

Single-molecule force spectroscopy of supramolecular heterodimeric capsules†

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The specific interaction of a supramolecular binding motif was quantitatively evaluated by dynamic single-molecule force spectroscopy (SMFS) using an atomic force microscope (AFM). The supramolecular capsule forms by two different cavitands stitched together by four hydrogen bonds between carboxylic acid and pyridyl groups. The tetra(carboxyl)cavitand is monofunctionalized at the *lower rim* with a flexible poly(ethylene glycol) linker and attached to the AFM sensor tip. Single-molecule association experiments are accomplished using a diluted self-assembled monolayer (SAM) of the tetra(pyridyl)cavitand on a gold substrate. The measured single-molecule dissociation forces of the heterodimeric capsule represent the mechanical stability of the supramolecular system and allow a quantitative evaluation of the interaction according to the Bell–Evans model yielding dissociation rate constant $k_{\text{off}} = (0.14 \pm 0.14) \text{ s}^{-1}$, reaction length $x_{\beta} = (0.56 \pm 0.076) \text{ nm}$ and an estimated value of $\Delta G^0 = -27 \text{ kJ mol}^{-1}$.

1. Introduction

The stability of supramolecular binding motifs is of major importance for the generation of complex, functional structures.^{1,2} It defines the degree of reversibility during self-assembly and therefore the ability for self-correction.³ Furthermore, it ensures the integrity of the structure and controls the adaptability to environmental changes.⁴ With the investigation and development of biological and synthetic molecular machines the interest in mechanical forces acting in and between molecules was sparked.^{5–10} Atomic force microscopy single-molecule force spectroscopy (AFM-SMFS) has been developed to an outstanding technique providing quantitative data on mechanical stabilities of interactions and mechanochemical effects.^{11–13} Moreover, a connection between mechanical forces, kinetic rate constants and thermodynamic data has been established within the Bell–Evans model and used for the characterization of biological complexes.^{14–16} Fundamental interactions like hydrogen bonding, coordination bonding, hydrophobic interactions, π – π interactions and host–guest interactions have been studied using diverse basic supramolecular systems.^{17–24} The investigation on specific host–guest interactions between ammonium cations and cavitands by Anselmetti *et al.* showed that AFM-SMFS is especially well suited to analyze kinetic off-rate constants in a broad affinity range of $K_{\text{d}} = 10^{-5} \text{ M}$ (for the ammonium–cavitand complex) to $K_{\text{d}} = 10^{-15} \text{ M}$ (for biotin–streptavidin).²³ Besides simple host–guest systems,

complexes of photoswitchable receptors based on calix[4]-arenes that represent optomechanical switches have been characterized by AFM-SMFS.²¹ Vancso *et al.* studied the association of the quadrupole hydrogen-bonded UPy–UPy-dimer (UPy = 2-ureido-4(1*H*)-pyrimidinone) and a supramolecular polymer derived from this binding motif.¹⁷ The results highlight that this technique can yield detailed information even about complex systems containing a number of supramolecular bonds. Although established ensemble measurements can provide parameters as the kinetic dissociation rate constant more precisely, the broad range of affinities that can be analyzed and the substantial additional information only accessible by AFM-SMFS make this technique valuable and complementary to other methods.

The design and properties of supramolecular capsules have attracted considerable interest in the last decade.^{25–29} This intensive research activity is motivated by the opportunity to investigate effects of compartmentalization and processes in confined spaces provided by the discrete, well-defined environments of the inner cavity of these sophisticated architectures.³⁰

To realize technical applications such as detection and stabilization of encapsulated molecules or their use as nano-sized reaction vessels, precise control of important factors such as size, porosity of the walls and stability is mandatory. Furthermore, their use as supermolecular building blocks (SBB) of metal–organic materials (MOM) and supramolecular polymers has inspired material sciences.^{31–33} Metal–ligand interactions, hydrogen bonding, and electrostatic and hydrophobic interactions have been used to construct supramolecular capsules. Supramolecular dimeric capsules based on calixarenes and cavitands have been established as valuable systems to study the basic principles of molecular encapsulation.³⁴ They follow a uniform molecular design, consisting of the two bowl-shaped cyclophane moieties non-covalently connected by a variety of bridging units. Therefore, such supramolecular capsules represent excellent structures to quantitatively evaluate

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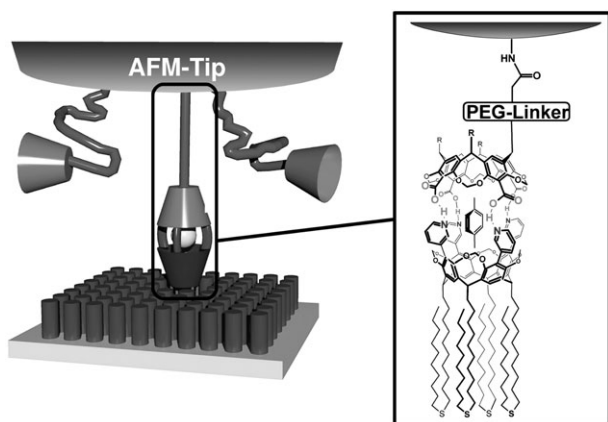


Fig. 1 Setup of the SMFS experiment: the supramolecular capsule is formed by association of the immobilized building blocks in *p*-xylene.

the stability of supramolecular binding motifs without changing the basic design of the system.

Recently, Janshoff *et al.* reported stretching experiments on elegantly designed oligo calix[4]arene catenanes–calix[4]arene dimers to investigate the rupture and rejoining of calix[4]arene capsules.²⁴ However, separation of forces resulting from ruptures of hydrogen-bonded calix[4]arene capsules and opening of a sterically locked conformation of the covalent linkages is demanding and impedes quantitative evaluation of the obtained data.

Here, we present the quantitative evaluation of the interactions in a hydrogen-bonded supramolecular capsule introduced by Kobayashi *et al.* in 2003.^{35,36} By association of a tetra-(carboxyl)cavitand and a tetra(pyridyl)cavitand in *p*-xylene heterodimeric capsules are formed selectively. The cavitands are connected *via* four hydrogen bonds between the carboxylic acid groups and the pyridyl groups, while one solvent molecule is incorporated in the interior of the aggregate.³⁵ To probe the interaction between the capsule's halves, the building blocks were immobilized at the AFM force sensor (cantilever) and a gold substrate, respectively (Fig. 1). The tetra(carboxyl)cavitand is tethered to the cantilever *via* a flexible poly(ethylene glycol) linker (PEG linker) to add steric freedom to the system that allows capsule formation to take place and reduces non-specific interaction.³⁷ In addition, the use of PEG linkers yields characteristic stretching curves in SMFS experiments, facilitating discrimination of single-molecule from multiple dissociation events and the evaluation of molecular elasticities and single-molecule dissociation forces.

This experimental setup allows the quantitative investigation of the mechanical stability of the complex supramolecular system. Furthermore, the kinetic dissociation rate constant k_{off} and detailed information about the binding energy landscape of the interaction are obtained. We show that SMFS can be used to characterize supramolecular binding motifs and gives important insights into the dynamic properties of self-organized assemblies.

2. Experimental section

2.1 Functionalization of AFM cantilevers

Si_3N_4 AFM cantilevers (MSCT, nominal tip radius ≈ 10 nm, Veeco Instruments, California, USA) were activated for 30 s in

concentrated nitric acid, rinsed with water, ethanol and acetone and dried in a stream of N_2 . Silanization was carried out in a solution of (3-aminopropyl)triethoxysilane (1%) in dry toluene (3 h, room temperature). After washing extensively with toluene, acetone, ethanol and water and drying in a stream of N_2 , the cantilevers were placed in a solution of poly(ethylene glycol) substituted cavitand **8** (7.8 mg), TBTU (1.2 mg, 3.7 μmol) and *N,N*-diisopropylethylamine (1.3 μL , 0.93 mg, 7.2 μmol) in dry dimethyl sulfoxide (300 μL) for 4 h at room temperature. They were rinsed with dimethyl sulfoxide, ethanol and acetone and dried in a stream of N_2 . Remaining free amino groups were blocked by storing the cantilevers for 24 h at room temperature in a solution of *N*-hydroxysuccinimidyl acetoacetate (30 mg, 0.15 mmol) and *N,N*-diisopropylethylamine (54 μL , 40 mg, 0.30 mmol) in dry dimethyl sulfoxide (300 μL). After rinsing extensively with dimethyl sulfoxide, ethanol and acetone the cantilevers were placed in formic acid (10 mL) for 1 h at 40 $^\circ\text{C}$ to cleave the acid-labile *p*-methoxybenzyl ester groups.

2.2 Preparation of substrates

Gold-coated sample substrates (Arrandee, Werther, Germany) were extensively cleaned by repetitive treatments in an ultrasonic bath with water, ethanol, acetone and chloroform and placed immediately in a solution of the sulfide (1 mM) for 16 h at 60 $^\circ\text{C}$, which is essential for effective SAM formation.^{38,39} To obtain substrates suited for the observation of single-molecule interactions, the concentrations of tetra(pyridyl)cavitand **12** at the surface were reduced by preparation of a mixed SAM containing cavitand **12** and decyl sulfide (see below). Best results were achieved using substrates prepared by placing the sample into a solution of tetra(pyridyl)cavitand **12** (2.1 mg, 0.99 μmol) and decyl sulfide (31 mg, 99 μmol) in chloroform (100 mL) for 16 h at 60 $^\circ\text{C}$, rinsing with chloroform and drying it in a stream of N_2 .

2.3 AFM force spectroscopy and data analysis

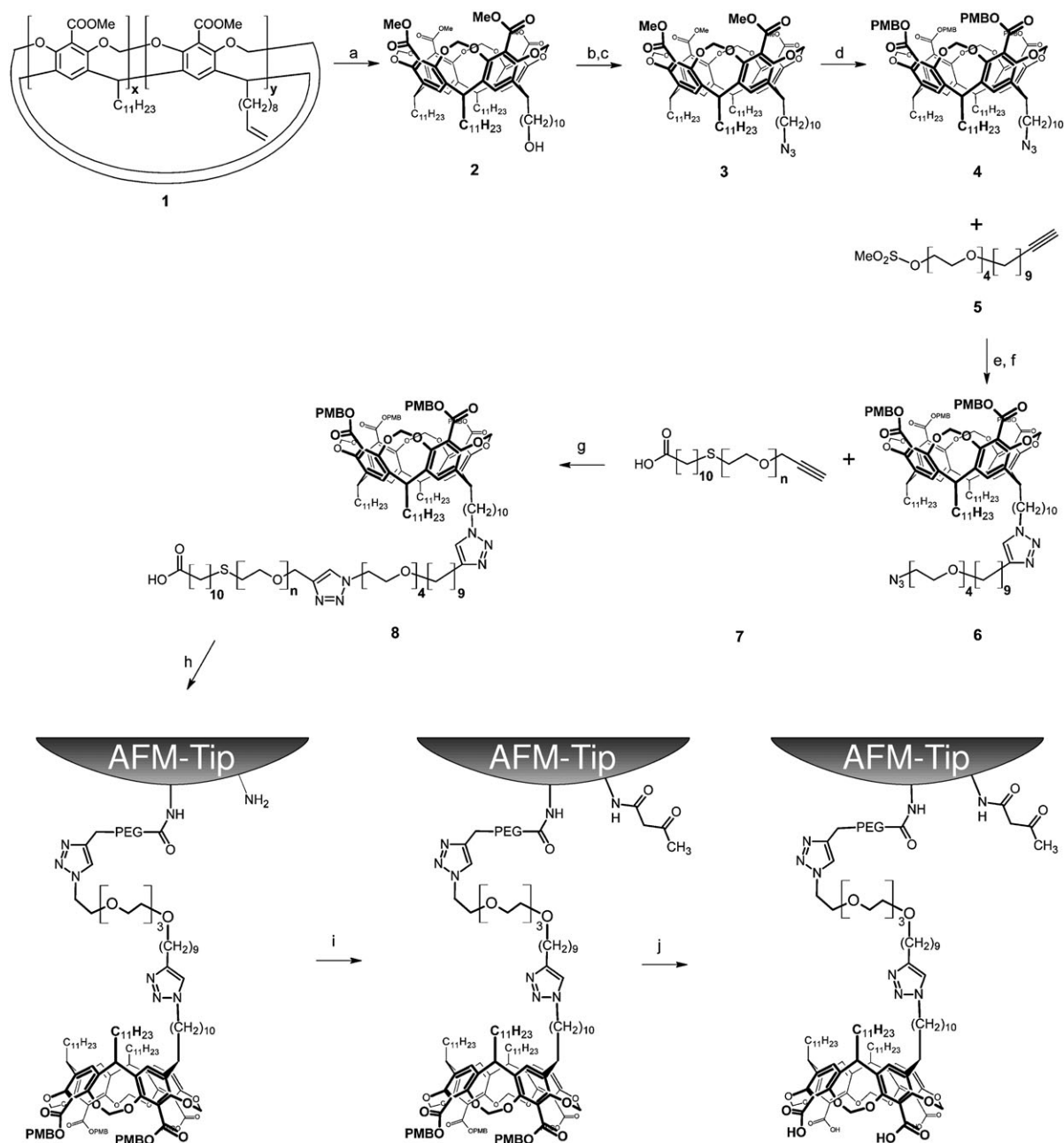
Single-molecule force spectroscopy measurements were performed with a custom-made AFM force spectroscopy setup based on a Multimode IIIa AFM (Veeco Instruments, California, USA) at 25 $^\circ\text{C}$. The AFM cantilever deflection force signal and the vertical movement of the piezoelectric transducing element were controlled by a 16 bit DA/AD card (PCI-6052E, National Instruments, Austin, Texas, USA) and a high-voltage amplifier (600H, Nano-TechTools, Echandens, Switzerland) *via* an in-house software based on Labview (National Instruments). Si_3N_4 cantilevers with spring constants ranging from 17 to 22 pN nm^{-1} were calibrated by the thermal fluctuation method with an absolute uncertainty of approximately 10%.⁴⁰ In dynamic, loading rate-dependent force spectroscopy experiments, the retract velocity of the piezo was varied from 50 nm s^{-1} to 2000 nm s^{-1} while the approach velocity was kept constant (2000 nm s^{-1}). Measured force–distance curves were quantitatively analyzed with a custom-made Matlab program (MathWorks, Natick, Massachusetts, USA) and numerically fitted with a commercial software (Origin, OriginLab Corporation, Northampton, USA).

3. Results and discussion

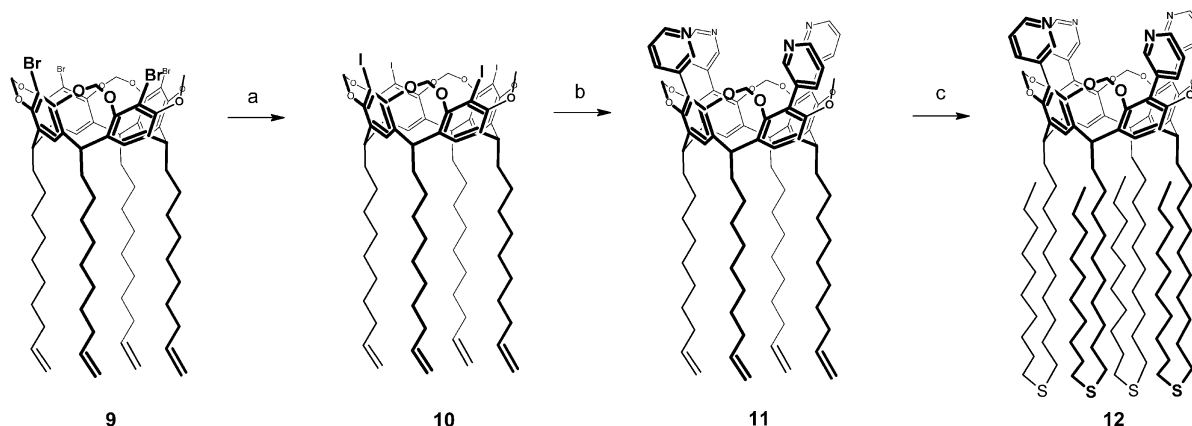
3.1 Synthesis

While functionalization of cantilevers with less complex ligands is possible using commercially available hetero-bifunctional PEG linkers,^{23,41} the tetra(carboxyl)cavitand could not be immobilized this way. Therefore, a new strategy was developed for the tethering to the cantilever *via* a flexible PEG linker, using orthogonal coupling reactions and ester protecting groups (Scheme 1).

Starting with a mixture of cavitands **1** with four methyl ester groups and up to four terminal alkenes (denoted by $x = 0-4$ and $y = 4 - x$), introduction of a hydroxyl group allowed the isolation of the cavitand **2** monofunctionalized at the *lower rim*. After replacement of the hydroxyl group by an azido group, transesterification yielded cavitand **4** with *p*-methoxybenzyl ester moieties,⁴² which can be cleaved under mild acidic conditions. The short connecting unit **5** was attached and the conjugate **6** could successfully be tethered to the long PEG linker **7**. The introduction of **5** was necessary,



Scheme 1 Synthesis and immobilization of the monofunctionalized tetra(carboxyl)cavitand. *Reagents and conditions:* (a) 1. 9-BBN, THF; 2. NaOAc, H₂O₂; (b) methanesulfonyl chloride, triethylamine, dichloromethane; (c) sodium azide, DMPU; (d) *p*-methoxybenzyl acetate, sodium *tert*-butoxide, 4-*tert*-butylphenol; (e) sodium ascorbate, copper sulfate, THF, ethanol, water; (f) sodium azide, DMPU; (g) sodium ascorbate, copper sulfate, TBTA, THF, ethanol, water; (h) TBTU, DIPEA, DMSO; (i) NHS-acetoacetate, DIPEA, DMSO; (j) formic acid.



Scheme 2 Synthesis of the tetra(pyridyl)cavitand. *Reagents and conditions:* (a) 1. *n*-butyllithium, THF; 2. iodine; (b) 3-pyridineboronic acid pinacol ester, Pd[PPh₃]₄, potassium hydroxide, tetrabutylammonium bromide, toluene, water; (c) 1-decanethiol, 9-BBN, THF.

because several attempts to couple cavitands with four carboxyl groups at the *upper rim* with the long PEG linker **7** via copper-catalyzed 1,3-dipolar cycloaddition failed.⁴³ For the force spectroscopy experiments, cavitand **8** was linked to the amino-functionalized cantilever surface by amidation. After blockage of remaining free amino groups, the *p*-methoxybenzyl ester moieties were cleaved to set free the carboxylic acid groups.

To immobilize the tetra(pyridyl)cavitand on a gold substrate, thioether groups were attached at the *lower rim* (Scheme 2). After substitution of the bromine atoms, the tetra(pyridyl)cavitand **11** with four terminal alkenes was obtained by Suzuki coupling. Addition of 1-decanethiol with catalytic amounts of 9-BBN yielded the thioether-substituted cavitand **12**.⁴⁴

The formation of self-assembled monolayers (SAM) of thioether-functionalized cavitands on gold is a well-established procedure, which needs elevated temperatures to obtain adsorbates of high quality.³⁸ Immobilization of the tetra(pyridyl)cavitand was carried out by keeping gold substrates in a solution of **12** in chloroform at 60 °C for 16 h. To lower the concentration of cavitands on the surface, mixed SAMs were prepared under similar conditions using solutions containing decyl sulfide and cavitand **12**.

3.2 Single-molecule force spectroscopy experiments

To investigate the bonding at the single-molecule level, functionalization of the cantilever tip or the substrate at low concentrations is mandatory. Aminofunctionalization of silicon nitride cantilevers with 3-aminopropyltriethoxysilane can yield tips that fulfill the requirements of SMFS.¹⁸ Therefore, initial force spectroscopy experiments were carried out in *p*-xylene by moving the cantilever modified with the tetra(carboxyl)cavitand towards a substrate with a SAM of the undiluted tetra(pyridyl)cavitand **12** and pulling back after capsule formation has taken place. In the obtained AFM force curves always a strong adhesion due to multiple binding interactions between tip and sample was detected (Fig. 2a). This high binding activity clearly indicates that multiple

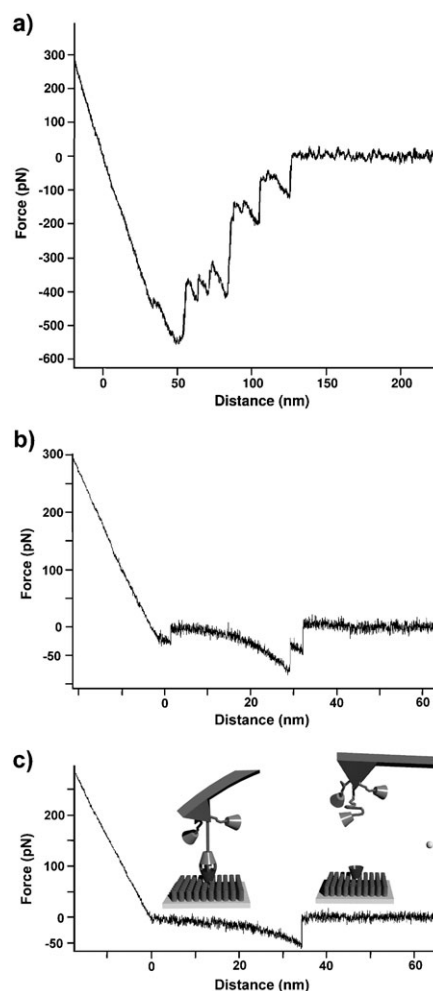


Fig. 2 Typical force curves obtained in force spectroscopy experiments. (a) Multiple dissociation events detected in measurements on an undiluted tetra(pyridyl)cavitand SAM. (b) Discarded force curve containing a multiple adhesion event measured on a diluted SAM. (c) Single-molecule dissociation event with characteristic non-linear force curve measured on a diluted SAM. Inset: scheme of SMFS experiment.

tetra(carboxyl)cavitands attached to the cantilever tip interact with tetra(pyridyl)cavitands at the substrate.

To realize single-molecule experiments, reduction of the concentration of binding partners at the surface was necessary. Besides using low concentrations of 3-aminotriethoxysilane and short reaction times during aminofunctionalization,^{19,22} the employment of mixed self-assembled monolayers on the substrate containing only a low proportion of molecules with binding capacity is an important strategy to reduce the binding activity in force spectroscopy experiments.^{21,23,45} This approach represents a straightforward way for the required reduction of the binding activity while keeping the more complex functionalization of the cantilever surface constant. Diluted SAMs were prepared from solutions containing the tetra(pyridyl)-cavitand **12** and decyl sulfide in a ratio of 1 : 100.⁴⁶ Results obtained in subsequent force spectroscopy experiments using these mixed SAMs are markedly different compared to the results from the first experiment. While multiple rupture events were detected in all of the measurements on the undiluted SAM, the binding activity was significantly reduced using the diluted SAM. Characteristic non-linear force curves that could be assigned to the elastic stretching of the chain-like polymer were observed. The observation of this specific curve form is a reliable criterion to discriminate wanted and specific single-molecule dissociation events from unspecific or multiple adhesion events (Fig. 2b and c). Furthermore, the dissociation events are detected well-separated from the surface (tip–surface distance 20 nm–80 nm), which simplifies identification and allows precise determination of the dissociation force.

Due to the stochastic nature of the thermally activated dissociation process the measured dissociation forces display a typical distribution that can be fitted *e.g.* with a Gaussian distribution.^{14,15} Plotting these forces in a histogram, the most probable dissociation force (f^*) that is characteristic for the supramolecular complex at a given loading rate can be obtained (Fig. 3a). The specificity of the interaction was verified in control experiments, where excess of free tetra(carboxyl)cavitand was added to the diluted SAM of the tetra(pyridyl)cavitand (Fig. 3b). As a result, a strongly reduced binding activity was detected, because capsule formation between the cantilever-bound tetra(carboxyl)cavitand and the tetra(pyridyl)cavitand at the surface was effectively blocked. The high selectivity of the heterodimerization reaction accounts for the exclusive formation of heterodimeric capsules.³⁵

According to the Bell–Evans model in a dynamic force spectroscopy experiment the measured dissociation forces can be related to kinetic data and information about the length scale of the interaction can be extracted. Here, the most probable dissociation force (f^*) depends logarithmically on the loading rate ($r = \text{pulling velocity} \times \text{molecular elasticity}$) according to $f^* = (k_{\text{B}}T/x_{\beta})\ln(x_{\beta}r/k_{\text{B}}Tk_{\text{off}})$.^{14,15} Plotting f^* —determined for different retract velocities—vs. $\ln(r)$, a linear dependence can be observed (Fig. 4). Analysis of the obtained data yields $k_{\text{off}} = (0.14 \pm 0.14) \text{ s}^{-1}$ that corresponds to an averaged complex lifetime of 7.3 s ($\tau = 1/k_{\text{off}}$).

The reaction length x_{β} , which corresponds to the width of the binding potential, was determined to be $x_{\beta} = (0.56 \pm 0.076) \text{ nm}$. Upon assuming a fast formation of the capsule ($k_{\text{on}} = 10^4 \text{ M}^{-1} \text{ s}^{-1}$) observed for similar systems,⁴⁷

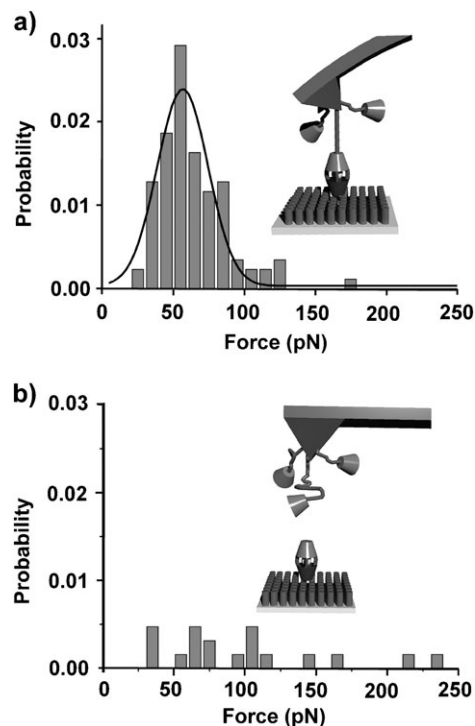


Fig. 3 (a) Histogram of dissociation forces of single heterodimeric capsules and Gaussian fit to the distribution. (b) Control experiment: addition of free tetra(carboxyl)cavitand results in a strongly suppressed binding activity between tip and sample. Inset: schematic representation of the competition experiment.

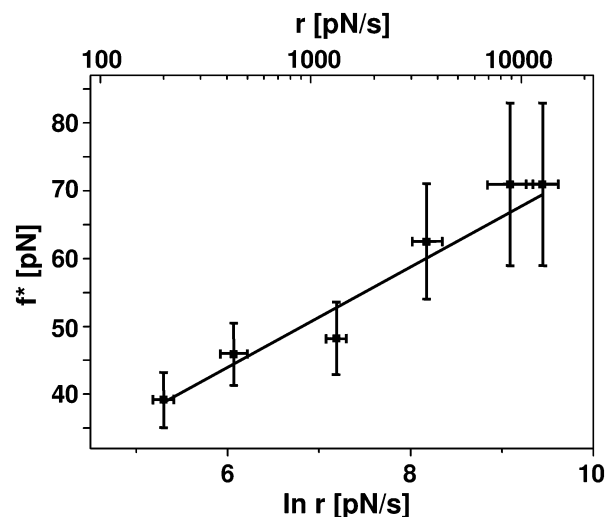


Fig. 4 Dynamic SMFS plot and fitting analysis of the most probable rupture forces f^* determined at different retract velocities vs. loading rate $\ln(r)$.

the equilibrium constant of association can be estimated to be $K_{\text{a}} = 7.1 \times 10^4 \text{ M}^{-1}$, yielding $\Delta G^0 = -RT \ln K_{\text{a}} = -27 \text{ kJ mol}^{-1}$ for $T = 293 \text{ K}$.

The dissociation rate constant measured in this single-molecule study is in very good agreement with values obtained for related hydrogen bonded supramolecular capsules based on calix[4]arenes and cavitands.^{47–49} Homo- and heterodimeric

supramolecular capsules held together by sixteen hydrogen bonds have been analyzed by NMR and FRET techniques.^{48,49} Here, depending on the solvent and the substituents at the urea and the *narrow rim* of the calix[4]arenes, disassembly was found to take place in a relatively slow manner with dissociation rate constants in the range between $k_{\text{off}} = 0.26 \text{ s}^{-1}$ – $5 \times 10^{-6} \text{ s}^{-1}$. Information on the self-assembly dynamics of supramolecular capsules based on cavitands held together by eight bifurcated hydrogen bonds have been obtained from FRET measurements.⁴⁷ While the exchange of subunits in toluene takes place with $k = 1.9 \times 10^{-3} \text{ s}^{-1}$ (with capsule dissociation as the rate-limiting step), appropriate guest molecules stabilize the aggregate substantially.

Even though the stability of the capsule investigated in this report is lower due to the reduced number of hydrogen bonds connecting the cavitands, we were able to gain important information using AFM-SMFS, which impressively demonstrates the sensitivity of this technique. In the future, we will quantify the effect of an incorporated guest molecule on the complex stability. Due to the increasing number of capsules based on cavitands connected *via* hydrogen bonding, coordination bonding and electrostatic interactions, they are especially suited to study interactions in multiple bonded supramolecular systems. Moreover, the accessibility of cavitands with one to four functional groups at the *upper rim* affords the opportunity to quantitatively investigate the effect of multiple bonding on the complex stability.

4. Conclusions

In conclusion we demonstrated that complex supramolecular systems like heterodimeric capsules can be quantitatively studied at the single-molecule level using AFM-SMFS. The mechanical stability of the evaluated system stitched together by multiple hydrogen bonds can be determined and detailed information on the kinetics of the thermally activated dissociation, the energetic landscape and an estimation of the binding energy can be obtained. Our results are in very good agreement with the data from well established ensemble measurements showing the reliability of the molecular design and the experimental setup. In the rapidly expanding field of sophisticated and functional supramolecular architectures, single-molecule force spectroscopy is a sensitive technique that gives access to the stability of important binding motifs and their mechanochemical properties.

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