9
$\Delta H^0_1 [kcal mol^{-1}]$	-11.6 (0.3)	-29.6 (0.2)	-8.3 (0.2)	-	-
$\Delta H^0_2 [kcal mol^{-1}]$	-13.7 (1.7)		-14.7 (2.5)	-	-
$\Delta G^0_1 [kcal mol^{-1}]$	-7.2 (0.3)	-15.4 (0.1)	-6.7 (0.1)	-7.6	-6.8
$\Delta G^0_2 [kcal mol^{-1}]$	-6.8 (0.1)		-6.4 (0.2)	-8.0	-6.7
$T\Delta S^0_1 [kcal mol^{-1}]$	-4.3 (0.5)	-14.2 (0.3)	-1.5 (0.2)	-	-
$T\Delta S^0_2 [kcal mol^{-1}]$	-6.9 (2.2)		-8.3 (3.3)	-	-

Notes: (a) Data obtained from the simultaneous fitting of calorimetric enthalpograms at three different CB[8] concentrations. The average of three independent ITC experiments at 25 °C in PBS (10 mM phosphate buffer, 2.7 mM potassium chloride and 137 mM sodium chloride, pH 7.4) is reported for each peptide. Standard deviations are given in parentheses. The value of K_{ter} was calculated from the product of K_1 and K_2 . The entropic contributions $T\Delta S^0$ were calculated from the difference of the Gibbs free energy and the enthalpy. (b) Data reported for the overall ternary complex HG₂ (for three ITC experiments titrating 2 mM of PheGly₂ into 0.1 mM CB[8] in 10 mM sodium phosphate, pH 7.0 at 27 °C).¹³ (c) Data obtained from the simultaneous fitting of the distributions of the different species H, HG and HG₂ as determined at 25 °C in D₂O by ¹H-NMR during one titration experiment per peptide.

Another observation from our calorimetric results is that, when comparing the thermodynamic data for the two peptides, a stronger binding affinity was found for PheGly₂ with respect to PheGly₆, arising from differences for both the first and second guest binding steps. In particular, the first PheGly₆ seems to have a weaker

interaction with the host (less favorable ΔH^0_1). Moreover, our results reveal a slightly weaker overall binding than the one reported in the literature¹³ for the overall ternary complexation of the peptide PheGly₂ with CB[8] (see K_{ter} in Table 2.1), which can be explained by a higher concentration of cations competing with the guest for the binding to the host in our buffer.²² The crystal structure of the complex¹³ shows that the shorter PheGly₂ can assume a circular conformation to maximize its dipole-dipole interactions of the amidic protons with the carbonyl on the CB[8] rims. This cannot be achieved for a longer chain in the case of PheGly₆, which may explain the observed difference in affinity. Unfortunately, the X-ray structure of the complex CB[8]·(PheGly₆)₂ is not available to confirm this hypothesis. Our observations are in agreement with calorimetric experiments on heteroternary complexes of CB[8]·paraquat with TrpGly₂ or TrpGly₅ that have shown a tighter binding for the short peptide compared to the long one.²¹

Taken together, the calorimetric data indicate that the most realistic scenario is the non-cooperative binding of the peptides. However, further narrowing the range of possible K_1/K_2 values could not be achieved by ITC alone, due to both the restricted operative concentration range (see above) and the convolution of the heat effects arising from the first and the second binding events. To overcome the latter limitation, ¹H-NMR was used to provide direct spectroscopic insight into the (relative) concentrations of all participating species separately. This technique has a relatively low sensitivity, so fairly high concentrations are preferred. In an earlier study, however, the CB[8] concentration that was used exceeded the solubility limit.¹³ Therefore, we determined the speciation at CB[8] concentrations below the solubility limit.

To this end, a titration experiment was performed at a constant total CB[8] concentration (in D₂O) of 50 μ M while titrating from 0.5 to 4 equivalents of the peptides (Fig. 2.3a/d). The three species G, HG and HG₂ were distinguished based on the signals of the aryl protons of the guests.¹³ Upon the first complexation, the upfield shifts of the phenyl protons of the Phe residue showed the shielding of the surrounding CB[8] host molecule. With the second complexation, the interaction among the two guests in the cavity of the CB[8] caused an additional upfield shift.²³ Even though less sensitive than calorimetry, the ¹H-NMR titrations allow to monitor

the formation of each species participating in the equilibrium (Fig. 2.3a/d). Specifically, under non-saturation conditions for CB[8], the HG complex is well visible at low concentrations for both peptides, thus excluding a strongly positive cooperative system, in contrast to what has been described in an earlier study.¹³

Fig. 2.3. ¹H-NMR titrations of CB[8] (50 μM) with PheGly₂ (a) and PheGly₆ (d) in D₂O at 25 °C. The guest concentrations in the species free G, HG and HG₂ (data points) determined experimentally are simultaneously fitted to a model varying K_1 and K_2 (solid lines) for (b) PheGly₂ and (e) PheGly₆. Plots of the normalized fit error calculated at fixed values of the ratio K_1/K_2 for (c) PheGly₂ and (f) PheGly₆. The non-cooperative value of $K_1/K_2 = 2$ is indicated by a red line, and the areas highlighted in green represent the acceptable ranges of K_1/K_2 in terms of error (within 20% of the minimum error).

By monitoring the signals of the aromatic protons (Fig. 2.3a/d), the distributions of all species G, HG and HG₂ were determined for each titration step (Fig. 2.3b/e). These distributions were fitted to a model expressing the calculated distributions of species as a function of the fitting parameters K_2 and K_1 (see Experimental section). The calculated data are shown as lines in Fig. 2.3b/e for the peptides PheGly₂ and PheGly₆, respectively.

Table 2.1 summarizes the values found for the optimized parameters K_1 , K_2 , the corresponding free energies ΔG^0_1 , ΔG^0_2 , and the overall binding constant K_{ter} . Higher overall binding affinities (K_{ter} in Table 2.1) with respect to the ones found

by calorimetry were determined. This was expected because the cations in the PBS solutions used for ITC can compete with the guest for the interaction with the host thus destabilizing the complex,²² while these salt effects are absent in the solvent (D_2O) used for the 1H -NMR experiments. In agreement with what has been observed by ITC, CB[8] binds more strongly with the shorter peptide PheGly₂ ($3.0 \times 10^{11} M^{-2}$) than the longer PheGly₆ ($7.1 \times 10^9 M^{-2}$, Table 2.1). The optimal fits gave $K_1/K_2 = 0.5$ and 1.2, for PheGly₂ and PheGly₆, respectively, indicative of non-cooperative or slightly positive cooperative binding.

In order to assess the sensitivity of the degree of cooperativity, and similarly to what was described for the ITC data, the graphs in Fig. 2.3c/f were obtained by optimizing K_2 (and the correlated K_1) at chosen values of the ratio K_1/K_2 . The values of the least-squares error for each K_1/K_2 ratio are reported for each peptide (Fig. 2.3c/f). A cut-off value of 20% from the minimum fit error was arbitrary chosen to find the acceptable range of degree of cooperativity. The values of K_1/K_2 are in a range between 0.2 and 1 for the shorter peptide PheGly₂, and between 0.6 and 10 for the longer PheGly₆. Notably, the minima by 1H -NMR are within the range of K_1/K_2 obtained by calorimetry, indicating a non-cooperative system.

Taken together, these results confirm a most probable scenario in which the ternary complexation between the peptides and CB[8] is non-cooperative. It should be noted, however, that these ternary complexes cannot be compared directly to, for example, the cooperativity observed in hemoglobin, because in the former case the first guest does not occupy one of two identical, well-spaced binding sites, but resides somewhere in the same cavity to which also the second one binds in the next step. As a result, the second guest experiences interactions with the first guest directly, as witnessed by the correlation between enthalpy and entropy.

2.3. Conclusions

In conclusion, combining the pieces of evidence from calorimetric and 1H -NMR titrations shown in this work, the most probable scenario to describe the homoternary complexation of phenylalanine-based peptides by CB[8] is a non-cooperative mode of interaction. This is independent of the tail length of the

peptides studied in this work. Remarkably, while the second guest experiences a stronger interaction with the host after the first complexation step, there appears to be a counterbalancing entropic contribution that leads to an overall non-cooperative behavior in affinity. This contrasts the normal non-cooperative behavior of well-separated binding sites, in which case the binding enthalpies of all steps are equal, and entropy differences arise solely from differences in statistical pre-factors.

The binding behavior of the homoternary peptide complexes resembles that observed for heteroternary complexes. The PheGly binding motif offers the synthetic flexibility and biocompatibility of peptides, and can have an active role in natural functional structures as well, such as in nuclear membrane pores.²⁴ The insights in the complexation between peptides and CB[8] allow for a rational design of more complex self-assembled systems built on this powerful interaction motif.

2.4. Acknowledgments

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2.5. Experimental section

2.5.1. General methods

Chemicals were purchased from Sigma Aldrich unless differently specified and used as received. UV-Vis spectroscopy was performed using a Perkin Elmer UV-Vis spectrophotometer (Lambda 850). High performance liquid chromatography (HPLC) was performed on Water (2535) setup equipped with analytical and preparative XBridge C₁₈ columns. Mass spectrometry analysis was performed using a Waters ESI(+)-ToF spectrometer (Micromass LCT). Calorimetry measurements were conducted on a microcalorimeter (Microcal). ¹H-NMR spectra were recorded in D₂O on an Ultrashield 600 (Bruker, ¹H-NMR 600 MHz) spectrometer. Residual solvent protons were used as an internal standard, and chemical shifts are given in units of parts per million (ppm) relative to trimethylsilane (TMS).

The peptide PheGly₆ was synthesized using an automatic solid phase peptide synthetic robot (MultisynTech), following standard Fmoc protocols.²⁵ Purification of the peptide was done by reversed phase HPLC in a gradient of H₂O/acetonitrile (10/90% to 0/100% in 70 min), and characterization by analytical HPLC and mass spectrometry. ESI-MS [M+H]⁺ = 507.26 (calc. 507.22); [M+Na]⁺ = 529.24 (calc. 529.21).

2.5.2. Concentration assessment of CB[8] solutions

Solutions of CB[8] for calorimetry studies were freshly prepared in PBS buffer (Dulbecco's Phosphate Buffered Saline, Sigma Aldrich, 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25 °C). Solutions of CB[8] for ¹H-NMR spectroscopy experiments were freshly prepared in D₂O. In both cases, the solid CB[8] was solubilized always for more than 2 h under ultrasonication at approximately 80 °C. The obtained solutions were then filtered through a 200 nm pore diameter membrane filter and the concentration of CB[8] was accurately determined by titrating an aliquot of each solution, according to a UV-Vis spectroscopy procedure reported by Kaifer and coworkers.²⁶ The resulting titer of the solutions of the batch of CB[8] used in this work was 82±3%.

2.5.3. Isothermal titration microcalorimetry

Calorimetry measurements were conducted at 298 K. Solutions were freshly prepared in PBS buffer and degassed prior to use. ITC measurements were made in triplicate and at three different sets of concentrations as described in the main text, with solutions of CB[8] between 10 and 50 μM in the cell, and of the peptide PheGly₂ or PheGly₆ in the syringe at a concentration equal to 20 times the one of the host in the cell (Fig. 2.4). As a reference, the peptide PheGly₆ was titrated in PBS (in the absence of CB[8]). This blank curve has not been subtracted from the enthalpograms of complexation in the presence of CB[8], but a constant dilution heat is assumed and co-optimized in the fitting procedure. The measured heat values matched the heat of dilution considered in the fitting (Fig. 2.5).

Fig. 2.4 (a-c) Enthalpograms for solutions 10, 20 and 30 μM CB[8] respectively titrated with 0.2, 0.4 and 0.6 mM PheGly₂. (d-f) Enthalpograms for solutions 10, 20 and 50 μM CB[8] respectively titrated with 0.2, 0.4 and 1 mM PheGly₆.

Fig. 2.5 Enthalpograms for a PBS solution at 0 μM CB[8] titrated with 1 mM PheGly₆.

2.5.4. Fitting model for calorimetric and ^1H -NMR data

Considering the equilibria described in the main text in Fig. 2.1, the distributions of all species can be derived from the following:

$$\begin{cases} K_1 = \frac{[\text{HG}]}{[\text{H}][\text{G}]} \\ K_2 = \frac{[\text{HG}_2]}{[\text{HG}][\text{G}]} \\ H_{\text{tot}} = [\text{H}] + [\text{HG}] + [\text{HG}_2] \\ G_{\text{tot}} = [\text{G}] + [\text{HG}] + 2[\text{HG}_2] \end{cases}$$

By substitution,

$$\begin{cases} [\text{HG}] = K_1[\text{H}][\text{G}] \\ [\text{HG}_2] = K_2[\text{HG}][\text{G}] \\ H_{\text{tot}} = [\text{H}](1 + K_1[\text{G}] + K_1K_2[\text{G}]^2) \\ G_{\text{tot}} = [\text{G}](1 + K_1[\text{G}] + 2K_1K_2[\text{H}][\text{G}]) \end{cases}$$

Thus,

$$\begin{cases} [\text{HG}] = K_1[\text{H}][\text{G}] \\ [\text{HG}_2] = K_2[\text{HG}][\text{G}] \\ [\text{H}] = H_{\text{tot}}/(1 + K_1[\text{G}] + K_1K_2[\text{G}]^2) \\ [\text{G}] = G_{\text{tot}}/(1 + K_1[\text{G}] + 2K_1K_2[\text{H}][\text{G}]) \end{cases} \quad (\text{I})$$

For fitting of the heat of complexation, the calorimetric experimental data Q_{exp} were fitted according to the equation:²⁷

$$Q_{\text{calc}} = Q_{\text{dil}} + V(\Delta H_1[\text{HG}] + \Delta H_2[\text{HG}_2])$$

where Q_{calc} is the calculated heat, Q_{dil} is the heat of dilution and V is the volume.

For the fitting of the species distributions as quantified by ^1H -NMR measurements, the system (I) was used directly to express the calculated distribution of species in function of the parameters K_1 and K_2 .

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3. Targeting protein-loaded supramolecular nanoparticles to cells

3

A supramolecular amphiphile, constituted by the ternary complex between cucurbit[8]uril (CB[8]), an alkylated viologen and a tetraethylene glycol-functionalized azobenzene, self-assembles into vesicles of ca. 200 nm in diameter. The vesicles could be used as nanocarriers of fluorescent proteins for imaging purposes. Cell targeting peptide ligands were readily introduced by host-guest chemistry and promoted receptor-mediated uptake of the loaded nanoparticles.

3.1. Introduction

Self-assembly of molecules into ordered structures is a powerful approach for the design of novel and functional materials.^{1, 2} For example, supramolecular nanocarriers enable the incorporation of multiple characteristics, such as for example targeting, drug delivery and imaging, in the same system *via* reversible, yet robust non-covalent interactions.³⁻⁸ In this context cucurbit[8]uril (CB[8]) is a particularly interesting candidate to consider owing to its biocompatibility and its ability to form ternary complexes by including two guests simultaneously with high binding affinities in water.^{9, 10} The last property provides synthetic versatility to self-assemble amphiphilic ternary complexes into vesicles.¹¹⁻¹⁶ These vesicles can be exploited as supramolecular nanocarriers in which multiple entities can be readily incorporated by host-guest interactions or by loading the vesicles.^{14, 15} Release of these entities from the nanocarriers can be achieved by reversing the formation of the amphiphilic ternary complex through the use of thermo-¹⁵ and pH-responsive guest moieties, or by adding a competing guest.^{12, 14}

Recently, two types of guests, lipidated *trans*-azobenzene and methylviologen, formed vesicles in the presence of CB[8].¹⁷ Upon irradiation with UV light the photostationary state of the azobenzene switches to the *cis*-isomer, which is sterically unable to form the ternary complex and hence the vesicles disassembled.¹⁸ This type of ternary complex was also attached to the surface of silica nanoparticles. In the *trans*-isoform cargo was retained in the pores, however upon photo-irradiation the *cis*-isoform leads to the disruption of the ternary complexes and hence the pores are open to release the cargo.¹⁹ In addition, a peptide ligand, which recognized macrophages, was simply ratiometrically co-assembled with the *trans*-azobenzene guest to form the ternary complex.¹⁹

In this chapter, vesicular supramolecular nanoparticles (SNPs) are formed by the self-assembly of an amphiphilic ternary complex formed between CB[8], a hydrophobic alkyl substituted methylviologen (MV) and a hydrophilic oligo ethylene glycol chain substituted azobenzene (Azo), which also provides light-responsiveness to the SNPs (Fig. 3.1). We introduce ratiometric amounts of cell

targeting ligands into the SNPs and load the SNPs with fluorescent proteins to ease the visualization of the cellular targeting.

Fig. 3.1. (a) A supramolecular amphiphile is formed as a ternary complex between CB[8], a methylviologen with a hydrophobic alkyl chain (MV) and an azobenzene with a hydrophilic oligo ethylene glycol chain (Azo). This amphiphilic ternary complex self-assembles in water into photo-responsive vesicles. (b) Cell targeting is possible by including a small percentage of AzoRGD ligands, while the vesicles can be loaded with protein cargo (here: teal fluorescent protein, TFP). (c) Structures of the molecules used in this study.

3.2. Results and discussion

3.2.1. SNPs characterization

The three components MV, CB[8] and Azo were mixed in an equimolar ratio at a final concentration of the ternary complex of 16 μM (see Experimental section). These components form a supramolecular amphiphile that self-assembles in

aqueous solutions into vesicular nanoparticles. The formation of spherical SNPs was verified using high resolution scanning electron microscopy (hrSEM, Fig. 3.2a). Inspection of these SNPs using transmission electron microscopy (TEM) showed that the particles are hollow, suggesting that the SNPs are vesicles (Fig. 3.2b and Experimental section). Considering the amphiphilic nature of the building blocks of the SNPs, a bilayer structure is hypothesized for the shell of the vesicles, where two supramolecular ternary amphiphiles form a bilayer where the hydrophilic oligo ethylene glycol chains interact with the aqueous inside of the SNPs and the solution surrounding the SNPs, while shielding the hydrophobic alkyl tails and CB[8] that reside inside this bilayer. From the TEM images, the thickness of this bilayer is estimated to be 8 ± 3 nm and this value is in close agreement with the calculated width of such a bilayer structure (Fig. 3.6 in the Experimental section). The average overall diameter of the SNPs was determined by hrSEM (128 ± 39 nm averaging 300 particles) and the average hydrodynamic diameter was determined by dynamic light scattering (DLS) (204 ± 38 nm, 3 separate measurements). Due to the drying of the sample, the sizes measured by hrSEM are smaller than the hydrodynamic diameters measured in solution by DLS. In the case of all negative controls, in absence of CB[8] or using its smaller homologue CB[7], which is unable to complex both guests simultaneously, no particles were observed (Fig. 3.2c).

Importantly, the formation of particles was found to be dependent on the ratio of the three components. DLS was used to monitor samples prepared by mixing a constant concentration of Azo ($16 \mu\text{M}$) with increasing concentrations of a preformed dimeric complex of CB[8] and MV ranging from 0 to $16 \mu\text{M}$. The closer the concentration of the dimer MV-CB[8] approached that of Azo, the higher the amount of particles formed (Fig. 3.2d-e). This confirmed that particles were exclusively formed by the self-assembly of the amphiphilic ternary complex.

Fig. 3.2. (a) SNPs formed at an equimolar ratio of MV, CB[8] and Azo (16 μM) imaged by hrSEM (scale bar 400 nm) and by (b) TEM (negative staining with 1% uranyl acetate, scale bar 40 nm). (c) Hydrodynamic diameter size distributions as measured by DLS of SNPs formed in a mixture of Azo:CB[8]:MV at 1:1:1 ratios, or when the CB[8] was replaced by CB[7] (AzoCB[7]MV). As a reference the signal measured for water is reported. (d) The formation of SNPs is monitored by DLS for concentrations of MV:CB[8] between 0 and 16 μM, while keeping the concentration of Azo constant at 16 μM. The mixing ratios between (MV:CB[8]) and Azo are given. (e) Areas of the DLS size distributions plotted relative to the mixing ratios. (f) UV-Vis absorption spectra of SNPs after irradiation with UV light and time dependent *cis-trans* interconversion in ambient light. The legend denotes the duration of irradiation with ambient light. (g) Absorbance (at λ = 342 nm) of the *trans*-azobenzene monitored over 2 cycles of alternating exposure with UV and ambient light (30 min each). (h) DLS size determination of SNPs formed with MV, CB[8] and Azo at 1:1:1 ratio, after UV irradiation (1 h, 104 mW, with a shortpass filter <392 nm), and after subsequent irradiation with ambient light (15min).

Furthermore, the light responsiveness of the Azo guest when included as guest in the SNPs was investigated. The kinetics of re-equilibration of the photostationary state of the azobenzene upon UV irradiation (λ < 392 nm, 1 h,

r.t.) and, subsequently, under ambient light were monitored using UV-Vis spectroscopy (Fig. 3.2f-g). Upon UV irradiation the *cis*-isomer causes the disassembly of the amphiphilic ternary complexes triggering the expected disassembly of the particles as was observed by DLS and only about 20% of the particles remained after UV irradiation by DLS. The exposure to ambient light for 15 min prompted the reassembly of the particles as concluded by an increase in the amount of SNPs, which indicates that the azobenzene unit converted back to the *trans*-isomer that is able to form the amphiphilic ternary complexes (Fig. 3.2h).

3.2.2. Protein loading

Next, the feasibility to load the SNPs with cargo was explored. As model cargo, fluorescent proteins were chosen for their ease of detection using fluorescence spectroscopy.²⁰ To this end, a Förster resonance energy transfer (FRET) experiment was performed. SNPs were prepared from solutions containing, either a yellow fluorescent protein (YFP, donor), or a red fluorescent protein (TagRFP, acceptor) or both (all proteins at 18 μ M final concentration, see Experimental section). The same solutions, prepared in the absence of the SNPs components, were used as references. In Fig. 3.3a the normalized spectra of pure donor and acceptor, as well as their mixtures, are shown, both in the presence and the absence of SNPs. Surprisingly, although the proteins were used at semi-diluted (μ M) concentrations and have short (ns) lifetimes, considerable FRET occurred already in the protein mixture of YFP and TagRP without SNPs, which was attributed to protein aggregation.²¹

To estimate the magnitude of FRET in solutions with SNPs, we tried to quantify the change in FRET when SNPs were formed. The volume fraction actually encapsulated in SNPs, was estimated to be 0.01% of the total volume (see calculation in the Experimental section). The solution enclosed in the resulting SNPs will have the same concentration as the solution outside the SNPs as a result of the uniform protein distribution throughout the solution. This means that the fraction of enclosed proteins is equal to the enclosed volume fraction, *i.e.* 0.01%, which is not expected to give rise to any observable FRET effect or

change in FRET. However, we find marked changes in the spectra in solutions where SNPs could form (Fig. 3.3a-b). To analyze whether the effects can be attributed to FRET, first, the donor and acceptor components of the emission spectra of the mixtures (Fig. 3.3a-b, YFP and TagRFP with and without SNP, black lines) were deconvoluted using Lorentzian fits (Fig. 3.3c-d). Then, each acceptor component was corrected by subtracting the emission that originates from direct excitation of the acceptor at the excitation wavelength of the donor (Fig. 3.3a-b, TagRFP without SNP, red lines). Finally, the intensity ratios (donor/acceptor emission) of the resulting deconvoluted and corrected spectra of the mixtures with and without SNPs were evaluated to be 0.62 and 0.34, respectively. The fact that the donor/acceptor intensity ratio doubles upon SNPs formation cannot be caused solely by the encapsulated fraction of 0.01%, even if assuming a 100% FRET efficiency between these donor and acceptor. This indicates that the interaction of the mixed proteins caused by the presence of SNPs is much larger than can be explained by the effects expected for purely statistical inclusion, indicating the loading of the SNPs with proteins.

Fig. 3.3. (a) Normalized and (b) non-normalized fluorescence emission spectra ($\lambda_{\text{ex}} = 440$ nm) of: YFP/TagRFP mixtures (YFP+TagRFP), pure TagRFP or pure YFP. The sum of the normalized emission spectra of the single proteins is also given (sum). Each condition was measured for proteins (18 μM) mixed with MV, CB[8] and Azo (+SNP, solid lines) and only proteins (-SNP, dashed lines). (c) Lorentzian fits of the non-normalized fluorescence spectra ($\lambda_{\text{ex}} = 440$ nm) of YFP/TagRFP mixtures (YFP+TagRFP) with SNPs and (d) without SNPs.

Therefore the observed spectral changes are explained by significant protein loading of the SNPs, causing an enhanced local protein concentration that in turn causes the observed FRET enhancement.²² However, there are other effects that could cause the FRET enhancement upon SNPs formation. In the next section a more detailed discussion of the FRET experiments is presented.

3.2.3. Discussion of the FRET enhancement

As shown in Fig. 3.3b, the (non-normalized) emission spectra of the pure proteins changed in the presence of SNPs, in particular for YFP. This indicates that the SNPs have an effect on the individual proteins, which could be due to several factors. Firstly, the change of the refractive index of the medium surrounding the proteins in proximity of the supramolecular amphiphiles, that would in turn affect the lifetimes (and therefore quantum yields) of the excited

state of the fluorophores.²³ Secondly, the rupture of aggregates of proteins in the presence of SNPs that would reduce self-quenching and increase their emission. Thirdly, a combination of both factors can occur. The effect of the SNPs on the emission of the pure proteins means that the donor and acceptor emission could not be directly used to quantify the energy transfer. In order to compare the FRET in absence and presence of SNPs, the emission spectra of the mixtures of proteins with and without SNPs were corrected for the effects of the SNPs on the pure proteins. In particular, the spectra of the mixed proteins were deconvoluted by fitting Lorentzian curves to the emission peaks of TagRFP and YFP (Fig. 3.3c-d). The integrals of the raw spectra of the pure proteins and the Lorentzian fits for the proteins in the mixtures should be linearly correlated to the number of photons emitted (cps). The integrated emission intensities were hereafter used to quantify the FRET with and without SNPs.

Assuming that the effects of the SNPs on YFP are the same for the pure protein as for the protein in the mixture, we calculated that the SNPs led to an increase of the YFP emission by a factor 1.32. Applying this factor to the YFP component of the emission of the mixture without SNPs (7130 cps) yields an expected emission of YFP in the mixture with SNPs of 9410 (7130×1.32) cps. The emission measured for YFP in the mixture without SNPs is 7540 cps, thus 1870 cps less than the maximum possible (expected) value. This difference of 1870 cps represents the maximum possible increase in FRET that is only due to the increase of donor intensity, *i.e.* not a direct effect of the SNPs on the efficiency of the energy transfer.

Next, the TagRFP acceptor emission needed to be corrected because the TagRFP emission band in the mixture still contained a fraction of directly excited emission (2300 cps with SNPs, 2140 cps without SNPs, Fig. 3.3 red lines), not originating from the energy transfer with YFP. These values were subtracted from the respective emission values of the TagRFP component spectra, resulting in TagRFP emission solely due to FRET of 4700 cps with SNPs and 2450 cps without SNPs. The difference between 4700 and 2450 cps of 2250 cps represents the actual enhancement of FRET in presence of SNPs. This value is higher than maximum number of photons contributed by the increased donor emission (1870

cps, see above). The discrepancy between the expected maximum YFP emission and the actual increase of the TagRFP emission in the presence of SNPs indicates that an increase in FRET is related to the presence of SNPs.

Clearly, the encapsulation of 0.01% (see Experimental section) of the proteins in solution via statistical inclusion cannot lead to such a significant increase in FRET due to SNPs formation. The results of our calculations, therefore, indicate an interaction of proteins with the bilayer structure of the SNPs. If this interaction indeed exists and would lead to closely packed proteins, it could cause a strong energy transfer between proteins. Considering an average diameter of the β -barrel of the fluorescent proteins of 2.4 nm and a Förster radius of 5.7 nm for YFP with TagRFP (see Experimental section), the FRET efficiencies could theoretically become close to 100%. Moreover, the close proximity of the proteins on the SNPs would cause a change of the refractive index of the medium surrounding the proteins, which in turn would cause changes in lifetimes and consequently quantum yields,²³ which in turn could explain the increase in emission intensity of the pure proteins in the presence of SNPs. Taken together, all observed spectral changes can be explained by assuming protein binding to SNPs.

3.2.4. Cellular targeting and delivery of TFP

The next step was to introduce cellular targeting groups on the protein-loaded SNPs. For this purpose an Arg-Gly-Asp (RGD) motif was grafted onto an azobenzene guest moiety (AzoRGD). RGD is a ligand for integrin receptors on the cellular membrane,²⁴ which are overexpressed in tissues where angiogenesis is stimulated, making RGD ligands a promising tool in cancer therapies and imaging.²⁵⁻²⁷ Particles were prepared by mixing a teal fluorescent protein (TFP, 0.5 μ M) with the main three components of our photo-sensitive ternary complex - MV, CB[8] and Azo, resulting in SNPs loaded with TFP. The targeting moiety RGD was introduced by replacing 1% of Azo with AzoRGD (Fig. 3.1 and Experimental section). C2C12 cells were seeded into standard tissue culture plates and allowed to adhere for 2 h. Cells were then stained with a nuclear dye (Hoechst) and incubated for 10 min with the TFP-loaded SNPs. After washing, the cells were

directly imaged using a fluorescence microscope (Fig. 3.4a). Controls confirmed the specific roles of the SNPs and the RGD ligands (Fig. 3.4b). The TFP green fluorescence intensity was quantified as averages of triplicated experiments ($75 \leq n \leq 220$ cells) and the data is reported in Fig. 3.4c. In the absence of the particles, *i.e.* omitting the structural building blocks (MV, CB[8] and Azo) of the supramolecular amphiphiles, the green fluorescence intensity decreased significantly, independently from the presence of the targeting ligands AzoRGD. More notably, when the targeting ligand AzoRGD was missing on the SNPs very limited TFP emission was observed. The absence of TFP emission for samples treated with SNPs without TFP confirmed that the residual green fluorescence in the other negative controls is related to non-specific adsorption of the TFP on the cells. Moreover, the specificity of the delivery of TFP by SNPs bearing RGD was confirmed by the trend in TFP emission recorded when lowering the percentage of AzoRGD in the particles. When the percentage of AzoRGD in SNPs was lowered to 0.05% green emission inside the cells was observed that was significantly less intense when compared to cells treated with SNPs with 1% AzoRGD. Their emission intensity when using SNPs with 0.05 % AzoRGD was still significantly larger than the negative controls (Fig. 3.8 in the Experimental section). Interestingly, the overlap of the bright field micrographs of the cells showed that the TFP emission is localized in the proximity of the nuclei, rather than dispersed over the whole cell membrane or in the cytosol (Fig. 3.4a). Multivalent RGD-functionalized particles are known to induce the active endocytosis of the RGD-integrin complex, and with it, the SNPs, resulting in the observed accumulation in a perinuclear compartment²⁸ as it has previously been employed for imaging and drug delivery.²⁶ Overall, the results presented here confirm the specific interaction between AzoRGD-functionalized and TFP-loaded SNPs with C2C12 cells. This in turn confirms the interaction between TFP and the SNPs, as discussed above.

Fig. 3.4. a) Epifluorescence and bright field micrographs of C2C12 cells treated with SNPs loaded with TFP (green) and functionalized with AzoRGD ligands for integrin receptors. (b) As negative control, C2C12 cells were imaged after incubation with only AzoRGD and TFP (top-left), only TFP (top-right), TFP-loaded SNPs without AzoRGD (bottom-left) and SNPs with AzoRGD but not loaded with TFP (bottom-right). Cells were stained with live cell stain for the nuclei (Hoechst, blue). Scale bars 50 μm . (c) The green fluorescence intensity was measured using the ImageJ software package²⁹ for all conditions and plotted as the average over $75 \leq n \leq 220$ cells collected in three independent experiments. The error bars are the standard deviations of the distributions and ** indicates a significant difference with a 95% level of confidence.

3.3. Conclusions

In conclusion, we obtained protein-loaded supramolecular nanoparticles for cellular targeting.

Upon complexation within CB[8], methylviologen and azobenzene guests, equipped with an aliphatic chain and a hydrophilic chain respectively, formed supramolecular amphiphiles which self-assembled in aqueous media to form light-responsive nanometer-sized vesicles. The applicability of SNPs in biological systems was explored by loading the particles with fluorescent proteins and by demonstrating the feasibility of incorporating targeting ligands into the nanoparticles. Cell targeting peptide ligands, equipped with an azobenzene moiety were readily introduced in the particles by host-guest chemistry and promoted receptor-mediated uptake of the loaded nanoparticles. The shown above results demonstrate that CB[8] supramolecular nanoparticles offer a highly versatile, modular approach for the assembly of highly-ordered systems having the potential of introducing the desired characteristics to biomedical applications.

3.4. Acknowledgments

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3.5. Experimental section

3.5.1. General methods

Chemicals were purchased from Sigma Aldrich unless differently specified and used without further purification. Each batch of cucurbit[8]uril (Sigma Aldrich) was assessed by UV-Vis titration with cobaltocenium according to a literature procedure.³⁰ Cell culture grade water (Water, Sigma Aldrich) and Dulbecco's Phosphate Buffered Saline (PBS, Sigma Aldrich) with pH 7.4 at 25 °C containing 0.01 M phosphate buffer, 2.7 μ M potassium chloride and 0.137 M sodium chloride were used. Fmoc-protected amino acids and agents for peptide synthesis were purchased from Novabiochem and Multisynthet GmbH Chemicals. Cell culture buffers and staining reagents were purchased from Invitrogen or Thermo Fisher Scientific. Reactions were monitored using thin-layer chromatography (TLC), which was performed on 0.2 mm Merck precoated silica gel 60 F254 aluminum sheets. Spots were visualized using a basic KMnO_4 solution unless otherwise specified. Column chromatography was carried out on silica gel 60 (0.063-0.2 mm, Merck). High performance liquid chromatography (HPLC) was performed on Water (2535) setup equipped with analytical and preparative XBridge C_{18} columns. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker spectrometer (Ascend 400). Mass spectra were recorded with a Waters ESI-ToF spectrometer in positive mode (Micromass LCT). Dynamic light scattering (DLS) analysis was carried out on an Anaspec particle analyser (Nanotrac operating with

a Microtrac FLEX Operating Software). Particles were further characterized by high resolution scanning electron microscopy (hrSEM) on a Carl Zeiss microscope and by transmission electron microscopy (TEM) on an analytical FEG-TEM (Phillips CM 30). hrSEM samples were prepared on silicon wafers, while TEM samples were prepared on Formvar-carbon coated grids and stained with 1% w/v uranyl acetate in water. UV-Vis and fluorescence spectroscopy were performed using a Perkin Elmer UV-Vis spectrophotometer (Lambda 850) and a Perkin Elmer fluorimeter (LS 55) respectively. An Olympus microscope (IX71) with appropriate filter settings was used for recording bright field and fluorescence images.

3.5.2. Synthetic procedures

Synthesis of MV

Following a literature procedure³¹ 0.35 g (1.3 mmol) bromododecane was added to a stirred solution of 0.20 g (0.67 mmol) methylviologen in 25 mL of dry acetonitrile. The resulting solution was stirred at 70°C for 48 h followed by cooling to room temperature. The resulting precipitate was filtered and carefully washed with cold acetonitrile. The product (4, 4'-bipyridinium, 1-dodecyl-1'-methyl-, bromide iodide, MV) was obtained as orange solid. The yield, ¹H and ¹³C-NMR data match the reported values in literature.

Synthesis of Azo

The synthesis of 2-(2-(2-hydroxyethoxy)ethoxy)ethyl 4-methylbenzenesulfonate and Azo 2-(2-(2-(2-(4-(phenyldiazenyl)phenoxy)ethoxy)ethoxy)ethoxy)ethanol was carried out as described in literature.³² To a stirred solution of 10.0 g (51.0 mmol) of tetraethyleneglycol, 25 mg (cat.) of DMAP and 1.9 mL (13.3 mmol) of Et₃N in 100 mL of DCM, 2.4 g (13.0 mmol) of p-toluenesulfonylchloride was added keeping the temperature below 5°C. Stirring was continued for 1 h at this temperature and the solution was further stirred at room temperature for 24 h. The solution was then extracted with 1 M HCl (2 × 50 mL), water (2 × 50 mL) and brine (2 × 50 mL). The organic phase was dried over MgSO₄ and the solvent was removed in vacuo. The residue was purified by silica gel column chromatography to yield 2-(2-(2-hydroxyethoxy)ethoxy)ethyl 4-methylbenzenesulfonate. The product was obtained as colorless oil. The yield, ¹H and ¹³C-NMR data match the

reported values in literature. Next, 1.1 g (5.3 mmol) of 4-phenylazophenol in 15 mL of acetonitrile was added to a stirred solution of 1.7 g (55.0 mmol) of 2-(2-(2-hydroxyethoxy)ethoxy)ethyl 4-methylbenzenesulfonate, 3.1 g (25.0 mmol) of K_2CO_3 and a catalytic amount of LiBr in 0.55 L of dry acetonitrile. The reaction mixture was refluxed for 2 days under argon. It was then allowed to cool to room temperature and the solvent was removed in vacuo. The residue was dissolved in DCM (30 mL), washed with water (30 mL) and brine (3 x 30 mL). The organic phase was dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by silica gel column chromatography to yield Azo. The product was obtained as orange oil. The yield, 1H and ^{13}C -NMR data match the reported values in literature.

Synthesis of AzoRGD

First a carboxylic acid-functionalized azobenzene (ethyl 2-(4-(phenyldiazenyl)phenoxy)acetic acid) was synthesized.³³ To a solution of 0.37 g (1.87 mmol) 4-phenylazophenol in 20 mL of dry acetone, 0.67 mL (6 mmol) of ethyl bromoacetate and 0.93 g (6.76 mmol) of K_2CO_3 were added. The reaction mixture was refluxed for 15 h and then allowed to cool to room temperature. After removal of the solvent under vacuum, the mixture was taken up in EtOAc and washed with water (3 x 50 mL). The organic layer was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The crude product was purified by silica gel chromatography to afford ethyl 2-(4-(phenyldiazenyl)phenoxy)acetate as an orange solid. The 1H and ^{13}C -NMR data are in agreement with reported values in literature. Next, to a solution of this product (1.55 mmol) in 20 mL of methanol, 4 mL of sodium hydroxide (1 N) was added and stirred for 2 h at room temperature. Then, methanol was removed under reduced pressure and 15 mL of EtOAc was added to the remaining solution. The aqueous layer was acidified to a pH of 2 with 1 M HCl. The aqueous phase was separated and the organic layer was further washed with brine (3 x 50 mL), dried over $MgSO_4$ and concentrated in vacuo to yield the desired product 2-(4-(phenyldiazenyl)phenoxy)acetic acid. The product was obtained as orange solid. The yield, 1H and ^{13}C -NMR data match the reported values in literature. Peptide Azo-GSGGRGDSG (AzoRGD) was synthesized using a Multisynth automatic solid

phase peptide synthetic robot (Syro II) following standard Fmoc protocols.³⁴ The carboxylic acid-functionalized azobenzene was added to the N-terminal of the peptide on the resin. Purification of the peptide was done by reversed phase HPLC in a gradient of H₂O/acetonitrile (10/90% to 0/100% in 70 min) and characterized by analytical HPLC and mass spectrometry (Fig. 3.5). $[M+H]^+ = 985.88$ (calc. 986.39); $[M+2H]^{++} = 493.49$ (calc. 493.69).

Fig. 3.5 Analytical HPLC chromatogram (top) and ESI-ToF MS spectrum (bottom) of the purified AzoRGD.

3.5.3. Nanoparticles preparation and characterization

To form nanoparticles MV, Azo and CB[8] stock solutions were prepared in cell culture grade water at 50 μ M and mixed to obtain a 1:1:1 ratio for the ternary

complex at final concentrations of 16 μM , each. After a sonication step (1 h, approx. 40 $^{\circ}\text{C}$) the samples were degassed under vacuum on a membrane pump for 15 min and the particles were then characterized by hrSEM, TEM and DLS. Fig. 3.6 shows the TEM micrographs on negatively stained samples of particles and the theoretical extended conformations of two supramolecular ternary complexes. For UV switching studies, freshly prepared particles were irradiated for 1 h with UV light (104 mW, with a short pass filter $< 392\text{ nm}$) and immediately measured by DLS or UV-Vis spectroscopy. Fluorescent proteins were loaded inside the nanoparticles by adding the proteins to the MV, Azo and CB[8] mixture prior to vesicles formation. No further purification was performed. Three different fluorescent proteins were used as cargo for the particles. Firstly, a FRET pair consisting of yellow and red fluorescent proteins (YFP and TagRFP) was used (Fig. 3.7). Secondly, teal fluorescent protein (TFP) was used for cell studies to take advantage of its limited nonspecific adsorption to cells. The pipetting error was determined by calculating the standard deviation of three independent experiments at equal nominal concentrations of TFP and resulted in a standard deviation of 0.77% of the maximum emission fluorescence intensity.

Fig. 3.6 TEM images of particles formed at equimolar ratios of MV, CB[8] and Azo (16 μM). Scale bars 100 nm, negative staining with 1% uranyl acetate. The schematic representation of two supramolecular ternary complexes in their extended conformation is also reported.

Fig. 3.7 Normalized absorption (dashed lines) and fluorescence (solid lines) spectra of YFP (blue, 18 μ M, λ_{ex} = 440 nm) and TagRFP (red, 18 μ M, λ_{ex} = 500 nm).

3.5.4. Calculation of the theoretical encapsulated protein fraction

To estimate the total volume fraction of proteins encapsulated inside the SNPs ($V_{\text{SNP}, \text{tot}}$), a spherical vesicular nanoparticle was considered with an outer diameter of 204 nm and an inner diameter of 190 nm. The corresponding inner and outer spheres delimit the shell of the SNP corresponding to the walls, which thickness is set at 7 nm considering the bilayer structure of the supramolecular amphiphiles. For one SNP this yields to:

$$\text{Outer surface area} = A_{\text{out}} = 4\pi r^2 = 1.31 \times 10^5 \text{ nm}^2$$

$$\text{Inner surface area} = A_{\text{in}} = 4\pi r^2 = 1.13 \times 10^5 \text{ nm}^2$$

$$\text{Total particle volume (including walls)} = V_{\text{out}} = (4/3)\pi r^3 = 4.45 \times 10^6 \text{ nm}^3$$

$$\text{Enclosed particle volume (excluding walls)} = V_{\text{in}} = (4/3)\pi r^3 = 3.59 \times 10^6 \text{ nm}^3$$

$$\text{Walls volume} = V_{\text{walls}} = V_{\text{in}} - V_{\text{out}} = 0.86 \times 10^6 \text{ nm}^3$$

Assuming the highest possible density in the plane, *i.e.* an hexagonal packing with a packing density equal to 0.907, and approximating that the footprint of each ternary complex projected onto respective sphere's surface to be the maximized cross section through the CB[8] molecule ($A_{\text{CB}[8]} = 0.61 \text{ nm}^2$), then the number of molecules comprising the wall of one nanoparticle is:

$$\text{Molecules forming the outer sphere} = (A_{\text{out}} / A_{\text{CB}[8]}) * 0.907 = 1.95 \times 10^5$$

$$\text{Molecules forming the inner sphere} = (A_{\text{in}} / A_{\text{CB}[8]}) * 0.907 = 1.68 \times 10^5$$

Thus, their sum (bilayer) gives:

$$\text{Molecules per particle} = 3.63 \times 10^5$$

Next, the number of molecules forming ternary complexes MV·CB[8]·Azo at the equilibrium (at 1:1:1 ratios) in solution needs to be calculated. The overall equilibrium binding constant K_{ter} is:

$$K_{ter} = \frac{[MV \cdot CB[8] \cdot Azo]}{[MV] \cdot [CB[8]] \cdot [Azo]}$$

and, according to literature:^{18, 35}

$$K_{ter} = K_{MV \cdot CB[8]} \cdot K_{(MV \cdot CB[8]) \cdot Azo} = 8.5 \times 10^5 \text{ M}^{-1} \cdot 1.4 \times 10^4 \text{ M}^{-1} = 1.2 \times 10^{10} \text{ M}^{-2}$$

Assuming that the azobenzene is fully present as *trans* isomer, the initial concentrations of the host and guests are known:

$$[MV]_i = [CB[8]]_i = [Azo]_i = 16.6 \times 10^{-6} \text{ M}$$

and therefore the problem can be solved analytically, setting the equilibrium concentration of the complex to $[MV \cdot CB[8] \cdot Azo] = x$ and the equilibrium concentrations of the free reagents as $[MV] = [CB[8]] = [Azo] = (16.6 \times 10^{-6}) - x$. Substituting these equilibrium concentrations in the formula for K_{ter} , a cubic equation in x is obtained with only one real solution for $x = 7.87 \text{ } \mu\text{M}$ which is the concentration of the ternary complex at the equilibrium, *i.e.* $[MV \cdot CB[8] \cdot Azo]$. Therefore, having a known total volume of the solution of $V_{tot} = 0.6 \text{ mL}$, the number of ternary complexes for $x = 7.87 \text{ } \mu\text{M}$ is equal to 2.84×10^{15} .

Next, the average number of particles in the solution can be calculated from the ratio of the total number of ternary complexes in solution at the equilibrium (2.84×10^{15}) and the average number of ternary complexes per particle (3.63×10^5), yielding $n_{tot} = 7.82 \times 10^9$.

Finally the total volume enclosed in the particles is calculated as:

$$V_{in, tot} = V_{in} \cdot n_{tot} = 2.81 \times 10^{16} \text{ nm}^3 = 0.028 \text{ } \mu\text{L}$$

The total volume occupied by the walls of the nanoparticles results:

$$V_{walls, tot} = V_{walls} \cdot n_{tot} = 6.72 \times 10^{15} \text{ nm}^3 = 0.007 \text{ } \mu\text{L}$$

Assuming a uniform distribution of the protein molecules in the solution, which means roughly excluding interactions of the proteins with the walls of the particles, the total volume available for protein encapsulation, arbitrarily defined by the walls and the cavity of the particles, yields:

$$V_{SNP, tot} = V_{in, tot} + V_{walls, tot} = 3.48 \times 10^{16} \text{ nm}^3 = 0.035 \text{ } \mu\text{L}$$

This corresponds to approx. 0.01% of the total volume ($V_{\text{tot}} = 0.6 \text{ mL}$) of the solution that is available for protein encapsulation.

3.5.5. Calculations of the Förster radius

Non-radiative fluorescence resonance energy transfer (FRET), between a donor molecule in its excited state (D^*) and an acceptor molecule (A) in the ground state, can be characterized by the Förster radius R_0 , defined by:

$$R_0 = \sqrt[6]{\frac{9000 \ln 10}{128 * \pi^5 * N} \frac{\kappa^2 * Q_D * J(\lambda)}{n^4}} = 0.211 \sqrt[6]{\frac{\kappa^2 * Q_D * J(\lambda)}{n^4}}$$

where κ^2 describes the relative orientation of the transition dipole moments of the donor and the acceptor, which is assumed to be equal to 2/3 for randomly oriented molecules in solution. Q_D is the quantum yield of the donor (= intensity of fluorescence/intensity of the absorbed radiation) and for YFP equal to 0.61. N is Avogadro's number and the refractive index of the solvent is $n = 1.333$ for water at 25 °C.

The degree of spectral overlap between the donor and the acceptor is described by the overlap integral ($J(\lambda)$). The absorption spectrum of the acceptor expressed as variations of the extinction coefficient as a function of λ ($\epsilon_A(\lambda)$). The emission spectrum of the donor was normalized so that the value of the integral over λ from 0 to "infinity" was equal to 1 ($F_D(\lambda) = 1$). These spectra were used to calculate the overlap function and thus the overlap integral $J(\lambda)$:

$$J(\lambda) = \int_{\infty}^0 F_D(\lambda) * \epsilon_A(\lambda) * \lambda^4 d\lambda$$

where $\epsilon_A(\lambda)$ is given in units of $\text{M}^{-1}\text{cm}^{-1}$ and λ is in nm, thus $J(\lambda)$ is in $\text{M}^{-1}\text{cm}^{-1}\text{nm}^4$. The overlap integral yielded $J(\lambda) = 4.39 * 10^{15} \text{ nm}^4\text{M}^{-1}\text{cm}^{-1}$, and the resulting Förster radius R_0 was determined to be 56.7 Å.

3.5.6. Cell experiments

C2C12 cells (murine myoblast cell line) were cultured in DMEM medium with 10% fetal bovine serum, L-glutamine and penicillin-streptomycin. Cells between passages 15 and 25 were used and kept below 90% confluence for sub-culture. For experiments, cells were seeded in tissue culture 96-well plates at densities

between 8,000 - 10,000 cells cm^{-2} and left for at least 2 h in the incubator (37 °C with 5% CO_2). Cells were then washed once with Live Cell Imaging Solution (LC-buffer), counterstained with Hoechst (1 $\mu\text{g}/\text{mL}$, 5 min) and rinsed twice with LC-buffer. Cells were then incubated with nanoparticles loaded with TFP (0.5 μM) and targeted with AzoRGD (0.05-1% of the total content of Azo was replaced by AzoRGD, *e.g.* resulting in 1.6 μM AzoRGD, 14.5 μM Azo and 16 μM CB[8] and MV for the 1% AzoRGD condition) for 10 min in the incubator. Finally cells were washed twice with LC-buffer to remove unbound particles. Live cells were then directly imaged by (fluorescence) optical microscopy. The green fluorescence intensity was quantified after background subtraction using the ImageJ software package.²⁹ Experiments in Fig. 3.4 were reproduced on three separate days and on each day each condition was duplicated. Experiments in Fig. 3.8 were duplicated during one day.

Fig. 3.8. The green fluorescence intensity from C2C12 cells treated with TFP-loaded SNPs functionalized with different amounts of AzoRGD ligands (0.05-1% of the total content of Azo) was measured using ImageJ software for all conditions and plotted as averages of $25 \leq n \leq 220$ cells collected in three independent experiments. The error bars are the standard deviations of the distributions. The ** indicates a significant difference with a 95% level of confidence.

3.6. References

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4. Photo-responsive cell adhesion employing cucurbit[8]uril ternary complexes

4

The photo-responsiveness of a cucurbit[8]uril (CB[8]) ternary complex was used for influencing the cell adhesion on surfaces. Monolayers on gold surfaces and patterned gold lines on glass were functionalized with a ternary complex consisting of a surface-bound methylviologen (MV), CB[8] and an azobenzene derivative that was functionalized with a cell adhesive Arg-Gly-Asp (RGD) peptide (AzoRGD). The AzoRGD binds to the preformed MV-CB[8] complex on the surface with an affinity of $2.4 \times 10^4 \text{ M}^{-1}$, which was measured using quartz microbalance (QCM) and is similar to the affinity observed in solution. Mouse myoblast (C2C12) cells adhered specifically on the substrates presenting the RGD ligands in a ternary complex. Upon photo-isomerization of the azobenzene moiety, the ternary complex was partially dissociated and as a consequence the lamellipodia of the adhered cells were partially retracted.

4.1. Introduction

The controlled display, patterning and release of bioactive ligands on surfaces is of paramount importance in developing new strategies for guiding cell behavior in biomaterials.¹ For example cell adhesion depends on the recognition between cellular receptors and surface-bound ligands. The possibility of including a moiety in the molecular structure of the ligand that is sensitive towards an external stimulus, such as light, is attractive to provide control over the strength of the interaction between the ligand and cellular receptor.^{1, 2}

Photo-labile moieties have been used to covalently link cell adhesive ligands to surfaces. Photo-irradiation of the linker releases the adhesive ligands away from the surface leading to a reduction in cell adhesion.³⁻⁵ Alternatively, photo-deprotection of surface-bound ligands could induce cell adhesion to surfaces.^{6, 7}

An interesting way to change the adhesive properties of a surface-bound ligand in a reversible manner made use of the photo-induced conformational change of azobenzene as part of the linker. The conformational change of the ligand that accompanies the *trans* to *cis* photo-isomerization of azobenzene masks the ligands and reduces cell adhesion. This conformational state can be reversed by exposure to heat or visible light and thereby re-expose the ligands to cells.⁸⁻¹⁰

Alternatively, reversible photo-responsive molecular switching can be achieved on supramolecular surfaces.¹¹ The *trans*-azobenzene forms host-guest complexes with α - and β -cyclodextrins (CDs)¹²⁻¹⁴ as well as with cucurbit[7]uril (CB[7])¹⁵ and heteroternary complexes with cucurbit[8]uril (CB[8]) and methylviologen.^{16, 17} For example, the host-guest complex between β -CD and *trans*-azobenzene modified with an RGD ligand formed a self-assembled monolayer (SAM) for photo-controlled and reversible supramolecular cell adhesion.¹⁸ Recently, in a similar design also cell migration could be controlled through both the photo-isomerization of the azobenzene guest and the competition with a stronger binding guest (adamantane).¹⁹ In previous work from our group, β -CD monolayers on gold and glass surfaces were used to control by light the display of azobenzene-modified glycoconjugates and to investigate the recognition properties of these ligands towards lectins and bacteria.²⁰ Alternatively CB[8] ternary complexes with

viologen and azobenzene derivatives offer the opportunity of orthogonally switching the self-assembly by either electrochemically reducing the viologen or by photo-isomerization of the azobenzene.¹⁷ Even though a CB[8] ternary complex between methylviologen and a mannose-modified azobenzene showed photo-controlled release of bacteria on lipid bilayers,²¹ the ability to employ a dual responsive surface based on CB[8] ternary complexes for changing the ligand presentation is still a challenging target in developing new strategies for guiding cell behavior. In our group, SAMs of CB[8]·methylviologen were employed to construct ternary complexes for supramolecular adhesion of living organisms.^{22, 23} Dissociation of the ternary complex by electrochemical reduction of the methylviologen caused the release of cell adhesive ligands resulting in the detachment of the cells from the substrates.²² However, the introduction of a light-responsive guest such as azobenzene in these surfaces to create a potentially dual-responsive surface for cell adhesion has not been explored yet.

In this chapter we investigate whether the use of a ternary complex between CB[8], surface-bound methylviologen and a photo-switchable azobenzene RGD ligand (AzoRGD) can provide photo-responsive cell adhesion (Fig. 4.1).

Fig. 4.1. Schematic representation of the photo-responsive CB[8]-mediated cell adhesion on a mixed SAM on gold. C2C12 myoblasts cells adhere specifically onto monolayers presenting RGD ligands *via* the ternary complex MV·CB[8]·AzoRGD. UV irradiation ($\lambda \approx 350$ nm, 14 mW/cm²) of the surface leads to dissociation of the ternary complex and thus reduces cell adhesion.

4.2. Results and discussion

The formation of the ternary complex between paraquat·CB[8]·AzoRGD was investigated in aqueous solution using UV-Vis spectroscopy (Fig. 4.2). Paraquat was

used instead of MV as a solution model for the ternary surface complex MV·CB[8]·AzoRGD. As expected, at room temperature and under ambient light, the spectrum of the pure AzoRGD showed a photostationary state of the two isomers, the - more stable - *trans* and the *cis*, with a maximum absorbance at $\lambda = 350$ nm and at $\lambda = 425$ nm, respectively (Fig. 4.2a).¹⁶ The exact distribution of the two isomers was determined using ¹H-NMR (D₂O, 100 μ M, room temperature) and of the total amount of azobenzene, 36% was in the *cis* isomer under ambient light (see Experimental section).

Upon adding AzoRGD to the preformed 1:1 complex between paraquat and CB[8] under ambient light and room temperature, the formation of the charge transfer ternary complex was evidenced by a decrease of the intensity of the *trans*-azobenzene absorbance ($\lambda = 350$ nm) and by a simultaneous increase of the maximum absorbance of the methylviologen ($\lambda = 260$ nm) (Fig. 4.2a). Notably, the increase of absorbance at 260 nm was higher than the sum of the spectra of the single components.^{12, 16}

Fig. 4.2. (a) UV-Vis spectra of paraquat/CB[8] (1:1 ratio), AzoRGD alone and paraquat/CB[8]/AzoRGD (1:1:1 ratio) were measured directly after sample preparation under ambient light (final concentration 100 μ M in milliQ water at room temperature). (b) UV-Vis spectra of the solution of the complex measured in this sequence: before and after 30 min of UV irradiation (104 mW, with a short pass filter < 392 nm), after keeping the sample in the dark for 50 min and after keeping the sample under ambient light for 10 min.

The response of the ternary paraquat·CB[8]·AzoRGD complex to photo-irradiation was firstly studied by exposing the solution of the complex to UV light for 30 min. This led to a decrease of the absorption band of the *trans* isomer at $\lambda = 350$ nm and an increase of the absorption band of the *cis* isomer that and at $\lambda =$

425 nm (Fig. 4.2b). The distribution of *trans-cis* isomers after UV photo-irradiation was 3:7, as determined by $^1\text{H-NMR}$ (see Experimental section). These results indicated the partial photo-isomerization of the *trans*-AzoRGD to the *cis* isomer. The absorbance intensity of the peak corresponding to the paraquat ($\lambda = 260$ nm) also decreases after exposure to UV light. This was expected and indicated that the *cis* isomer dissociates from the ternary complex.¹⁶ Further, the photostationary state remained relatively stable over a 50 min interval at room temperature (dark 50 min, Fig. 4.2b) and only small changes were observed in the absorbance spectra. Exposure to ambient light promoted instead quick (5-10 min) re-formation of *trans*-AzoRGD, verified by the increase of the absorbance band of the *trans* isomer around $\lambda = 350$ nm and a decrease of the band of the *cis* isomer around $\lambda = 425$ nm (Fig. 4.2b). The reversible photo-isomerization of the azobenzene moiety resembles the behavior reported before for unfunctionalized azobenzene,¹⁶ suggesting that the functionalization of the azobenzene with an RGD ligand does not hamper the photo-isomerization.

In order to develop a CB[8]-mediated supramolecular monolayer to promote specific cell adhesion, the formation of the ternary complex at the surface was investigated using quartz crystal microbalance with dissipation (QCM-D) (Fig. 4.3). To this end, a mixed SAM was formed on a gold substrate using undecane disulfides bearing either tetra(ethylene glycol) chains as the anti-fouling component (99 mol%) or a maleimide hexa(ethylene glycol) functionality as the reactive component (1 mol%).²⁴ The maleimide functionality was used in a Michael addition reaction with thiolated methylviologen (MV, Fig. 4.1) to install a MV-modified monolayer suitable for CB[8] mediated assembly of guests. Upon binding of CB[8] to the methylviologen SAM, a change of the resonance frequency was observed by QCM-D (Fig. 4.3) in agreement with previous studies.²⁵ Subsequent titration of the CB[8]·MV SAM with increasing concentrations of AzoRGD gave a step-wise decrease of the resonance frequency indicating that the ternary complex was formed on the surface. Finally, the complex dissociated upon rinsing the surface with the buffer (Fig. 4.3). The difference in frequency (relative to the frequency observed for the CB[8]·MV SAM) recorded for each concentration of AzoRGD were used to derive the equilibrium binding constant of $2.4 \times 10^4 \text{ M}^{-1}$ using a Langmuir binding model. This

value is in agreement with the reported value in aqueous solution¹⁶ and suggests that the MV·CB[8] complexation with AzoRGD on the surface is comparable to the case in solution.

Fig. 4.3. QCM-D sensogram of an MV SAM in water, followed by flowing with a 50 μM CB[8] solution (●), and subsequently titrated with solutions of AzoRGD at 5, 10, 20, 50 μM in the presence of 50 μM CB[8] (*), finally followed by rinsed with water (♦). All solutions were in milliQ water, 25 $^{\circ}\text{C}$, ambient light.

To verify whether the supramolecular presentation of RGD functionalities on the CB[8] surface can promote cell adhesion, C2C12 myoblast cells were seeded onto the Azo SAMs – prepared as described above – and were allowed to adhere for 1 h, after which the cells were fixed and stained for actin and the focal adhesion marker vinculin (see Experimental section). To verify the specificity of the supramolecular adhesion, C2C12 cells were seeded on control SAMs that presented only MV or MV·CB[8] (Fig. 4.4). Cells spread over the SAM that displayed the supramolecular ternary complex MVCB·[8]·AzoRGD, while rounded, smaller cells were found for the negative controls. Focal adhesions (stained for vinculin) were exclusively observed on fluorescence micrographs recorded on SAMs of MV·CB[8]·AzoRGD demonstrating the specificity of the cell adhesion in the presence of the AzoRGD (Fig. 4.4). These results indicate that cells recognized and interacted with the azobenzene-modified RGD tethered to the MV·CB[8] SAM through specific interactions. C2C12 cells were observed to attach and spread on this type of supramolecular surface in about 10 min from the seeding, in line with results found in literature,²⁶ and therefore in a faster time scale than the dissociation of the RGD ligands from the surface.²⁷ In our case the clear contrast observed between cell spreading in presence and absence of AzoRGD on the supramolecular monolayers confirmed these earlier observations.

Fig. 4.4. Bright field and fluorescence micrographs of C2C12 cells seeded on surfaces displaying (a) the ternary complex MV-CB[8]·AzoRGD, (b) only MV, (c) only MV-CB[8], or (d) with covalently attached RGD (Cov, GGCGGRGDS). Cells were stained for actin (red), nuclei (blue) and the focal adhesion marker vinculin (green). Scale bars represent 100 μm (left and center columns) and 50 μm for the magnified images (right column).

Attempts to use light to release cells from the monolayers showed no or little retraction (results not shown) and therefore substrates with smaller adhesion areas were evaluated. To this end, we proceeded to test whether the MV-CB[8]·AzoRGD functionalization can be achieved on thin gold line patterns on glass. CB[8]-mediated display of RGD-naphthol guests on such patterned substrates has been previously employed in our group for inducing local changes in cell adhesion by

electrochemical reduction of methylviologen.²² Here, substrates were treated with piranha, and the glass areas were passivated with poly(ethylene glycol) (PEG) by silane chemistry.²² Any physisorbed PEG was rinsed off, and the gold lines were functionalized subsequently with maleimide, MV, CB[8] and AzoRGD, similarly to the steps performed for functionalizing the full gold substrates described above. As a negative control for light responsive cell retraction, an RGD peptide (Cov, GGCGGRGDS) was covalently attached to the maleimide SAM by a Michael addition reaction. Cells adhered and spread to a similar extent on these types of SAMs (Fig. 4.4). C2C12 myoblasts were seeded for 1 h on the patterned surfaces and subsequently stained (see Experimental section). Cells were observed on all SAMs, however, larger numbers of cells were observed on the RGD-functionalized SAMs (results not shown). More importantly, cells were spreading over multiple, up to 4, gold lines in the case of RGD-functionalized samples, whereas cells were restricted to 1 or 2 lines in the case of MV or MV-CB[8] lines (Fig. 4.5). The fluorescence micrographs showed dense assemblies of vinculin exclusively on the RGD-functionalized lines indicating that cells were specifically adhering to the lines that display the RGD ligands (Fig. 4.5). These results indicate the specific interaction of cells with the AzoRGD line patterns.

Fig. 4.5. Bright field and fluorescence micrographs of C2C12 cells seeded on line patterned surfaces displaying (a) the ternary complex MV-CB[8]-AzoRGD, (b) only MV, (c) only MV-CB[8], or (d) with covalently attached RGD. Cells are stained for actin (red), nuclei (blue) and the focal adhesion marker vinculin (green). Scale bars represent 100 μm (left and center columns) and 50 μm for the magnified images (right column).

Finally, the effect of photo-isomerization of immobilized AzoRGD on cell adhesion was monitored by bright field microscopy (Fig. 4.6). For these experiments, surfaces with gold lines were functionalized with the RGD-modified AzoRGD, as described above. C2C12 myoblast cells were seeded for 1 h upon which the sample was mounted on the optical microscope for cell release experiments. A bright field microscopy setup was used to monitor the cell responses, and the fluorescence setup of the microscope was used simultaneously to irradiate the

surface at a wavelength around 350 nm. As a control, cells adhered to lines functionalized with the covalently bound RGD peptide were subjected to the same UV treatment. Neither cell death nor cell detachment were visible for 30 min under the experimental conditions applied, as expected for values (14 mW/cm^2) of the power used here.³

When cells were adhered onto lines that were supramolecularly functionalized with AzoRGD, a modest but significant retraction of the lamellipodia was observed within 10 min of UV photo-irradiation (Fig. 4.6) and no further changes were recorded when the exposure time was prolonged up to 20 min. The differences in cell spreading along a gold line with MV-CB[8]-AzoRGD were measured before and after UV irradiation and compared with the observed responses on gold lines with covalently attached RGD (Fig. 4.6f). The average reduction of the linear extension of the cells adhered to supramolecularly AzoRGD-modified surfaces was $3.5 \pm 2.1 \text{ }\mu\text{m}$, while for cells on covalently modified RGD gold lines this reduction was close to zero (0.2 ± 1.1). For each of both surfaces, the photo-induced response observed for cells adhered on only one electrode was not significantly different compared to the response of cells spanning on multiple lines. The difference in spreading behavior observed between covalent and supramolecular adhesion is therefore attributed to the differences in cell adhesion: the covalently attached RGD surfaces do not change, hence give no change in cell spreading, whereas the supramolecular binding of AzoRGD and the concomitant *trans*-to-*cis* photoswitching is expected to provide a release of RGD ligands and thus a decrease in cell attachment. These results indicated that the extent of cell spreading can be changed by switching part of the RGD ligands by photo-isomerization of the AzoRGD moieties.

Fig. 4.6. (a-d) Representative bright field images of C2C12 cells seeded on gold lines functionalized with MV-CB[8]-AzoRGD complex before (a and c) and after (b and d) photo-irradiation (14 mW/cm², 10 min, $\lambda \approx 350$ nm). Scale bars represent 20 μm . Yellow and green pointers indicate cell lamellae before and after photo-irradiation respectively. (e) Depiction of the data analysis method of the cell length measured parallel to the gold lines, and (f) plot of the observed changes of the cell length, with $\Delta L = b - a$ (in μm). Each diamond represents ΔL for a single cell and the lines represent the mean value. The difference of the means is significant at the 0.005 level. (AzoRGD, $n = 27$ measured on 22 cells in 5 different areas of 2 samples; covalent, $n = 17$ measured on 14 cells on 3 different areas of 2 samples).

4.3. Conclusions

In conclusion, a strategy for the selective, CB[8]-mediated, photo-responsive cell adhesion on patterned surfaces has been developed. Complexation on surfaces

has been studied by QCM and a binding affinity of $2.4 \times 10^4 \text{ M}^{-1}$ was found, similarly to the value in solution. We showed that the CB[8]-mediated control over cell adhesion on an RGD-modified gold surfaces was selective on both gold SAMs and on thin gold lines on glass. Moreover, the retraction of cells observed on gold lines modified with the ternary complex MV·CB[8]·AzoRGD was significant upon photo-irradiation when compared to covalently attached RGD. We believe that these CB[8] surfaces are potentially suitable for a dual-responsive platform for controlling cell adhesion by electrochemistry and photochemistry.

4.4. Acknowledgments

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4.5. Experimental section

4.5.1. General methods

Chemicals were purchased from Sigma Aldrich unless differently specified and used without further purification. Each batch of cucurbit[8]uril (Sigma Aldrich) was assessed by UV-Vis titration with cobaltocenium according to a literature procedure.²⁸ Dulbecco's Phosphate Buffered Saline (PBS, Sigma Aldrich) with pH 7.4 at 25 °C containing 0.01 M phosphate buffer, 2.7 μM potassium chloride and 0.137 M sodium chloride was used. Triton X-100 was purchased from Acros Organics. Maleimide-terminated disulfide (4-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-N-(1-hydroxy-3,6,9,12,37,40,43,46,49,52-decaoxa-24,25-dithiapentapentacontan-55-yl)butanamide) and tetra(ethylene glycol) disulfide (3,6,9,12,37,40,43,46-octaoxa-24,25-dithiaoctatetracontane-1,48-diol) were purchased from ProChimia. Fmoc-protected amino acids and agents for peptide synthesis were purchased from Novabiochem and Multisynthetech GmbH Chemicals. Cell staining reagents were purchased from Invitrogen. NMR spectra were recorded

on a Bruker spectrometer (Ascend 400). The signal of the protonated solvents were used as reference. Chemical shifts are given in units of parts per million (ppm) and expressed relative to tetramethylsilane (TMS). Coupling constants (J) are reported in Hertz (Hz). High performance liquid chromatography (HPLC) was performed on Water (2535) setup equipped with analytical and preparative XBridge C₁₈ columns. Mass spectrometry (MS) was performed using a Waters ESI(+)-ToF spectrometer (Micromass LCT). UV-Vis spectroscopy was performed using a Perkin Elmer UV-Vis spectrophotometer (Lambda 850). An Olympus microscope (CKX41) was used for recording bright field images. An Olympus microscope (1X71) with filters was used for recording fluorescence images and bright fields images of cell release.

4.5.2. Synthetic procedures

Synthesis of AzoRGD. Peptide Azo-GSGGRGDSG was synthesized using a Multisynthtech automatic solid phase peptide synthetic robot (Syrro II) following standard Fmoc protocols.²⁹ A carboxylic acid-functionalized azobenzene was reacted on the resin in the last step. Purification of the peptide was done by reversed phase HPLC in a gradient of H₂O/acetonitrile (10/90% to 0/100% in 70 min) and characterized by analytical HPLC and mass spectrometry (see Chapter 3). $[M+H]^+ = 985.88$ (calc. 986.39); $[M+2H]^{++} = 493.49$ (calc. 493.69).

Synthesis of MV. The synthesis of MV was adapted from Rauwald, et al.³⁰ and González-Campo, et al.³¹

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

12-Bromo-1-(methyl-4,4'-bipyridinium)-undecane. 1,11-Dibromoundecane (2.07 g, 6.6 mmol) was dissolved in acetonitrile (15 mL) and DMF (3 mL). Methyl-

4,4'-bipyridinium iodide (500 mg, 1.68 mmol) was dissolved separately in a warm mixture of acetonitrile (10 mL) and DMF (2 mL) and added dropwise to the refluxing solution while stirring. Stirring and refluxing conditions were continued for 48 h. The hand warm solution was filtrated and the residue was washed with acetonitrile. Ether was slowly added to the clear filtrate while stirring. The product was collected by centrifugation (10 min at 7000 rpm) and decantation, and dried. Recrystallization from methanol and acetonitrile and drying *in vacuo* yielded an orange crystalline solid. This product was used for the following synthetic step without further characterization.

1-Methyl-4,4'-bipyridinium-undecanethioacetate. 12-Bromo-1-(methyl-4,4'-bipyridinium)-undecane (226 mg, 0.4 mmol) was dissolved in a mixture of Milli-Q (12.5 mL) and ethanol (5 mL). potassium thioacetate (114 mg, 1.0 mmol) was dissolved in Milli-Q (12.5 mL) and ethanol (5 mL) and added to the refluxing solution while stirring. The reaction mixture was kept under N₂ and refluxed for 48 h resulting in a light green-blue solution. NH₄PF₆ (0.26 g, 1.6 mmol) was added to the reaction mixture. The crème-white precipitate was filtered under vacuum and redissolved in acetonitrile. Any brown, solids were removed by filtration. The product was again precipitated by adding tetrabutylammonium chloride (0.44 g, 1.6 mmol) resulting in a white precipitate. The product was filtered under vacuum and further dried *in vacuo*. The product was obtained as a pale yellow powder. This product was used for the following synthetic step without further characterization.

1-Methyl-4,4'-bipyridinium-undecanethiol (MV). 1-Methyl-4,4'-bipyridinium-undecanethioacetate (44 mg, 72 µmol) was dissolved in the 1.25 M HCl solution in methanol (0.58 mL) while stirring. The mixture was refluxed overnight. The product was dried *in vacuo* and then dissolved in Milli-Q water (25 mL). The product was precipitated by adding NH₄PF₆ (0.53 g, 3.25 mmol). After vacuum filtration, the residue was dissolved in acetonitrile (10 mL) and again precipitated by adding tetrabutylammonium bromide (1.08 g, 3.25 mmol). Vacuum filtration and further drying *in vacuo* yielded the final product as a yellow-green solid. Yield: 19 mg (51%). ¹H-NMR (400 MHz, D₂O): δ = 9.12-9.05 (dd, 4H, CH); 8.56-8.52 (m, 4H, CH); 4.72 (t, 2H, CH₂), 4.50 (s, 3H, CH₃); 2.71 (t, 2H, CH₂), 2.08 (m, 2H, CH₂), 1.63 (m,

2H, CH₂), 1.36-1.26 (m, 14H, CH₂). ¹³C-NMR: (150 MHz, D₂O): δ = 23.74-38.45, 48.34, 62.21, 126.60, 126.93, 145.41, 146.31, 149.93 HRMS [M²⁺-H]⁺ = 357.2354 (calc. 357.2354), [M²⁺-C₁₁H₂₂SH]⁺ = 171.0896 (calc. 171.0917).

4.5.3. Quartz crystal microbalance (QCM-D)

All QCM-D measurements were performed using a Q-sense E4 module (BiolinScientific/Q-sense). The microsensors were cleaned with oxygen plasma for 15 min, washed in ethanol for 10 min, then incubated in a 1 mM ethanolic solution of the disulfides (1% maleimide, v/v) overnight under argon. Freshly after functionalization with MV a baseline was monitored in Milli-Q water until stable. All solutions were prepared with the same solvent. Flow rate was kept at 100 µl/min, temperature was kept at 25 °C.

Fig. 4.7. Partial ^1H -NMR spectrum of AzoRGD before (top) and after (bottom) UV irradiation in D_2O .

4.5.4. Surface functionalization

Gold substrates (1" glass, 20 nm Au, Sens BV) were cleaned in piranha for 20-30 s followed by thorough rinsing in Milli-Q water and drying with N₂. Substrates were immersed in a 1 mM ethanolic solution of the disulfides (1% maleimide, v/v) overnight under argon. MV was then reacted (200 μM, PBS pH 7.4, freshly prepared) by incubation for 1 h at room temperature. Further incubation with CB[8] or CB[8] and AzoRGD (all solutions 50 μM in 0.1x PBS) was done for 1 h at room temperature under ambient light conditions, after which the substrates were briefly dip-rinsed in MilliQ and dried with N₂.

Gold electrodes on glass (3 nm Cr and 17 nm Au) were cleaned with piranha for 20-30 s, rinsed thoroughly with Milli-Q and dried with N₂ just prior to modification of the glass with poly ethylene glycol (PEG), as previously described.²² Briefly, substrates were immersed in 2% (v/v) 2-methoxy (polyethyleneoxy) propyl trimethoxysilane in anhydrous toluene for 2 h, rinsed in toluene and baked at 120 °C for 2 h. Prior to further functionalization of the gold electrodes, substrates were sonicated 5 min in toluene, rinsed in toluene, rinsed in ethanol, sonicated in ethanol 5 min, rinsed in ethanol to remove any physisorbed PEG residues from the gold and directly incubated in a 1 mM ethanolic solution of the disulfides (1% maleimide, v/v) overnight under argon. Following rinsing in ethanol, surfaces were dried and further incubated with freshly prepared 200 μM MV in PBS for 1 h at room temperature. Subsequently surfaces were rinsed thoroughly in Milli-Q water and dried under N₂ stream. MV functionalized samples were further incubated in 50 μM AzoRGD-CB[8] in 0.1x PBS for 30 min at RT, briefly dip-rinsed in MilliQ and dried with N₂.

4.5.5. Cell culture

C2C12 mouse myoblasts were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with L-Glutamine, 1% v/v Penicillin/Streptomycin and 10% v/v Fetal Bovine Serum (FBS) and used between passage 10 and 30. Cells were sub-cultured on functionalized surfaces in serum depleted medium (without FBS).

Cells adhered to the surfaces (cell density 8000-10000 cells/cm²) were fixed directly with cold 4% paraformaldehyde (PFA) for 10 min at room temperature (RT)

and washed thrice in PBS. Cells were then permeabilized in 0.5% Triton X-100 and 5% Bovine Serum Albumin (BSA) in PBS for 10 min at RT and blocked with 0.1% Triton X-100 and 5% BSA in PBS (PBST) for 60 min at RT. Immunocytochemical labelling of cell proteins was done in PBST for 1 h at 1:200 for vinculin-FITC and 1:100 phalloidin 568 (Actin). Subsequently cells were washed with PBS twice and labelled with 4',6-diamidino-2-phenylindole (DAPI) 1:1000 in PBS. Immunolabelled cells were imaged using an inverted fluorescent microscope with corresponding excitation and emission filters.

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5. Directing cell adhesion through RGD-displaying knottins with tunable affinity for cucurbit[8]uril surfaces

5

In this chapter the results of a study are described in which cell adhesion on CB[8]-modified surfaces is directed through the assembly of multivalent knottins displaying RGD ligands with high affinity for integrin receptors. The multivalency in the knottins stems from the number of tryptophan amino acid moieties, between 0 and 4, that can form a heteroternary complex with cucurbit[8]uril (CB[8]) and surface-tethered methylviologen (MV). The binding affinity of the knottins with CB[8] and MV surfaces was evaluated using surface plasmon resonance spectroscopy. Specific binding occurred, and the affinity increased with the valency of tryptophans on the knottin. The surface coverage also increased with the valency. Cell experiments with mouse myoblast (C2C12) cells on the supramolecular knottin surfaces showed specific integrin recognition by the RGD-displaying knottins. Moreover, cells were observed to elongate more on the supramolecular knottin surfaces with a higher valency. Collectively, these results are promising for the development of platforms based on peptide-CB[8] ternary complexes for tunable interactions with cells.

5.1. Introduction

Cells are surrounded by the highly dynamic extracellular matrix (ECM) and respond to various factors in the ECM environment among which chemical and mechanical cues play a major role.^{1, 2} Such cues can initiate a cascade of instructions for adhesion, spreading, proliferation, migration and differentiation and eventually even drive the formation and the development of tissues. Among the cell surface receptors that signal these cues members of the integrin family recruit multiple proteins that eventually form so-called focal adhesions between the cell's cytoskeleton and the ECM.^{3, 4} A synthetic Arg-Gly-Asp (RGD) peptide motif, present on several ECM proteins such as fibronectin or vitronectin, has been identified to selectively bind to integrin receptors.⁵ The development of biomimetic materials incorporating this short RGD peptide sequence has resulted in much progress in understanding cell responses on biomaterials that mimic ECM cues.^{6, 7} The introduction of dynamic and stimuli-responsive molecular constituents in such biomimetic materials have only recently gained traction.⁸⁻¹⁰

Supramolecular chemistry offers a powerful approach to design systems in which ligands engage with their cell receptors in a dynamic, reversible and stimulus sensitive manner.¹¹ Biomimetic matrices have been reported based on host-guest chemistry using cyclodextrins and cucurbit[n]urils for the tunable, modular and responsive presentation of RGD peptide ligands for cell adhesion.¹¹⁻¹⁸ For example, we have reported the development of a self-assembled monolayer (SAM) for the dynamic display of linear RGD sequences through the formation of a CB[8] heteroternary complex between surface-bound methylviologen and RGD-naphthol guests.^{16, 19, 20} On these surfaces it was also possible to locally release ligands through electrochemical stimuli with subcellular resolution.^{16, 19} Surprisingly, recent FluidFM measurements indicated that cell-adhesion forces on such non-covalent surfaces are similar to surfaces where the RGD ligands were immobilized in a conventional covalent manner.²⁰ It was proposed that the linear RGD ligand used in this study had a relatively weak binding affinity ($K_d \approx 1\text{-}1000$ nM) towards integrins compared to the interactions that bound it to the surface ($K_d = 1\text{-}10$ μM).^{20, 21} It is known that the RGD

sequence in ECM proteins have a precise stereochemical conformation of the amino acid residues for optimal binding affinity and specificity to integrin receptors.²² We envisioned that supramolecular matrices able to mimic the natural ligand-receptor recognition would not be limited by the integrin affinity for the ligand. On the contrary, on supramolecular SAMs that displayed RGD with high affinity for integrin receptors, the interaction between the cell and the surface would be directed solely through the binding affinity of the host-guest complex.

In this direction, knottins seemed ideal candidates for the constructions of surfaces displaying bioactive ligands. This highly versatile class of miniprotein scaffolds have a characteristic cystine-knot structure that renders them highly stable against proteases and temperature denaturation. They contain solvent exposed loops through which they bind to target proteins with very high affinities. These loops have been determined to tolerate extensive modification, which has been widely used to engineer high affinity knottin binders against various medically relevant target proteins.²³ The Cochran group has developed a suitably high affinity ($K_d < 30$ nM) knottin binder towards integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$ ²⁴ and have successfully used this knottin for inhibiting tumor cell propagation.^{25, 26} We have recently reported the display of knottins on SAMs in a non-covalent and bivalent manner, yet with a moderately high affinity, ($K_d = 0.75$ μ M) mediated by heteroternary complexation between two tryptophan moieties on the knottins, CB[8] and methylviologen on the surface. Surface-tethered knottins were found to specifically recognize its target binding enzyme that was still active.²⁷

We demonstrate in this chapter that cell behavior can be directed by a library of integrin-targeting knottins with varying affinities towards our dynamic CB[8]-SAM surfaces. To this end, CB[8]-SAMs were prepared on an antifouling layer on gold displaying 1% maleimide functionalities that were reacted with a thiolated methylviologen (MV). Subsequently a ternary complex between MV, CB[8] and the knottins is formed. The differences in binding affinities for these CB[8]-SAMs of a series of knottin constructs, given by the number of available tryptophan (W)

residues on the knottins, as well as the effect of the knottins' valency on cell adhesion were evaluated (Fig. 5.1).

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5.2. Results and discussion

Our genetically engineered knottin constructs that are able to simultaneously bind integrins and CB[8] were designed with the features as depicted in Fig. 5.1. A knottin miniprotein (**W**) engineered by Cochran and coworkers²⁴ to bind integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$ with high affinity ($K_d < 30$ nM) was selected for the current study. To either one of the *N*- and *C*-termini of this knottin, zero to two CB[8]-binding motifs (SGGWGGS) were genetically fused, resulting in five different knottin constructs (W0 – W4) as shown in Fig. 5.1b. For the constructs with less than four SGGWGGS motifs, tryptophan was replaced by a serine resulting in a non-binding SGGSGGS motif which was placed at the remaining sites. These motifs were separated from the knottin by flexible linkers composed of glycines and serines with a maximum length of about 2.2 nm, which is a sufficient distance to allow for host-guest interactions to occur between adjacent hosts on the surface and tryptophans residing on one end of the knottin.²⁰ At the *N*- and *C*-termini of the entire constructs, zwitterionic GEGK peptide sequences were included to improve their solubility in aqueous solutions. As a negative control to the integrin-binding RGD motif, the functional loop of the W4 knottin was mutated to RGE resulting in construct WE (Fig. 5.1b).

Fig. 5.1. (a) Schematic representation of one of the integrin-binding knottin constructs (W2) non-covalently bound to a CB[8]-SAM through forming two ternary complexes. (b) Cartoon representing the library of knottin constructs prepared in this study. Different regions of the knottins indicate GEGK (green), SGGSGGS (brown), SGGWGGGS (yellow), SGGSGG (blue), RGD (pink) and RGE (grey).

These knottin constructs were genetically fused to the C-terminus of a teal fluorescent protein (TFP), separated by an enterokinase cleavage site (EKCS) linker and expressed in *E. coli* to prevent inclusion body formation as described previously.²⁷ Once expressed the knottins were cleaved from TFP using enterokinase and purified them using centrifugal filters (see Experimental section for details). SDS-PAGE was performed using the mixture obtained after the cleavage reaction and before centrifugation where bands corresponding to TFP (~30 kDa), enterokinase (~25 kDa) and the knottin constructs (~10 kDa) were observed (Fig. 5.7 in the Experimental section). The bands were visualized by enhancing the fluorescence of the tryptophan using Bio-Rad Stain-free precast gels (see Experimental section). Accordingly, the W0 band was not visible, while the intensity of the bands corresponding to the other constructs seemed to depend upon the number of tryptophans they contained.

Through MALDI-ToF measurements, the masses of the purified knottin constructs were determined (Fig. 5.2a and Fig. 5.8). Surprisingly, the recorded masses were always 378 Da lower than the theoretically calculated masses. This mass difference corresponded exactly to that of the GEGK sequence at the termini of the knottin constructs. The enterokinase cleavage site (DDDDK) lies adjacent to the N-terminal GEGK sequence and enterokinase is normally expected to specifically cleave the protein at the C-terminal of the lysine. In our construct, we suspect that the enterokinase detected its specific cleavage site however performed the cleavage after the lysine residue in the GEGK sequence as well.²⁸ Subsequently, the masses of the knottin constructs calculated by subtracting the mass of the GEGK sequence correspond to the experimentally determined masses. In the theoretically determined masses, we also took into account the 6 Da loss during the formation of all disulfide bridges within the knottin core. Since the experimental masses corresponded to these theoretical values, it can be assumed that all disulfide bridges were formed in our constructs. These results indicated that we were able to successfully isolate our knottin constructs using *E. coli*.

To determine the concentrations of the cleaved and purified knottin constructs, UV-Vis absorbance spectra were recorded (Fig. 5.2b). An absorbance

peak was observed at $\lambda = 280$ nm, due to the presence of tryptophans and another peak appeared at $\lambda = 250$ nm due to the presence of cystines. Using the absorbance values at $\lambda = 280$ nm and 250 nm of these curves and their ratios, along with theoretically determined extinction coefficients, the concentration of each sample was determined (calculations presented in the Experimental section).

Fig. 5.2. (a) Masses (m/z) of the cleaved and purified knottin constructs determined using MALDI-ToF and compared to calculated values. (b) UV-Vis absorbance values at $\lambda = 280$ and 250 nm (left y-axis) and their ratios (right y-axis) for the cleaved and purified knottin construct solutions.

The differences in binding between the knottin constructs to well-packed self-assembled monolayers (SAMs) of CB[8] were evaluated. The monolayers were prepared (Fig. 5.1a) on gold sensors for surface plasmon resonance (SPR) with a background layer of antifouling oligo(ethylenglycol) alkanethiols consisting of 1% maleimide groups, as described previously (Chapter 4).¹⁹ Thiolated methylviologen (MV) was conjugated to the maleimide groups and acted as the first guest for CB[8] to bind at the surface. In Fig. 5.3a, SPR measurements are shown that were performed in a flow cell using the MV conjugated sensors over which CB[8] was flowed and allowed to interact. Following this, increasing concentrations of each knottin construct, in the presence of CB[8], were sequentially injected and allowed to flow until equilibrium was reached. After the highest concentration, dissociation was allowed to happen by first flowing solutions with only CB[8] followed by buffer. It was observed that binding responses of the knottin constructs were generally higher in the constructs containing more SGGWGGS motifs. In the case of W0, negligible binding was

observed even when the highest concentration was used, supporting our claim that the knottin constructs bind to the CB[8] SAMs specifically through the tryptophan containing motifs. Much to our surprise the higher the valency of the knottins the higher the value where the SPR signal saturated indicating that more knottins were bound to the surface the more binding sites were available. At low concentrations of knottins the SPR response for W3 was unexpectedly higher than for W4. We tentatively relate this behavior to the ability of tryptophan to bind in the presence of CB[8] to either the surface-bound methylviologen in a heteroternary complex, or as homoternary complexes in solution.²⁹ Considering that the concentration of CB[8] was kept constant at 50 μM while the concentration of guests (knottins) was varied over the titrations from 50 nM up to 4 μM , the competition between the homo- and heterocomplexation prefers the former at low guest concentrations whereas the latter is preferred at high concentrations of guest. Moreover, depending on the number of tryptophan residues on the knottin constructs, a tryptophan on one knottin might dimerize intermolecularly with another tryptophan on a second knottin, or intramolecularly with another tryptophan linked by a flexible peptide chain on the same knottin. An intramolecular complexation is mostly probable on the tetravalent W4 in which the tryptophan residues have more chances of interaction, in respect to the trivalent W3. Therefore, at low concentrations the interaction of the tetravalent construct with the surface in a heteroternary complex might suffer from the competing formation of homoternary complexes in solution to a greater extent when compared to the W3 construct, explaining the apparent stronger response of W3 in comparison with W4 at low concentrations of knottin.

In Fig. 5.3b, the equilibrium level responses were plotted against their respective concentrations for each knottin construct apart from W0. The resulting plots were fitted with the four parameters logistic equation from which the slope coefficient (b) - indicating the steepness of the response curve at increasing knottin concentrations - and the dissociation constant (K_d) were derived.³⁰ This analysis shows clearly that the affinity of the knottin constructs towards CB[8]-SAMs increased the more SGGWGS motifs were present in the

knottin constructs, suggesting that binding occurred through multivalent interactions (Fig. 5.3b). The bivalent knotting W2 yielded a binding affinity value comparable to what has been reported with similar host-guest systems by us previously.^{27, 31} Interestingly, in the cases of W2 and W3, the parameter b remained very close to 1, indicating a conventional Langmuir adsorption. In contrast, in the case of W4, b increased to 1.81, indicating that the binding behavior changes with the knottin concentration. This observation confirms the hypothesis of a concentration-dependent scenario in which homodimerization in solution is competing with the formation of the heteroternary complex on the surface. Moreover, higher values of saturation were observed for W3 and W4, even though all SAMs presented comparable MV and CB[8] coverages (Fig. 5.3b). The most probable explanation is the formation of multilayered structures in which heteroternary complexes with the MV-CB[8]-modified surfaces anchor buckles of polyvalent knottins held together by homoternary tryptophan-CB[8]complexes.

Fig. 5.3. (a) SPR response plots observed when flowing different knottin constructs over CB[8] SAMs. $\bullet$ = buffer, $\circ$ = 50 μ M CB[8], 1 = 50 nM, 2 = 100 nM, 3 = 200 nM, 4 = 400 nM, 5 = 800 nM, 6 = 1 μ M, 7 = 1.6 μ M, 8 = 2.4 μ M, 9 = 3.2 μ M, A = 4.0 μ M of knottin construct in the presence of 50 μ M CB[8]. All plots were normalized to the response of CB[8] binding once it reached equilibrium at $\Delta\alpha = 0.06^\circ$. (b) The SPR response values of the knottin constructs (in the presence of 50 μ M CB[8]) were plotted against corresponding concentrations of knottins. The solid lines represent four parameters logistic regression fits of the data and the table summarizes the inflection point (K_d) and the slope values (b).

Having demonstrated the ability of our knottin constructs to bind specifically with MV-CB[8]-SAMs, we next proceeded to test cell adhesion on these surfaces.

To this end, SAMs were prepared as for the SPR experiments (see Experimental section) with the mono-, bi-, tri- and tetravalent knottins (W1-W4) having on an exposed loop an RGD-binding motif with sub-nM binding affinity for integrins (Fig. 5.1).²⁴ As variations of the cell environment in terms of ligand availability influence cell adhesion,³² two degrees of surface density were tested. The first set of conditions (high density) were prepared using 2 μ M knottin solutions resulting in saturation for all polyvalent knottin constructs but to different surface coverages depending on the valence. The second set (low density) was obtained by incubating the SAMs with knottin concentrations at which all constructs yielded approximately the same surface coverage ($\Delta\alpha = 0.085^\circ$ in Fig. 5.3a). Mouse myoblast (C2C12) cells were seeded and adhered to the SAMs for one hour after which the cells were fixed and stained for actin, vinculin and nuclei. The results at high and low surface coverage are presented in Fig. 5.4. To verify specific cell adhesion, the modified version of W4 was used in which the RGD motif was replaced by an RGE sequence (WE). Additionally, surfaces modified only with MV, or with MV and CB[8] were tested as negative controls. As positive controls for cell adhesion, fibronectin (Fb) coated surfaces and SAMs presenting a covalently attached RGD peptide (Cov) were included (Fig. 5.4). Cells adhered and spread on all protein surfaces at low and high density of RGD-knottins, while fewer and rounded cells were seen in SAMs only displaying MV or MV-CB[8] (Fig. 5.4). On SAMs of the control WE knottin construct spread cells were also observed, however cells were less elongated in respect to the positive controls.

Fig. 5.4. Fluorescence micrographs of C2C12 cells on fibronectin (Fb) surfaces or on gold SAMs modified with covalently attached RGD (Cov), or with MV, MV-CB[8] or MV-CB[8] and knottins (Ws). The star (*) indicates the density of knottin constructs, high when present and low when absent. Cells were stained for actin (red), nuclei (blue) and the focal adhesion marker vinculin (green). Scale bars represent 100 μm and 50 μm for the magnified images.

A quantitative analysis of the fluorescence micrographs was performed using CellProfiler software (Experimental section). Attempts of quantifying the extent of cell spreading considering the cell area gave no significant differences between the various SAMs, with the exception of the negative controls MV and MV-CB[8] (Fig. 5.5a). This was in agreement with the observations that these two negative controls gave drastically different cell adhesion from all other conditions. Further insights into the differences in cell morphology were achieved by comparing the cell circularity (eccentricity), which is a method approximating each cell by an ellipse and calculates the eccentricity as the ratio of the distance between the ellipse foci and the cell major axis length. An eccentricity value of 1 represents a perfect circle. The plot of the values of eccentricity are presented in Fig. 5.5b. Once more the negative controls MV and MV-CB[8] were significantly different from the positive controls. In addition this parameter allowed to delineate a trend of cell elongation when changing from the low valency W1 and W2 knottins towards the high valency W3 and W4 knottins. Significant differences between low and high valency knottins were found for the SAMs prepared at low density. To more clearly represent the cell elongation the ratio between the major and the minor cell axis length was evaluated and revealed to be more sensitive towards the differences in valency of the knottins (Fig. 5.5c). Significantly higher ratios, indicating larger elongation, were determined for cells seeded on surfaces presenting high valency knottin constructs in comparison with cells on low valency ones, on both high and low surface densities. No differences were observed between the positive controls and the tri- and tetravalent constructs, nor between all negative controls and the mono- and bivalent constructs at any surface density. This quantitative analysis confirmed that knottin constructs can be used for promoting supramolecular cell adhesion and that constructs with different binding behavior can actually elicit differences in cell morphology. Interestingly, supramolecular SAMs at low surface density of knottins yielded more marked differences in cell elongation with respect to surfaces with high surface density. The better performances of the surface at low knotting coverages could be due to an RGD ligands density closer to the optimum for C2C12 cells as well as to the formation

of more uniform monolayers when diluted solutions of knottins are provided for interaction with the surfaces. Unfortunately, no direct evidence was found to point to either of these hypotheses due to the complex assembly of the knottins on the surface described earlier. Also, differences between the positive controls and the supramolecular SAMs of W3 and W4 at low density did not allow any statistical conclusion to be drawn. However, the distributions of the ratio between major and minor cell axis length presented in Fig. 5.5c presumably indicates that cells on a supramolecular SAMs with high enough affinity resemble very well the cell elongation observed on Fb as natural mimic of the ECM.

Fig. 5.5. Box chart with binned data (30 bins) showing (a) cell area, (b) eccentricity and (c) the ratio between the major and the minor axis lengths of cells ($16 \leq n \leq 42$), analyzed with CellProfiler software. The black lines represent the median and grey lines the 25th and 75th percentiles. Mood's median test for non-normal distributions ($p \leq 0.05$), populations are significantly different (†) versus Fb, Cov, W3*, W4*, W3, and W4; (§) versus negative controls MV, CB[8] and WE; (#) versus mono and bivalent knottin constructs W1*, W2*, W1 and W2.

The green fluorescence of the vinculin marker for focal adhesions was also compared for cells seeded on knottin SAMs at low surface coverage (Fig. 5.6). Along with the increase of valency, more pronounced localized green fluorescence intensity were detected. Oppositely, the green fluorescence intensity was uniformly spread in all negative controls. This confirmed the correlation between the valence of the knottins and the formation of focal adhesion clusters.

Taken together, these results indicated the possibility of finely tuning cell behavior on supramolecular knottin constructs with different affinities for the surfaces.

Fig. 5.6. On the left panel, fluorescence micrographs of C2C12 cells on SAMs modified with knottin constructs WE and W1-W4 at low surface density. Cells were stained for the focal adhesion marker vinculin (green). Scale bars represent 50 μm . All images were taken with the same acquisition parameters. On the right panel, intensity profiles for vinculin.

5.3. Conclusions

In conclusion, in this chapter we have investigated the CB[8]-mediated assembly of polyvalent knottins displaying RGD ligands for cell adhesion. A library of knottin constructs was produced to this aim with a variable number of tryptophan guests for CB[8]. The interaction of the knottins on MV-CB[8] monolayers was characterized by SPR showing a stronger binding affinity for knottins with the larger number of tryptophan residues. An intricate concentration-dependent binding behavior was also observed at least for W4 and further studies are necessary for its understanding. Cell experiments on the supramolecular SAMs demonstrated the availability of the RGD ligand for specific integrin recognition on the knottins displayed on the supramolecular SAMs.

Moreover a correlation was observed between the elongation of cells and the valence of the knottins on the supramolecular SAMs. Tri- and tetravalent knottin constructs, especially at low surface density, yielded the largest extent of cell elongation behaving similarly as observed on fibronectin surfaces. This study is a first step in using knottins to promote dynamic and tunable cell-surface interaction. This approach can be further developed to become a biocompatible system fully based on homoternary complexes between peptides and CB[8] for both cell-surface or cell-cell interactions.

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5.5. Experimental section

5.5.1. General methods

Chemicals were purchased from Sigma Aldrich or from Acros Organics unless differently specified and used without further purification. The concentration of each batch of CB[8] (Sigma Aldrich) was assessed by UV-Vis titration with cobaltocenium according to a literature procedure.⁵³³ Dulbecco's Phosphate Buffered Saline (PBS, Sigma Aldrich) with pH 7.4 at 25 °C containing 0.01 M phosphate buffer, 2.7 µM potassium chloride and 0.137 M sodium chloride was used. Cell staining reagents were purchased from Invitrogen. NMR spectra were recorded on a Bruker spectrometer (Ascend 400). Polymerase chain reaction (PCR) was performed using a Peqlab Primus 25 advanced thermocycler. UV-Vis measurements to determine DNA and protein concentrations were performed using a Thermo Scientific Nanodrop 1000. Matrix assisted laser desorption/ionization time-of-flight (MALDI-ToF) analysis was performed with a Waters (Maldi Synapt) high definition mass spectrometer. UV-Vis measurements for trypsin inhibitor assays were performed using a PerkinElmer (Victor X3)

multiwell plate reader. Surface plasmon resonance (SPR) experiments were conducted using SPR gold substrates (50 nm thickness of gold) from Ssens BV on a Resonant-probes SPR. The reflectivity was measured at fixed angle at which point the linear region of the SPR curve stopped. Fluorescence microscopy was performed using an Olympus (1X71) microscope with appropriate filter settings.

5.5.2. Molecular cloning of knottin constructs

The W2 knottin gene was first constructed using assembly PCR. ssDNA sequences, with complementary overhangs, corresponding to the W2 knottin flanked by 5' enterokinase cleavage site and BsrGI restriction site sequences and 3' NheI restriction site sequence were designed using the Assembly PCR Oligo Maker tool³⁴ and ordered from Eurofins MWG Operon, Germany. Assembly PCR was performed using pfu DNA polymerase. This PCR product and a pET15b TFP-KO plasmid²⁷ were then digested with BsrGI and NheI restriction enzymes (NEB) and mixed together in a 5:1 insert:plasmid molar ratio for ligation using T4 DNA ligase (NEB). This resulted in the plasmid pET15b-TFP-W2, which was then transformed into Novablu ultracompetent cells (Novagen). The plasmid was extracted from selected colonies and the insert was sequenced by Eurofins MWG operon and verified to be correct. Site-directed mutagenesis was performed simultaneously with 4 sets of primers to mutate GGWGG sequences to GSGGG and vice versa. The Quick change lightning multi site-directed mutagenesis kit (Agilent) was used and mutated plasmids were transformed in XL 10 Gold ultracompetent cells. Several colonies were selected, grown in culture and the plasmids extracted from them were sent for sequencing. The plasmids pET15b-TFP-W0, pET15b-TFP-W1, pET15b-TFP-W3 and pET15b-TFP-W4 were thus obtained. One more site-directed mutagenesis was performed to mutate the RGD sequence in W4 to RGE resulting in the pET15b-TFP-WE plasmid. The six different plasmids were transformed into the expression host, Rosetta-Gami 2(DE3)pLysS competent cells (Novagen), and grown overnight on LB agar plates containing 34 mg/L chloramphenicol and 100 mg/L ampicillin. Individual colonies were grown in LB media containing the mentioned antibiotics. For long-term storage, 15%-glycerol bacterial stocks were

made and placed at -80 °C. The primers used for these constructs are presented here:

W2 Assembly PCR Primers	
W2 assem f1	AGTCAGTCAGTCAGTCAGTCTGTACAATGATGATGATGATAAAGGTGA GGG
W2 assem r2	AACCTCCGCTGCTGCCACCCGACCCTCCGCTCTTGCCCTCACCTTTATC ATCATCATC
W2 assem f3	GCAGCAGCGGAGGTTGGGGTGGGAAGCTCCGGAAGTGGTAGCGGAGG CTGCCCCG
W2 assem r4	CCTGGCTGCAGGTCAGCGGCGGGTTATCGCCGCGCGGGCGCGGGCA GCCTCCG
W2 assem f5	CTGACCTGCAGCCAGGATAGCGATTGCCTGGCGGGCTGCGTGTGCGG CCCGAAC
W2 assem r6	CAACCACCGCTACTACCGCTTCCACTACCGCCGAAAAGCCGTTCTGGG CCGCACA
W2 assem f7	CGGTAGTAGCGGTGGTTGGGGAGGGAGCAGCGGTGGATCGGGAGGG AGCGGCGAAG
W2 assem r8	CTTGGAACCTTGAACCTTGGCTAGCTTATTTGCCTTCGCCGCTCCCT C
Fwd	Agtcagtcagtcagtcagtc
Rev	Cttggaaccttgaaccttg
Mutagenesis primers	
S13W	Gcaagagcggagggtgggtggca
W20S	Ggcagcagcggaggttcgggtggaag
W72S	Cggtagtagcgggtggttcgggagggag
S79W	Ggagcagcggtagatggggagggga
RGD>RGE	Ccgcgcggcgagaacccgccgct

DNA sequence of the W2 knottin:

agtcagtcagtcagtcagtcTGTACAatgatgatgatgataaaggtgagggcaagagcggagggtcgggtggca
gcagcggaggttgggttggaagctccggaagtggtagcggaggctgcccgcgccgcgcggcgataaaccgccg
ctgacctgcagccaggatagcgattgcctggcgggtgcgtgtgcggcccgaacggctttgcggcggtagtgga

gcggtagtagcggtggttggggagggagcagcggtggatcgggagggagcggcggaaggcaataaGCTAGCca
aggttccaaggtccaag

5

Protein sequence of the W2 knottin:

DDDDKGEKSGSGSGSSGGWGGSSGSGSGGCPRPRGDNPLTCSQSDSCLAGCVCGPNGFC
GGSGSGSSGGWGGSSGSGSGSGEGK

5.5.3. Protein expression and purification

Five milliliters of bacterial starter cultures was grown overnight from glycerol stocks at 37 °C with shaking in LB media containing appropriate antibiotics. This was then transferred into 1 L of the same media, and the cultures were grown until they attained O.D._{600 nm} values between 0.4 and 0.8. Protein expression was then induced using isopropyl- β -D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM. These cultures were grown overnight at 18 °C with shaking. The cultures were then spun down at 6000 rcf for 10 min at 4 °C, and supernatants were discarded. Bacterial pellets were resuspended in 10 mL of BugBuster protein extraction reagent (Novagen) with 10 μ L of benzonase (30 U/ μ L, Novagen) and gently shaken for 20 min at 25 °C. TFP-fused knottin constructs were then purified using His-Select nickel affinity columns (Sigma-Aldrich) into an elution buffer of 50 mM NaH₂PO₄, 300 mM NaCl, and 250 mM imidazole at pH 8. The purified TFP-fused knottins were then rebuffed into pH 7.4 phosphate buffered saline (PBS, Sigma-Aldrich) using 30 kDa-cutoff centrifugal filter units (Amicon Ultra). Concentrations of these TFP-fused knottins were determined from the absorbance value at $\lambda = 467$ nm and an extinction coefficient of 64000 M⁻¹ cm⁻¹. Knottins were cleaved from the TFP using an enterokinase enzyme (EKMax, Life Technologies). The optimized reaction conditions required 50 ng of protein per 30 μ L of reaction with 0.1 units of enterokinase in pH 7.5 buffer containing 20 mM trisHCl, 10 mM NaCl, and 2 mM CaCl₂ at 37 °C for 16 h. SDS-PAGE analysis is shown in Fig. 5.7. The cleaved knottin was isolated from the other proteins using a 10 kDa-cutoff centrifugal filter unit (Amicon Ultra) followed by a 3 kDa-cutoff centrifugal filter unit (Amicon Ultra). Final knottin solutions were in PBS. At particular steps SDS-PAGE

analysis was performed using Bio-Rad Mini-PROTEAN TGX Stain-Free Precast 4-15% Gradient Gels in conjunction with a Mini-PROTEAN Tetra Vertical Electrophoresis Cell. Protein samples were prepared by mixing them in 1:1 volume ratio with the Bio-Rad Sample preparation buffer (2X) with β -mercaptoethanol added and heated to 95°C for 10 mins. The samples were loaded in the gel and 120 V potential was applied for electrophoresis. The gels were then activated and imaged using the Gel Doc EZ Gel Documentation System and a Stain-Free filter tray.

Fig. 5.7. SDS-PAGE analysis of the enterokinase cleavage of the knottin constructs from TFP.

5.5.4. Knottin concentration determination

The concentrations of the cleaved knottin constructs were determined using UV-Vis spectroscopy. The tryptophan (W) units exhibit their mean peak absorbance at $\lambda = 280$ nm (W_{280}) and the cystines (CC) exhibit their mean peak absorbance at $\lambda = 250$ nm (CC_{250}). W also has an absorbance contribution at 250 nm (W_{250}) and CC also contributes to the absorbance at 280 nm (CC_{280}).

The contribution of W at $\lambda = 250$ nm was determined from its spectral information³⁵ yielding:

$$W_{250}/W_{280} = 0.406$$

From W0, which contains no W units, the absorbance at $\lambda = 250$ nm and 280 nm arises purely due to the presence of the CC units and from this:

$$CC_{280}/CC_{250} = 0.324.$$

This value also corresponds with what was determined in literature.³⁶

These provide us with the set of linear equations:

$$CC_{250} + 0.406W_{250} = A_{250}$$

$$0.323CC_{250} + W_{280} = A_{280}$$

Where A_{250} and A_{280} are the total absorbance at 250 nm and 280 nm respectively as found in Figure 5.2d. Using these linear equations, it was possible to determine the absorbance purely due to W at 280 nm and CC at 250 nm.

Extinctions coefficients of W4, W3 and W2 at 280 nm were determined as $22375 \text{ M}^{-1} \text{ cm}^{-1}$, $16875 \text{ M}^{-1} \text{ cm}^{-1}$ and $11375 \text{ M}^{-1} \text{ cm}^{-1}$ respectively using Expasy's Protparam tool³⁷ assuming all cysteines form disulfides. These values allowed us to determine the concentrations of W4, W3 and W2 from their well-defined A_{280} absorbance peaks.

From this, the concentration:CC₂₅₀ ratio was determined and used to calculate the concentrations of W0 and W1 from the CC₂₅₀ values determined previously using the linear equations.

5.5.5. MALDI-ToF

For MALDI-ToF analysis, protein samples in water/buffer were mixed with equal proportions of acetonitrile + 0.1% TFA. Samples were prepared at a final protein concentration between 30 - 50 μM and this was mixed with sinapinic acid (σ)-matrix.

Fig. 5.8. MALDI-ToF mass spectra of the 6 different cleaved knottin samples.

5.5.6. Synthesis of MV

The synthesis of MV was adapted from Rauwald, et al.³⁸ and González-Campo, et al.³⁹ and it is described in Chapter 4.

5.5.7. Surface functionalization

Gold substrates (1" glass, 20 nm Au, Sens BV) were cleaned in piranha for 20-30 s followed by thorough rinsing in Milli-Q water and drying with N₂. Substrates were immersed in a 1 mM ethanolic solution of the disulfides (1% maleimide, v/v) overnight under argon. MV was then reacted (200 μM, PBS pH 7.4, freshly prepared) by incubation for 1 h at room temperature. Further incubation with CB[8] or CB[8] and knottins was done for 1 h at room temperature under ambient light conditions, after which the substrates were briefly dip-rinsed in MilliQ and dried with N₂. CB[8] was 50 μM in 0.1x PBS for all conditions. For covalently attached RGD, the same procedure was followed replacing MV with Cov.

For cell experiments knottin solutions were 2000 nM in 50 μM CB[8] for W1*-W4* at high surface density and WE while at low surface density knottins

solutions were 3600, 1000, 75 and 100 nM in 50 μ M CB[8] for W1, W2, W3, and W4 respectively. Fibronectin surfaces were prepared on glass slides cleaned with piranha for 1-5 minutes, incubated with 10 g/mL fibronectin at room temperature for 1 h. then rinsed with PBS for 0.5-1 h and used directly.

5.5.8. Surface Plasmon Resonance (SPR)

SPR measurements were carried out under conditions of constant flow (50 μ L/min) using a pH 7.4 buffer of 0.5 $\times$ PBS. Surfaces were prepared as described above. All knottin solutions also contained 50 μ M CB[8].

5.5.9. Cell culture

C2C12 mouse myoblasts were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with L-Glutamine, 1% v/v Penicillin/Streptomycin and 10% v/v Fetal Bovine Serum (FBS) and used between passage 10 and 30. Cells were sub-cultured on functionalized surfaces in serum depleted medium (without FBS).

Cells adhered to the surfaces (cell density 8,000-10,000 cells/cm²) were fixed directly with cold 4% paraformaldehyde (PFA) for 10 min at room temperature (RT) and washed thrice in PBS. Cells were then permeabilized in 0.5% Triton X-100 and 5% Bovine Serum Albumin (BSA) in PBS for 10 min at RT and blocked with 0.1% Triton X-100 and 5% BSA in PBS (PBST) for 60 min at RT. Immunocytochemical labelling of cell proteins was done in PBST for 1 h at 1:200 for Vinculin-FITC and 1:100 Phalloidin 568 (Actin). Subsequently cells were washed with PBS twice and labelled with 4',6-diamidino-2-phenylindole (DAPI) 1:1000 in PBS. Immunolabelled cells were imaged using an inverted fluorescent microscope with corresponding excitation and emission filters.

5.5.10. Data analysis

Data were analyzed by using Mood's median test for non-normal distributions comparing all groups of interest (OriginPro v.2015, OriginLab). Image analysis was performed using Image J⁴⁰ and Cell Profiler image analysis software.⁴¹ Quantification of cell shape factors were performed by using Cell Profiler algorithms.

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

6

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

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6.1. Introduction

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

Several methods have been described to install non-native motifs on cell membranes without interfering with natural cell receptors and signaling, e.g. by engineering the cell genome,¹⁶ by performing covalent chemistry on membrane proteins,¹⁷ by inserting artificial lipids into the cellular lipid bilayer,^{1, 18-24} or by metabolic labeling of the glycocalyx.^{25, 26} For example, non-natural sugars containing a bio-orthogonal azido group²⁷ have been metabolically incorporated into the glycocalyx structure on the cell membrane, as depicted in Fig. 6.1. These azido-modified cell membranes were reacted with DNA strands by either strain-promoted azide-alkyne cycloaddition (SPAAC), copper(I)-catalyzed Huisgen azide-alkyne cycloaddition (CuAAC) or Staudinger ligation. This elegant metabolic approach has enabled the site-specific assembly of living cells labeled with single DNA strands on surfaces displaying complementary DNA sequences,^{28, 29} but also multiple cell types have been used to form microtissues with controlled stoichiometry.³⁰

Fig. 6.1. At the top, schematic representation of a Jurkat cell modified *via* metabolic labeling and SPAAC to display naphthol moieties on the cell membrane. In the middle, the naphthol (guest) functionalized Jurkat cell assembles on a methylviologen (guest)-bearing supported lipid bilayer in the presence of CB[8] (host) by the formation of multiple ternary host-guest complexes. At the bottom, structures of the molecules used in this study.

Although DNA immobilization strategies hold great potential, the use of synthetic supramolecular host molecules provides access to cellular assemblies responsive to external stimuli and avoids susceptibility to nucleases. Among the supramolecular host molecules that are compatible with physiological conditions are the pumpkin-shaped macrocyclic cucurbit[n]uril (CB[n]) host molecules.^{6, 31-33} For example, we previously used CB[8] (n=8) as the host to anchor guest Trp-Gly-Gly-Arg-Gly-Asp-Ser (WGGRGDS) peptides *via* their *N*-terminal tryptophan to surface-bound guest methylviologens as ternary complexes.³⁴ Detachment of the cells that adhered to these supramolecular RGD surfaces occurred under electrochemical control. Clearly, this cellular self-assembly strategy relies on the ability of the RGD ligands to bind to their natural occurring integrin receptors on the cell membrane. This strategy is integrin-dependent and therefore lacks in flexibility when dealing with different cell types with different adhesiveness characteristics. Therefore, strategies are required that install non-native supramolecular motifs on cell membranes that enable programmable assembly protocols, orthogonal to natural cell receptor-ligand interactions. Very recently, we have incorporated CB[8]-binding motifs at the bacterial surface, by genetically modifying a transmembrane protein to form multiple intercellular ternary complexes leading to the assembly of bacteria.¹⁵

In this chapter we report the installation of supramolecular CB[8]-binding ligands on the cellular membrane using metabolic labeling and the subsequent CB[8]-mediated assembly on a supported lipid bilayer (SLB). To achieve specific CB[8]-mediated cell adhesion, this receptor-independent strategy was combined with the use of a suspension cell line, such as blood cells. Supramolecular naphthol (Nph) guest moieties were introduced on the membrane of white blood cells by using metabolic oligosaccharide engineering.²⁹ Administration of peracetylated *N*-azidoacetyl-*D*-mannosamine (MAN) to living Jurkat cells incorporates azido groups into the glycocalyx on the cell surface (Fig. 6.1). These azido-functionalized cells were then reacted with bicyclononyne-functionalized naphthols (NphBCN) in a SPAAC reaction²⁶ to install the naphthol groups on the cell surface. Finally we show that specific cellular supramolecular interactions are established by heteroternary complex formation between CB[8] and two different guests, *i.e.*

SLB-bound methylviologen and cell surface-bound naphthol causing cellular assembly at surfaces.

6.2. Results and discussion

The synthesis of the bicyclononyne derivative NphBCN is reported in Fig. 6.2 (for synthetic details see Experimental section).

Fig. 6.2. Synthesis route of NphBCN (5) starting from 2-naphthol.

The metabolic introduction of azido groups and the progress of the subsequent reaction with NphBCN was validated using flow cytometry. Jurkat cells were incubated for 3 days with 50 μ M MAN in cell culture medium and subsequently, after washing, a PBS solution of 48 μ M bicyclononyne functionalized fluorescein (fluoBCN) was added for 1 h at 37 $^{\circ}$ C (see Experimental section for details) for a SPAAC reaction. Subsequently, the cells were washed and analyzed by flow cytometry (Fig. 6.3a and b). As controls, cells were reacted either with only MAN (condition +MAN-fluoBCN), or with only fluoBCN (condition -MAN+fluoBCN), or with neither of these two (condition -MAN-fluoBCN). All controls consistently showed low fluorescence intensities with respect to the condition in which both MAN and fluoBCN (condition +MAN+fluoBCN) were present, indicating that the reaction

occurred specifically. Moreover, inspection of the cells that were treated with both MAN and fluoBCN using confocal fluorescence microscopy revealed green emission that was localized on the cellular membrane (Fig. 6.3c). These results confirm earlier findings that metabolic labeling procedures yield azido groups at the cell surface, that can react with fluorescent molecules carrying complementary reactive groups for the SPAAC reaction.²⁶

Fig. 6.3. Analysis of Jurkat cell surface functionalization after 3 day incubation with 50 μM MAN and subsequent SPAAC with fluoBCN. (a) Distributions of the green fluorescence intensity of cells labeled with 48 μM fluoBCN and controls as assessed by flow cytometry. (b) Average fluorescence intensities over three independent experiments is shown for all conditions: for each individual measurement the mean fluorescence intensity was normalized against a set of calibration beads (error bars = standard deviation). (c) Representative confocal image of fluoBCN labeled Jurkat cells. Jurkat cells were imaged after 3 day incubation with 50 μM MAN and subsequent SPAAC with fluoBCN (scale bar 50 μm). (d) Turnover study of azide (functional group of MAN) and of fluoBCN groups at the cell surface by flow cytometry. Turnover of azide (+MAN+1day+fluoBCN, $n=1$) was evaluated by letting cells in medium for 1 day between the metabolic labeling and the click reaction. Turnover of fluoBCN (+MAN+fluoBCN+1day, $n=2$) was evaluated by letting cells in medium for 1 day after the click reaction. For both turnover experiments the corresponding negative controls are presented as well. (e) FluoBCN concentration dependence of the fluorescence intensity of labeled Jurkat cells as measured by flow cytometry.

To verify the availability of functional groups after metabolic labeling and subsequent SPAAC, the change in mean fluorescent intensity in the flow cytometry data was followed as function of reaction time and storage time. After incubation of the cells with azido-functionalized mannose (MAN), cells were washed, kept in culture medium for one more day without MAN prior to SPAAC coupling with fluoBCN. Similarly, the azido-functionalized mannose was administered to cells and directly reacted with fluoBCN, and then kept in culture medium for an additional day without any reactant. In both cases, most of the fluorescence intensity was lost, yet remained higher than the intensity of their corresponding negative controls (Fig. 6.3d).

Next, we correlated the concentration of fluoBCN to the number of fluorophores on the cell. Flow cytometry (Fig. 6.3e) shows a linear trend of increasing fluorescence intensity of cells treated with increasing concentrations of fluoBCN in the range of 10 to 240 μM . This result indicates that the number of supramolecular CB[8]-binding ligands on the cell membrane can be modulated. In fact, the number of molecules at the cell surface can be determined quantitatively (see Experimental section for details). When using for example 50 μM of MAN and 48 μM of fluoBCN, the number of fluorescein groups per cell was determined to be $1.1 \cdot 10^6$, in agreement to values found in literature.⁷ As a result, this approach seems promising to understand the thermodynamic and kinetic parameters that control cell-cell and cell-surface interactions by defining the density of the supramolecular guest molecules on the cell surface.

We envisioned that such modified cells with artificial supramolecular naphthol guest moieties would interact in a multivalent fashion with a surface displaying methylviologen as complementary guest and CB[8] as the host that binds both Nph and MV groups in a ternary complex. We selected to introduce the complementary methylviologen guest moieties on a supported lipid bilayer to benefit from its excellent nonfouling characteristics, as we have previously demonstrated in forming heteroternary complexes between methylviologen and azobenzene-modified sugars at the SLB, as an example of cell-material interactions based on a naturally occurring receptor-ligand interactions.³⁵

SLBs were produced on glass from vesicles that adsorb, fuse and rupture, forming a SLB covering the surface. Vesicles were made of DOPC, a maleimide headgroup modified lipid (MCC, 0.5 mol%) and a DHPE lipid labeled with Texas Red (TR, 0.2 mol%) mixed in chloroform. After drying and rehydration in PBS buffer, the vesicles were extruded through a polycarbonate membrane with 100 nm pore size, in order to make their size distribution more homogeneous (see Experimental section for details). To incorporate methylviologen into the vesicles, 100 μ M of thiol-functionalized methylviologen was added to a suspension of the maleimide-functionalized vesicles during the rehydration step. The pH of the rehydration buffer was set to 7.4, which is suitable for the Michael addition reaction to occur.³⁶ After reacting for 2 h at room temperature, the lipid suspension was extruded and the resulting vesicles were used for the formation of SLBs bearing MV groups. The presence of MV on the surface of these vesicles was verified using zeta potential measurements (Fig. 6.4). Vesicles incorporating the doubly positively charged MV moieties showed a positive potential. A negative potential originating from the negatively charged MCC and TR lipids was retained in a control experiment where paraquat, which bears no thiol, was used during the reaction. A decreased in potential was detected after adding CB[8] to the MV-bearing vesicles indicative of complexation of CB[8] and MV, which would result in shielded positive charges. Together these results confirmed that MV was incorporated in the vesicles and that complexation with CB[8] can occur.

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

Jurkat cells were functionalized with naphthol ligands using the same procedure that was used to equip the Jurkat cells with fluorescein but now using NphBCN instead of fluoBCN. Prior to flowing the naphthol-functionalized cells, a solution

of 20 μM CB[8] was injected in the flow channel and allowed to interact with the methylviologen on the SLB by host-guest interactions.

Fig. 6.4. Zeta potential characterization of vesicles of DOPC:MCC:TR (99.3:0.5:0.2 mol%). On the left, a vesicle bearing MV before and after complexation with CB[8] is depicted schematically. On the right, zeta potential results are shown for vesicles reacted with MV (vesicles+MV) and added of CB[8] (vesicles+MV+CB[8]). As control, vesicles which were not reacted with MV but which were still containing negatively charged MCC and TR were measured in the presence of paraquat (vesicles+paraquat). Error bars are standard deviations based on 3 measurements.

The formed CB[8]-methylviologen complex constituted the artificial receptor for the naphthol-functionalized Jurkat cells. These cells were then loaded in the flow channel in the presence of 20 μM CB[8]. In a period of 10 min without flow the Jurkat cells settled on the SLB (Fig. 6.5). Subsequently, a low flow rate was applied and then increased stepwise while monitoring the adhesion of the cells in the chip using bright field microscopy (Fig. 6.5). As negative controls, the same procedure was applied but altered either by replacing the CB[8] with CB[7], or to have unmodified cells, or to have cells with MAN lacking naphthol moieties. Cells in all negative controls already started to detach from the SLB at flow rates as low as 0.25 $\mu\text{l/min}$ and beyond a flow rate of 1 $\mu\text{l/min}$ hardly any cells remained visible at the SLB (Fig. 6.5). On the contrary, in the presence of CB[8], cells displaying Nph ligands selectively adhered on the MV functionalities on the lipid bilayer and cells started to flow away from the SLB when flow rates were applied higher than 10 $\mu\text{l/min}$. Only beyond 100 $\mu\text{l/min}$ all of the initially adhered Jurkat cells could be flushed away. The results demonstrate that the display of CB[8]-binding moieties that we introduced *via* metabolic labeling and strain promoted azide-

alkyne cycloaddition seem sufficiently stable to provide supramolecular ligands at the cell surface within the time scale of the surface assembly, and that these ligands interact in a specific manner.

Fig. 6.5. Immobilization of Jurkat cells on a MV-modified SLB in a flow channel setup. Cells were metabolically labeled with MAN and reacted with NphBCN. Cells were loaded (10^6 cells in PBS, 5 pulses of 2 s at 100 $\mu\text{l}/\text{min}$) in the flow channel and after 10 min the flow was started again and its rate was increased stepwise. Experiments were performed in the presence of 20 μM CB[8] (+MAN+NphBCN+CB[8]) or 20 μM CB[7] as a control (+MAN+NphBCN+CB[7]). As additional controls, cells reacted with MAN but not with NphBCN in presence of CB[8] (+MAN-NphBCN+CB[8]) and unmodified cells (-MAN-NphBCN+CB[8]) were tested. Left panel, representative bright field images at increasing flow rates for the conditions (+MAN+NphBCN+CB[8]) and (+MAN+NphBCN+CB[7]). Inset, higher magnification. Scale bars 200 μm , and 40 μm for the inset. Right panel, dependence of the number of immobilized cells on the applied flow rate for the conditions (+MAN+NphBCN+CB[8]), (+MAN+NphBCN+CB[7]), (+MAN-NphBCN+CB[8]) and (-MAN-NphBCN+CB[8]). Between 300 and 700 cells were counted per condition using Image J software³⁷ in triplicate experiments.

6.3. Conclusions

In conclusion, in this chapter a system has been described that simplifies the investigation of cell/surface interactions through the use of synthetic ligands and receptors. Such a system provides a bioorthogonal modification of the cell surface independently from natural cell ligands and with numbers of supramolecular handles that can in principle be modulated. In our strategy, the dynamic and reversible host/guest chemistry recreates the natural ligand/receptor interactions while it also enables the specific control over cell adhesion, possibly even on a spatial and temporal scale. This artificial yet biomimetic approach paves the way for quantifying the relation between the number of receptors and the forces involved in the supramolecular cell adhesion.

6.4. Acknowledgments

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6.5. Experimental section

6.5.1. General methods

Chemicals were purchased from Sigma Aldrich or from Acros Organics unless differently specified and used without further purification. The purity of each batch of cucurbit[8]uril (Sigma Aldrich) was assessed by UV-Vis titration with cobaltocenium according to a literature procedure.³⁸ Dulbecco's Phosphate Buffered Saline (PBS, Sigma Aldrich) with pH 7.4 at 25 °C containing 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride was used. TR was purchased from Invitrogen and all other lipids as well as polycarbonate membranes were purchased from Avanti Polar Lipids. Cell staining reagents were purchased from Invitrogen. FluoBCN was purchased from Synaffix. Reactions were monitored using thin-layer chromatography (TLC), which was performed on 0.2 mm Merck precoated silica gel 60 F254 aluminum sheets. Spots were visualized using a basic KMnO₄ solution unless otherwise specified. Column chromatography was carried out on silica gel 60 (0.063-0.2 mm, Merck). NMR spectra were recorded on a Bruker spectrometer (Ascend 400). The signal of the protonated solvents were used as reference. Chemical shifts are given in units of parts per million (ppm) and expressed relative to tetramethylsilane (TMS). Coupling constants (J) are reported in Hertz (Hz). High-resolution mass spectrometry (HRMS) was performed using a Waters ESI(+)-ToF spectrometer (Micromass LCT) calibrated on the molecular ion of reserpine. The elemental analysis was performed on an Interscience analyzer (Flash 2000 CHN). Zeta potentials of lipid vesicles (0.5 mg/mL in 0.5x PBS) were measured in folded capillary cells at 25 °C on a Malvern Instrument system (Zetasizer NanoZS) using a

laser with a wavelength of 633 nm and backscatter detection at an angle of 173°. Fluorescence microscopy was performed using an Olympus (1X71) microscope. For confocal microscopy, a Nikon confocal (A1) microscope was used equipped with a 488 nm laser and a 500-550 nm emission filter. Flow cytometry measurements were carried out on BD bioscience flow cytometer (FACS Aria II) equipped with a 488 nm laser. For each measurement, the flow cytometer was calibrated using a Molecular Probe (L14821, 2.5 μ m) LinearFlow™ Green Flow Cytometry Intensity Calibration Kit. Fluorescence spectroscopy was performed using a Perkin Elmer fluorimeter (LS 55).

6.5.2. Synthetic procedures

Synthesis of NphBCN. The NphBCN (compound 5) was synthesized accordingly to Fig. 6.2. Compounds 1³⁹, 2⁴⁰ and 6²⁶ were synthesized according to known literature procedures.

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

Compound 5 was synthesized from compound 3 and 4. Compound 3 (100 mg, 0.19 mmol, 1 eq.) was dissolved in 5 mL of dry MeOH. The solution was cooled to 0 °C followed by the dropwise addition of acetylchloride (0.05 mL) under argon. Stirring was continued at room temperature for another 3 h. Completion of the reaction was monitored using TLC. After that time all solvent was evaporated and the product was obtained as a brown oil. The product 4 was used without further purification in the next step. Compound 4 (50 mg, 0.13 mmol, 1.3 eq.) was dissolved in 5 mL of DMF followed by the addition of NEt₃ (0.1 mL). After stirring for 5 min, 4 (30 mg, 0.10 mmol, 1 eq.) dissolved in 2 mL of DMF was added dropwise. The solution was stirred at room temperature for 24 h followed by evaporation of all solvent *in vacuo*. The residue was purified by silica column chromatography (CH₂Cl₂/MeOH: 9/1). The desired product was obtained as a pale yellow oil. Yield: 33 mg, (60%); ¹H-NMR (400 MHz, CDCl₃): δ = 7.70-7.76 (m, 3H, 3CH); 7.42 (t, J = 7.8 Hz, 1H, CH); 7.32 (t, J = 7.8 Hz, 1H, CH); 7.11-7.14 (m, 2H, 2CH); 6.12 (s, 1H, NH); 5.18 (s, 1H, NH); 4.14 (d, J = 7.9 Hz, 2H, CH₂); 4.09 (bs, 2H, CH₂); 3.48-3.59 (m, 10H, 5 CH₂); 3.36 (bs, 2H, CH₂); 2.21-2.30 (m, 8H, 4CH₂); 1.88 (s, 4H, CH₂); 1.52-1.58 (m, 2H, CH₂); 1.33 (q, J = 8.5 Hz, 1H, CH); 0.87-0.83 (m, 2H, CH₂). ¹³C-NMR (101 MHz, CDCl₃): δ = 172.87; 156.99; 156.92; 134.68; 129.47; 129.02; 127.73; 126.82; 126.46; 123.67; 119.01; 106.67; 98.91; 70.39; 69.67; 67.66; 67.39; 65.97; 63.72; 62.97; 61.78; 40.87; 40.13; 39.35; 38.80; 38.27; 37.15; 36.36; 30.37; 29.14; 28.86; 26.77; 22.59; 21.52; 20.22; 17.86; 15.39. Traces of ethyl acetate are present in the ¹H-NMR and ¹³C-NMR. HRMS [M+Na]⁺ = 573.2936 (calc. 573.2941), [M+K]⁺ = 589.2888 (calc. 589.2680), [M+2ACN+H]⁺ = 655.2214 (calc. 655.3471).

Synthesis of MAN. The procedure from Laughlin and Bertozzi⁴ for the synthesis of peracetylated *N*-azidoacetylmannosamine was used. For TLC, spots were visualized by treatment with 0.05 M solution of triphenylphosphine in toluene and subsequent staining with a 0.08 M ninhydrin solution in 1-butanol containing 0.5 M acetic acid. Yield: 91 mg (6.6%), white wax. ¹H-NMR: (400MHz, CDCl₃) δ = 2.00 (s, 3H), 2.06 (s, 3H), 2.11 (s, 3H), 2.12 (s, 3H), 3.83-3.87 (ddd, 1H, J_{5,6a} = 2.4, J_{5,6b} = 4.5, J_{5,4} = 9.4), 4.12-4.16 (dd, 1H, J_{6a,5} = 2.4, J_{6a,6b} = 12.0), 4.16 (s, 2H), 4.23-4.27

(dd, 1H, $J_{6b,5} = 4.5$, $J_{6b,6a} = 12.0$), 4.72-4.75 (ddd, 1H, $J_{2,3} = 1.4$, $J_{2,NH} = 3.6$, $J_{2,1} = 9.0$), 5.14-5.19 (app t, 1H, $J_{4,5} = 9.4$), 5.91 (d, 1H, $J_{3,2} = 1.4$), 6.85-6.88 (d, 1H, $J_{1,2} = 9.0$). ^{13}C -NMR: (101MHz, CDCl_3): $\delta = 20.6, 20.6, 20.6, 20.7, 50.1, 52.3, 61.7, 64.9, 71.4, 73.3, 90.1, 168.4, 168.7, 169.8, 170.3, 170.8$. Minor peaks in the ^1H -NMR and ^{13}C -NMR are attributed to the anomeric isomer. HRMS $[\text{M}+\text{Na}]^+ = 453.1248$ (calc. 453.1234), $[\text{M}+\text{K}]^+ = 469.0982$ (calc. 469.0973), $[\text{2M}+\text{Na}]^+ = 883.2366$ (calc. 883.2570), $[\text{2M}+\text{K}]^+ = 899.2091$ (calc. 899.2309). IR: 2100 cm^{-1} $\nu_{\text{as}} \text{N}_3$ (strong). Anal. Calcd. for $\text{C}_{16}\text{H}_{22}\text{N}_4\text{O}_{10}$: C 44.65, H 5.15, N 13.02; found: C 43.34, H 4.88, N 12.33.

Synthesis of MV. The synthesis of MV was adapted from Rauwald, et al.⁴¹ and González-Campo, et al.³⁹ and it is described in Chapter 3.

6.5.3. Cell culture

Jurkat cells were cultured in RPMI medium with 10% fetal bovine serum at a density of $10^5 - 10^6$ cells/mL at standard cell culture conditions (37°C with 5% CO_2). Cells between passages 11 and 25 were used. For metabolic labeling of cells, 2.5×10^6 cells in 10 mL of the same culture medium were incubated with MAN in the incubator for 3 days and then washed with PBS three times. After that 10^6 cells per condition were allowed to react for 1 h in the incubator with NphBCN (5% DMSO in PBS) or fluoBCN (PBS) depending on the experiments. Finally cells were washed with PBS three times. Cells for negative controls were treated according to the same procedures but replacing the addition of only solvents as described in the main text.

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

Fig. 6.6. Live-dead assay of Jurkat cells. Cells were exposed for 1 h in the incubator to percentages of DMSO between 0 to 10% (top panel) and of CB[8] between 0 and 50 μM in PBS (bottom panel). Scale bar 200 μm .

6.5.4. Flow cytometry

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

6.5.5. Determining cell coverage

Cells were labeled with fluoBCN. Their mean fluorescence was measured by flow cytometry. For each measurement, standard beads with various coverages of fluorescein were also measured. The average number of fluorescein molecules per cell can be calculated by quantifying the amount of fluorescein molecules per bead *via* a calibration obtained by fluorescence spectroscopy. Here, an example calculation is given for cells labeled with 50 μM of MAN and 48 μM of fluoBCN.

First, a calibration curve of known concentrations of fluorescein at concentrations between 0 and 0.1 μM in Milli-Q water was made using a fluorescence spectrophotometer. The linear fitting of the calibration curve results in the equation:

$$C_{\text{fluorescein}}(M) = 1.38 * 10^{-10} * I_{\text{max,fluorescence}}$$

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

The same set of beads was measured by flow cytometry as well. This resulted in Fig. 6.7, giving a mean intensity for each type of beads. The values of mean intensities from flow cytometry were correlated to the corresponding intensity measured by fluorescence spectroscopy as follows. Each stock solution of beads had a given density of $6 \cdot 10^7$ beads/mL. For fluorescence spectroscopy, samples were prepared by adding one droplet of the sonicated bead solution in 2 mL of PBS. The volume of the droplet was determined to be $38 \pm 1 \mu\text{L}$ (standard deviation, $n=3$) resulting in a final concentration of $1.1 \cdot 10^6$ beads/mL. With the concentration of fluorescein as well as the density of beads known, the coverages (number of fluorescein molecules/beads) of the different beads were determined.

Fig. 6.7. Fluorescence intensity obtained with flow cytometry for a commercially available set of fluorescein labelled beads. For beads A to F, the coverage of fluorescein at the surface of the beads increases.

Expressing these calculated fluorescein coverages per particle, as a function of their mean intensities from representative flow cytometry experiments, a linear dependence could be found according to the equation:

$$\theta \text{ (} 10^6 \text{ molecules/particle)} = 3.59 * 10^{-3} * I_{FlowCytometry}$$

The latter equation provides the average fluorescein coverage of the cells. For a representative cell experiment, the +MAN+fluoBCN sample had a mean intensity of 317.5, giving a coverage of $1.1 * 10^6$ (fluorescein molecules/cell). This is in the same order of magnitude as values obtained in similar studies using metabolic labeling.⁷

6.5.6. Vesicles preparation

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) was stored as a 10 mg/mL stock solution in chloroform at -20

°C and used

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

6.5.7. Supported lipid bilayer (SLB) fabrication

SLBs were fabricated in a flow channel setup which was built as previously described.³⁵ In short, the flow channel consisted of a standard glass microscope

slide on which a polydimethylsiloxane (PDMS) flow channel was attached with a width of 1.5mm and a height of 50µm. A bright field microscopy overview is presented in Fig. 6.8 a.

Fig. 6.8. (a) Stack view of the flow channel. Inlet and outlet are visible at the ends of the cell. Scale bar 500 µm. (b) Fluorescence images of the same SLB before and after an experiment with cells. Red color from 0.2% TR in the SLB composition. Scale bar 100 µm.

For repeated use of flow channels, the channels were rinsed with 1 wt% sodium dodecyl sulfate in MilliQ and the glass was activated for 1 h with a 1 M solution of sodium hydroxide in MilliQ. The channels were subsequently thoroughly rinsed with MilliQ. Supported lipid bilayer formation was achieved by dilution of the LUV solution to 0.1 mg/mL in PBS. Prior to LUV incubation, the channels were flushed briefly with PBS. The channels were then incubated with the vesicle suspension for at least 30 min to allow for vesicle adsorption and rupture to occur. Subsequently, the channels were washed with an excess of MilliQ water. From this point forth, care was taken to ensure no air bubbles entered the device.

The quality of the SLBs was checked always before and after each cell experiment *via* fluorescence microscopy by having a uniform red intensity from the TR present in the composition of the bilayer (Fig. 6.8 b). Moreover a negative control of untreated Jurkat cells (-MAN-fluoBCN-CB[8]) was flown on the SLB prior to using the same SLB for further experiments to further verify the consistent performance of different individual SLBs.

6.6. References

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Summary

Self-assembly allows for rationally designed molecular platforms that exploit specific, directional, tunable and reversible non-covalent interactions. The dynamic nature of such supramolecular interactions gives rise to self-assembled systems that can sense and respond to physiological cues, or that mimic the structural and functional aspects of biological signaling. In this thesis we discuss the properties of a family of macrocycles called cucurbit[n]urils (CB[n]), with strong emphasis on the 8-membered macrocycle (CB[8]). The special characteristic of the CB[8] host is the ability of forming reversible ternary inclusion complexes with two guest molecules. The binding of CB[8] with two guests to form a ternary complex has been studied in solution in detail and this complexation unequivocally occurred non-cooperatively. Peptides and small aromatic guests can promote the formation of ternary complexes within the cavity of CB[8] displaying structural and targeting functionalities. In this thesis various applications of this type of ternary complexes have been described where amphiphilic ternary complexes have been used to form supramolecular nanoparticles with targeting and imaging ligands, where photosensitive and cell adhesive ternary complexes on self-assembled monolayers have been used to modulate the adhesion of cells and where ternary complexes have been established on supported lipid bilayers to trap non-adhesive cells carrying metabolically introduced guest moieties.

We envision that the results described in this thesis will further contribute to the development of bioanalytical platforms and biomedical materials in which the self-assembly of biomolecules is driven by CB[8]-mediated interactions. Describing and studying the scope and limitations of these interactions under conditions that are relevant for this type of applications is of crucial importance.

Samenvatting

Zelf-assemblerende moleculen geven de mogelijkheid tot het rationeel ontwerpen van moleculaire systemen doordat specifieke, directionele, moduleerbare en reversibele niet-covalente interacties worden gebruikt. Het dynamische karakter van zulke interacties, die ook supramoleculaire interacties worden genoemd, creëert zelf-geassembleerde systemen die fysiologische signalen kunnen opvangen en erop kunnen reageren, of die structurele en functionele aspecten van de biologische signalering kunnen nabootsen. Het werk dat is beschreven in dit proefschrift gaat in op de eigenschappen van een serie sterk verwante macrocyclische gastheer verbindingen die sterk lijken op pompoenen en ernaar vernoemd zijn cucurbit[n]urils (CB[n]) waarbij n de grootte van de ring weergeeft. De studies in dit proefschrift zijn uitgevoerd met voornamelijk de macrocyclische CB[8] doordat gastheer CB[8] de bijzondere eigenschap beschikt om twee gastmoleculen reversibel en tegelijkertijd in te sluiten. Deze ternaire complexvorming kan plaatsvinden onder fysiologische omstandigheden en dus is het een geschikte kandidaat om te bestuderen of hiermee biologische functionaliteit kan worden nagebootst. Echter, hoewel de vorming van een ternair CB[8] insluitingscomplex in oplossing in detail reeds onderzocht is, hebben gedetailleerde NMR en calorimetrische metingen nu ondubbelzinnig het niet-coöperatieve karakter van de complex vorming laten zien zoals in hoofdstuk 2 beschreven is. Aromatische peptiden of simpele aromaten kunnen als gast in de holte van CB[8] ternaire complexen vormen en daarmee structurele en doelgerichte biologische herkenning en functionaliteit verkrijgen. In dit proefschrift zijn diverse toepassingen van dergelijke ternaire complexen beschreven: amfifiele ternaire complexen die supramoleculaire nanodeeltjes vormen in water en voorzien van liganden die integrine eiwitten herkennen en medische beeldvorming. Ook is de zelf-assemblage van ternaire complexen beschreven waarin lichtgevoelige liganden zijn ingevangen waarmee de aanhechting van cellen te moduleren was. Verder is het aanbrengen van gastgroepen op niet-klevende cellen door middel van een metabolisch proces

beschreven en gebruikt om dit type cellen op oppervlakken te laten hechten via ternaire CB[8] complex vorming.

Wij voorzien dat deze resultaten zullen bijdragen aan de verdere ontwikkeling van bioanalytische toepassingen en biomedische materialen waarin CB[8]-gestuurde interacties de zelf-assemblage van biomoleculen bepaald. Het beschrijven en het bestuderen van de mogelijkheden en de beperkingen van dit type interacties onder omstandigheden die relevant zijn voor dit soort toepassingen is voor deze ontwikkeling van cruciaal belang.

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About the author

Emanuela Cavatorta was born in Parma (Italy) on January 7th, 1986. In the same city, she attended the Liceo Classico Romagnosi, where she obtained her pre-university education degree.

In 2007 she received her bachelor degree in Food Science at the University of Parma, presenting a thesis about “Allergenic protein extraction from biological matrices”, based on a four months project at the Molecular Biology Department, under the supervision of Prof. dr. C. Folli. Two years later she received her bachelor degree in Chemistry with a thesis about “Ionic liquids and their use as heterogenic catalysts in Knövenagel reaction”, after working five months in Prof. dr. F. Bigi’s group at the Organic Chemistry Department at the same university.

During her master course in Chemistry, she spent six months in the Molecular NanoFabrication Group at the University of Twente as an exchange student, working on her master project. In April 2012 she defended her master thesis entitled “Surface-immobilized Peptide-Nucleic Acids (PNAs) for sensing applications”, under the supervision of Prof. dr. R. Corradini of the University of Parma.

Since May 2012, she works as a PhD in the groups of Prof. dr. ir. P. Jonkhøj and Prof. dr. ir. J. Huskens. The results of her work are described in this thesis.

List of publications

shared first authorship

Brinkmann[#], J., Cavatorta[#], E., Sankaran[#], S., Schmidt[#], B., Van Weerd[#], J., Jonkheijm, P., About supramolecular systems for dynamically probing cells. *Chem. Soc. Rev.* 2014, **43**, 4449-4469.

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Cavatorta, E., Jonkheijm, P., Huskens, J., Ternary peptide-cucurbit[8]uril complexation assessed to be non-cooperative. (manuscript in preparation)

Cavatorta, E., Voskuhl, J., Brinkmann, J., Wasserberg, D., Huskens, J., Jonkheijm, P., Targeting protein-loaded supramolecular nanoparticles to cells. (manuscript in preparation)

Cavatorta, E., Sankaran, S., Brinkmann, J., Huskens, J., Jonkheijm, P., Photo-responsive cell adhesion employing cucurbit[8]uril ternary complexes. (manuscript in preparation)

Sankaran, S., Cavatorta, E., Brinkmann, J., Huskens, J., Jonkheijm, P., Directing cell adhesion through RGD-displaying knottins with tunable affinity for cucurbit[8]uril surfaces. (manuscript in preparation)

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