

Molecular Addressability of Lipid Membrane Embedded Calixarenes towards Cytochrome C

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Abstract

By means of the atomic force microscopy techniques we studied the surface topography of the supported bilayer lipid membranes (sBLM) composed of 1,2-sn-glycerodimyristoylphosphatidylcholine (DMPC) with incorporated calixarenes 'Oct [6]CH₂COOH (CX) specific to cytochrome c (cyt c). It is supposed that cyt c interacts with CX through amino groups of lysine residues at its surface. Therefore we also applied single molecule force spectroscopy (SMFS) to analyze the mechanisms of interaction of cyt c with the CX. In later case cyt c or individual NH₂ group have been connected to the AFM tip through special linker. The topography of bare sBLM in a gel state (T=19°C) revealed relatively smooth surface ($R_{\text{RMS}}=0.18$ nm) and thickness ~5.1 nm which agrees well with previous studies. Incorporation of CX into DMPC bilayer resulted in increase of the surface roughness ($R_{\text{RMS}}=0.39$ nm) and in increase of thickness in average by 0.5 nm. The incubation of the layer with 30 nM of cyt c resulted in a surface smoothing ($R_{\text{RMS}}=0.32$ nm) and in a further increase of the thickness between 0.7 to 1.2 nm. The SMFS experiments with cyt c modified AFM tips approved its specific binding to CX and allowed us to determine the binding parameters k_{off} (1.14 ± 0.59 s⁻¹) and x_{p} (3.98 ± 0.63 Å). SMFS experiments with an amino-ended linker also resulted in highly specific interactions with comparable values for k_{off} (2.74 ± 0.66 s⁻¹) and x_{p} (5.91 ± 2.55 Å). This suggests that both electrostatic and amino group specific interactions between cyt c and CX cavity exist.

Keywords: Supported bilayer lipid membranes; Calixarene; Cytochrome c; AFM; Single molecule force spectroscopy; Molecular recognition

Introduction

Cytochrome c (cyt c), a small water soluble membrane heme-protein found in the intermembrane space of mitochondria, is known to play a key role in physiological and pathological processes. Cyt c is a crucial regulator in the electron transport in mitochondrial respiratory chain and is also connected to apoptosis which is initiated by release of cyt c from mitochondria into the cytosol [1-5]. Cyt c has the ability for membrane-induced formation of amyloid fibers [6-8]. The storage of cyt c in mitochondria is closely related to the special lipid cardiolipin (CL) that is found nearly only in the inner lipid membrane of mitochondria [9]. The interaction of CL with cyt c is expected to be based on electrostatic interaction between the polycationic cyt c and the anionic CL.

These mitochondrial lipid bilayers are known to bind cyt c effectively but have rather complex structure consisting of numerous lipid components onto which the integrated proteins are incorporated and peripheral proteins, like cyt c are embedded at membrane surface. There, the zwitterionic glycerophospholipids are the most common compounds of the membrane. For modeling the lipid bilayer and for simplification of the system (e.g. for artificial cyt c based sensors) the limitation to one kind of glycerophospholipids is beneficial. Among them 1,2-sn-glycero-dimyristoylphosphatidylcholine (DMPC) is very convenient. DMPC has main transition temperature at $T_{\text{M}}=23.9^{\circ}\text{C}$. Below the transition temperature DMPC bilayer is in a gel phase whereas above this temperature it is in the fluid phase. Artificial functional lipid membranes can easily be generated by embedding membrane proteins and/or other membrane molecules allowing monitoring their behavior.

Calixarenes, cyclic oligomers based on a hydroxyalkylation product of a phenol and an aldehyde, are here of substantial interest [10-13]

since they are known to bind cations, anions, nucleic acids, amino acids, catecholamines and other proteins highly specific [14]. An important example of such calixarenes is the calix[6]arene carboxylic acid derivative ('Oct[6]CH₂COOH). It shows high extractability for cyt c and also interacts strongly with the NH₃⁺ group of lysine [15,16]. There are many reports related to metal ion recognition with the calixarenes [17-19], but lesser works have been focused on the molecular recognition of proteins by calixarenes. In our recent work we showed that ('Oct[6]CH₂COOH) calixarene (CX) can be incorporated into the supported bilayer lipid membrane (sBLM) where it can serve as receptor for impedimetric detection of cyt c with limit of detection of 10 nM [20]. We investigated the interaction of cyt c with CX incorporated into the large unilamellar vesicles (LUV) composed of DMPC or into sBLM and compared this with not specific adsorption of cyt c to the LUV containing DMPC and anionic phosphatidic acid (PA) or sBLM composed of a mixture of DMPC and dimyristoylphosphatidic acid (DMPA). We showed that with increasing concentration of CX the average size of LUV increased and zeta potential become more negative as it is suggested from dynamic light scattering experiments. For PA containing LUV the increase in vesicle diameter was less expressed, but zeta potential decreased similarly like that of LUV contained CX. Cyt

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c did not affect significantly the LUV size, but reduced the negative zeta potential both for CX and PA containing vesicles. The analysis of binding process suggests that the main driving force for interaction of cyt c with CX incorporated in lipid bilayer is the electrostatic attraction of the positively charged cyt c molecules by the negative surface charge caused by CXs [21]. However, so far the topography of CX in a lipid bilayer and the molecular interaction forces between cyt c and CX have not been determined.

To overcome this gap of knowledge atomic force microscopy (AFM) seems to be the perfect candidate. On the one hand AFM has been proven to be a very useful tool in studying the surface topography of model lipid membranes [22-28] with high resolution and under near physiological conditions [29,30]. On the other hand the upgrade of the AFM tip to a mono-molecular biosensor allows to directly investigate the energy landscape of the molecular interaction. Using the AFM tip modified by certain ligand a complex rupture force with a surface-bound receptor can be measured at a single molecule level [31-35]. Ligands have been usually covalently coupled to the tip via long flexible spacer [36-39]. This linkage method allowing free movement and orientation of the ligand favors the complex formation with a receptor. The characteristic stretching of the spacer [40], preceding complex dissociation, allows us to better discriminate specific binding events from nonspecific adhesion. This is especially important for the study of low-affinity interactions between biomolecules.

In this work we applied gentle physiological AFM imaging to study the topography of lipid bilayers with incorporated CX and cyt c as well as single molecule force spectroscopy to analyze the binding between cyt c immobilized at AFM tip and CX incorporated into the lipid film.

Materials and Methods

Materials

All chemicals were of the highest purity commercially available:

37,38,39,40,41,42-hexakis(carboxymethoxy)-5,11,17,23,29,35-hexakis(1,1,3,3-tetra-methylbutyl) calix[6]aren ('Oct[6]CH₂COOH, CX) was a gift by Dr. T. Oshima (University Miyazaki, Japan). 1,2-sn-glycero-dimyristoylphosphatidylcholine (DMPC) was purchased from Avanti Polar Lipids Inc. (USA). Cytochrome c from bovine heart (cyt c), phosphate buffer saline tablet (PBS), HEPES, chloroform, ethanol, triethylamine (TEA), amino-propyltriethoxysilane (APTES), Neutravidin, and d-biotin were from Sigma Aldrich (Germany). The heterobifunctional crosslinkers NHS-PEG-biotin [41] and NHS-PEG-Acetal [42] were synthesized in house as described earlier. Biotin-T₁₅-NH₂ was purchased from Thermo-Fisher Scientific GmbH (Germany). The buffers used were of following composition: PBS buffer: 2 mM KH₂PO₄, 10 mM Na₂HPO₄·2H₂O, 137 mM NaCl, 2.7 mM KCl at pH 7.4, HEPES buffer: 10 mM HEPES, 150 mM NaCl, 20 mM MgCl₂, pH 7.4.

Preparation of liposomes from DMPC

The liposomes were prepared according to bath sonication method developed by Johnson et al. [43] which is widely used also in recent to studies [44] with modest modification. First, small amount of DMPC (2 mg) has been added in a glass vessel and dissolved in 1 ml of chloroform. The chloroform was evaporated using a continuous nitrogen gas stream by rotating the vessel. The flask with the evaporated film was inserted into a water bath having a constant temperature of 50°C for 60 min. Again the vessel was floated with nitrogen gas for 10 min. Afterwards 10 mM HEPES buffer with 150 mM NaCl, 20 mM MgCl₂, pH of 7.4 was

added to achieve a final concentration of 0.5 mM DMPC in a buffer. To obtain small unilamellar liposomes, the suspension was sonicated in a bath type sonicator DT-31 (Bandelin Electronic, Germany) 20 times during 30 sec at 35-40°C until the solution was clear. For spreading the liposomes to a cleaved mica surface 100 µL of the solution were applied with a deposition time of 35-40 min. Finally, the sample was washed thoroughly three times with PBS buffer. Similar method has been used for preparation of liposomes containing CX. In this case a mixture of DMPC and CX dissolved in chloroform in a molar ratio of 10:1 was prepared. After the formation of lipid bilayer without or with CX the cyt c dissolved in a PBS (concentration 30 nM) has been added in a small volume (100 µl) that spreaded over the sample surface, after an incubation lasting 30 minutes the surface was gently washed with PBS.

Physiological AFM imaging

All AFM measurements were performed in PBS buffer using an Agilent 5500 AFM (Agilent Technologies, USA). All AFM imaging measurements were carried out in magnetically actuated tapping mode (MACMode, Agilent, USA) to ensure lowest possible indentation force on the investigated surface. Thus, magnetically coated silicon nitride AFM cantilevers (Type VII MACLevers, length: 125 µm, width: 35 µm, resonance frequency in water 18 kHz, nominal spring constant: 0.14 N/m, Agilent Technologies, USA) were used for MAC mode imaging of the DMPC layer. For all images 512 data points per line were set. The scan area of imaging was varied from 10x10 to 1x1 µm. A temperature controller Model 331 (Lakeshore Cryotronics, USA) including a current booster N9656A (Agilent, USA) was used for adjusting and controlling a definite temperature with a peltier element was placed below the support.

Images of DMPC on mica with and without CX before and after addition of cyt c were acquired at 19°C, i.e. when the lipid bilayer was in gel state, and at 32°C, when the lipid layer was in liquid phase. Image processing, cross section analysis and statistical evaluation of the images were done using Gwyddion software (Gwyddion 2.34; <http://gwyddion.net>).

Tip functionalization

The upgrade of inert silicon nitride tips of commercially available AFM cantilevers (MSCT, Bruker) for single molecule recognition force spectroscopy experiments was done in a multi-step procedure. For tethering both, cytochrome c as well as amino-functionalized T₁₅ DNA, the first step was to generate amino-groups on the inert tip material. For this, the cantilevers were cleaned in ethanol (3 times) and chloroform (3 times) followed by gas phase deposition of APTES as described earlier [45]: two trays, one filled with 60 µL freshly distilled APTES, the other one filled 20 µL triethylamine (TEA) as catalyst, were placed in a 5 l Argon flooded desiccator containing the tips for coating and allowed to react for 120 minutes. Afterwards APTES and TEA were removed, the desiccator was rinsed extensively with argon gas and the tips stayed there for 2 days for curing at room temperature. Afterwards, the tips used for coupling cytochrome c were functionalized with NHS-PEG-Acetal (2 mg/mL in chloroform containing 0.5 vol% TEA as catalyst, 120 min. reaction time) following the protocol of Wildling et al. [42]. Directly before incubation with cyt c the acetal function was deprotected by immersion in citric acid (10 min) resulting in amino-reactive aldehyde residues on the outer end of the tip bound poly(ethyleneglycol) linker. Finally the coupling of cyt c was performed by immersing the aldehyde-tips in a PBS buffered solution containing 0.2 mg/mL cyt c for 120 minutes. These tips were washed in PBS and used immediately for force spectroscopy experiments. For coupling

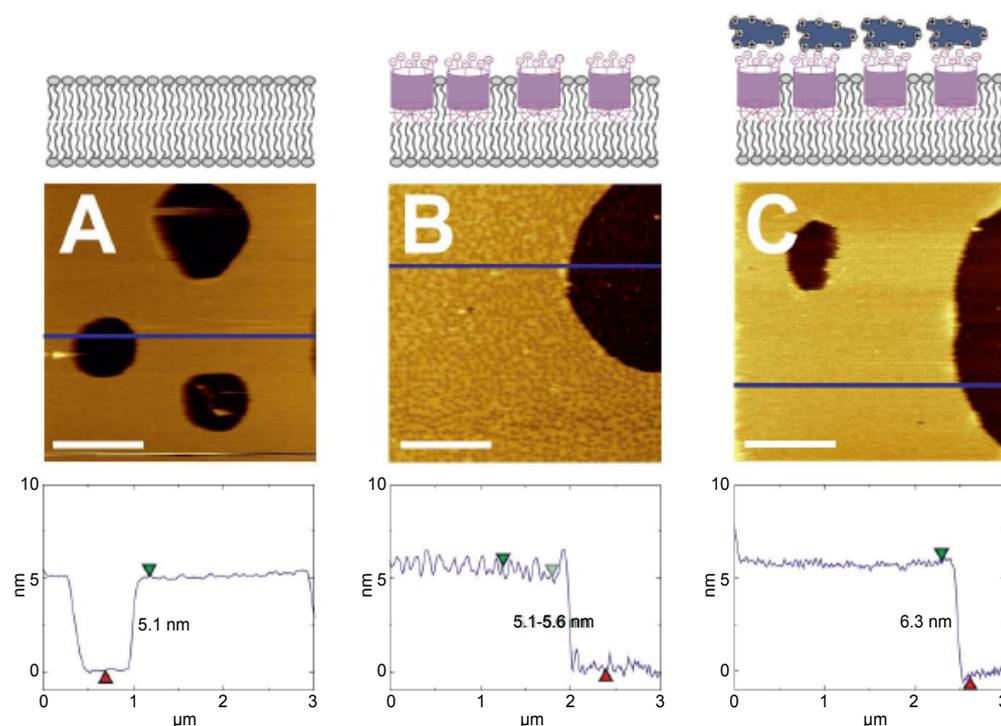


Figure 1: Topographical investigation of the DMPC self-assembled monolayer performing AFM imaging. (A) Bare DMPC bilayer on mica. The height difference between bilayer and mica is 0.5 nm. The RMS of the surface roughness on the layer is 0.18 nm. (B) DMPC layer with embedded Calix[6]arene molecules. Two phases are visible. The lower phases have a height of 5.1 nm whereas the higher areas have a height of 5.6 nm. The RMS of the surface roughness on the layer is 0.49 nm. (C) DMPC layer with embedded Calix[6]arene molecules after the addition of Cytochrome c. The height difference increased to 6.3 nm and the surface roughness had a RMS of 0.32 nm without detectable phase separation. z-Scale for all images is 12 nm.

amino-functionalized T_{15} DNA-biotin to the APTES coated tips 1 mg NHS-PEG-biotin was dissolved in 0.5 mL chloroform and 0.5% (v/v) TEA were added. The APTES tips were immersed in this solution for 120 minutes and carefully rinsed in CHCl_3 . After drying in a gently stream of argon gas the biotinylated tips were placed into a solution of 0.1 mg/mL neutravidin in PBS and allowed to react for 60 minutes. These tips were then carefully rinsed with PBS to remove unbound neutravidin. Finally the biotinylated T_{15} DNA (containing an amino residue on the other end) was allowed to react overnight followed by careful rinsing with PBS buffer. All washing steps were performed three times for five minutes each. Also these tips were used immediately afterwards for force spectroscopy experiments.

Force spectroscopy

Single molecule force spectroscopy experiments were carried out with bio-functionalized tips (see chapter 2.4.) in PBS buffer. At each pulling velocity at least 1000-5000 force distance cycles were performed. Typically after 250 force distance cycles (FDCs) the position on the support was changed to minimize position dependent artefacts. The measured deflection at the moment of rupture was translated into an unbinding force by multiplying the deflection with the spring constant. The spring constant itself was determined using the thermal noise method [46]. At each pulling force, all FDCs yielded in a typically 100-1000 unbinding forces distributed over a certain force range. To calculate the most probable rupture force out of these data, a probability density function [47] was drawn. In contrast to histograms data are weighted by their reliability and thus yield in a better resolution.

Results and Discussion

The small protein cytochrome c is important for both, physiological processes as well as artificial biosensing applications. This protein is released from inner membranes of mitochondria at cell apoptosis. Therefore its detection is important for diagnostics. For example, electrochemical biosensor based on calixarenes incorporated into sBLMs for cyt c detection has been reported by Mohsin et al. [20]. However, the mechanisms of interaction of cyt c with the calixarenes have not been studied yet in sufficient detail. It is assumed, that cyt c interacts specific with the calixarene $^{\text{Oct}}[6]\text{CH}_2\text{COOH}$ (CX) through amino groups of lysine residues at the surface of cyt c. The amino group is incorporated into the CX cavity. In addition negatively charged carboxyl groups at upper ring of the CX facilitate the electrostatic interaction with positively charged CX. Within this study we reveal the interaction properties of cyt c with a powerful synthetic anchor, the calix[6]arene molecules embedded into a supported bilayer. For simplification we only used DMPC bilayers immobilized on freshly cleaved mica. Pure DMPC bilayers have a well-defined transition temperature from gel to fluid phase ($T_m=23.9^\circ\text{C}$) [48]. All further measurements shown in this publication were performed below this temperature at 19°C adjusted with accurate temperature control equipment based on a Pelletier element embedded into the AFM liquid cell with an accuracy of $\pm 0.1^\circ\text{C}$. Although dense layer can be formed easily we adjusted the incubation time and the vesicle concentration low enough ensuring to see both, the lipid bilayer as well as the bare mica allowing determining height changes directly by cross-section analysis. In a first part of this work we analyzed the surface topography

roughness and determined the height profiles of lipid bilayers on mica surface without and with incorporated CX molecules as well as that with adsorbed cyt c. The second part was focused on measurement of forces between cyt c immobilised on AFM tip and the lipid bilayers at mica surface.

Topography of the supported lipid membranes on the mica surface

The results of AFM experiments focused on analysis of topography, roughness and height profile of supported lipid membranes are presented on Figure 1.

As shown in Figure 1A the formed bilayer (bright parts in the image) has a height of ~5.1 nm with a surface roughness of 0.18 nm (rms) relative to the bare mica surfaces (dark parts at the image). This is consistent with values reported in the literature of ~4.8 nm [44,49,50]. This result approves that the lipid film has the expected bilayer configuration. Thanks to negative surface charge of freshly cleaved mica the zwitterionic phosphatidyl groups of one monolayer of DMPC are in contact with the mica surface, while those of the outer monolayer are in contact with PBS. This allows directly comparing the effect of CX insertion. Liposomes to the above mentioned but with addition of embedded calix[6]arenes (1:10 content) were prepared and incubated on freshly cleaved mica. Figure 1B shows the topography and cross sectional plot of a DMPC bilayer with incorporated CX. These layers are accompanied with sporadic defects. Here also the dark regions correspond to the bare mica surface (Figure 1B). On the AFM scan and especially in the corresponding cross-section two different heights of the lipid layer are observable. While the lower height nicely corresponds to the bare DMPC bilayer a second and ~0.5 nm higher layer can be seen, most probable corresponding to an height increase introduced by the calix[6]arene molecules embedded into the lipid bilayer. Thanks to the negative charge of the carboxyl groups at upper ring of CX the electrostatic interaction with positively charged choline groups can affect the orientation of the zwitterionic dipoles which may cause rearrangement of the lipid bilayer structure [44,51]. Most probably the interaction of CX with phospholipids takes place at polar region of the bilayer. The results of our experiments on the study of the temperature phase behavior of liposomes containing CX using ultrasound spectroscopy method indicates that presence of CX does not affect the temperature of phase transition of phospholipids. This suggests that the hydrophobic part of bilayer is not significantly altered by calixarene molecules [52]. The analysis of the film roughness showed that the surface of DMPC-CX is significant rougher ($R_{\text{RMS}} = 0.39$ nm) in comparison to DMPC ($R_{\text{RMS}} = 0.18$ nm). Finally, in addition to the topographical investigation the addressability of the embedded calixarenes was proven by addition of cyt c molecules on the formed DMPC-CX layer at a concentration of 30 nM. The effect of the formed complexes can be seen in Figure 1C. In comparison to the DMPC-CX surface without cyt c (Figure 1B) we did not observe two different heights on the lipid layer and thus a lower surface roughness ($R_{\text{RMS}} = 0.32$ nm). Nevertheless, a clear increase in height from 5.6 nm (or 5.1 nm, respectively) to 6.3 nm, corresponding 0.7 to 1.2 nm, can be seen. Most probable this is caused by the coupling of cyt c to the membrane embedded calixarenes since it is known that cyt c interacts electrostatically with negatively charged carboxyl groups at upper ring of CX [15]. Measurements where cyt c was incubated on CX-free DMPC bilayers did not show any height increase (data not shown), only on the edges of the pure DMPC layer to uncoated mica a height increase was observable. This suggestion is based on bright rim between mica and DMPC that are clearly visible at the image. These results

suggest that cyt c absorbs on the mica surface on the edge of DMPC and cause apparent thickening of the membrane. Cyt c is probably not able to penetrate into the DMPC lipid membrane due to the positively charged residues on the surface and interacts strongly with negatively charged support [53-55]. The height of bright rim was found 3.5-7 nm. The average dimension of the globular cyt c molecule has been reported as 3.4 nm [56], which is in good agreement with the height of bright rims on lipid bilayer, where cyt c is probably localized. After washing of DMPC-cyt c surface with PBS no significant changes were observed (image not shown).

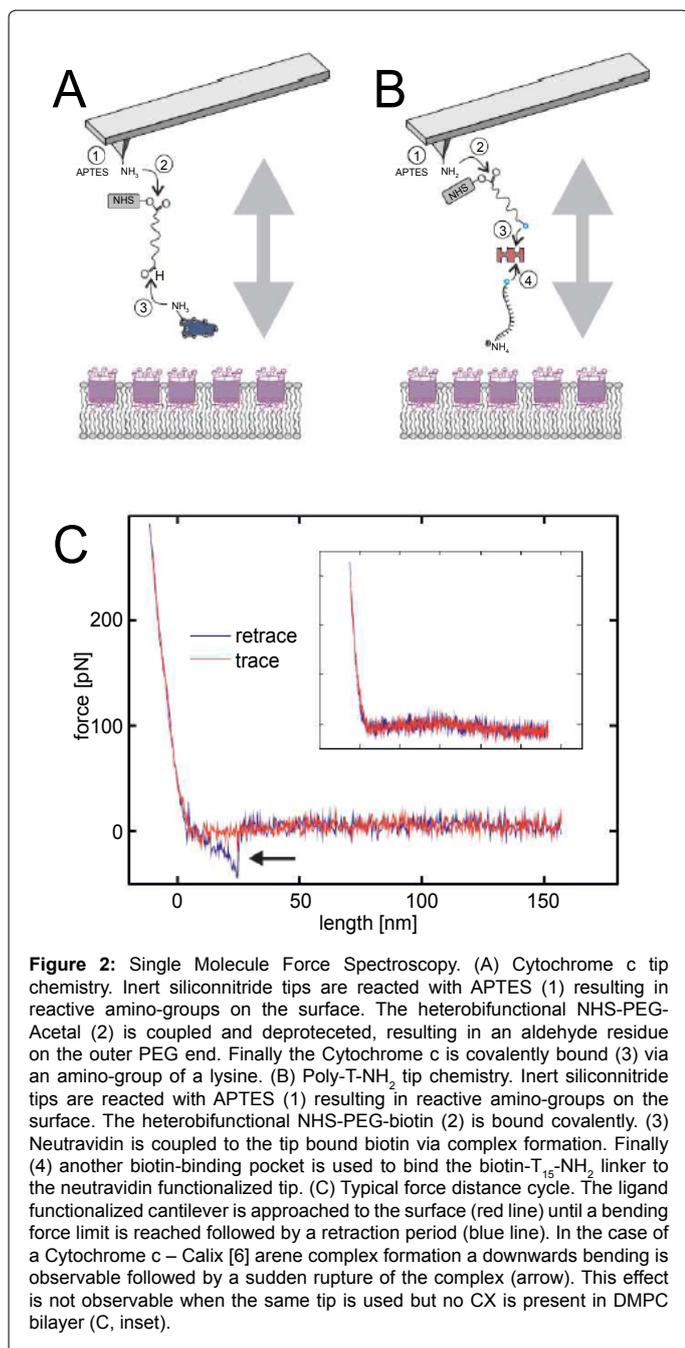
To sum up, it is proven that calixarenes embedded into a DMPC lipid bilayer can act as anchor for cyt c. Most probably electrostatic interactions are responsible for this.

Single molecule force spectroscopy analysis of the interaction between cytochrome c and the supported lipid membranes with incorporated calixarenes

For clarification the CX - cyt c interaction single molecule force spectroscopy (SMFS) [57] was used. SMFS allows measuring the interaction forces and dynamics of a ligand receptor pair at the real single molecule level [33,34,57-59]. For this, a single ligand molecule is tethered to the outer tip apex [39] whereas the corresponding ligand (or vice versa) is coupled to the surface (Figures 2A and 2B).

In so called force distance cycles (FDC) [57] the cantilever carrying the ligand functionalized tip is approached to the surface (Figure 2C, red line). No bending of the cantilever is observed until the tip touches the surface. Further approach results in an upwards bending, which is stopped when a previous set indentation force limit is reached. In the retraction part (Figure 2C, blue line) the cantilever gets removed from the surface resulting in a lowering of the bending. At the point of contact it reaches its resting position and stays there upon further retraction. In the contact time a receptor-ligand complex may be formed resulting in an additional (downwards) bending of the cantilever (Figure 2C, arrow) followed by a sudden rupture caused by dissociation of the complex. The maximum of the cantilever bending in the moment of complex rupture can be translated into a force according to Hook's law. The missing parameter, the cantilever spring constant, is measured for all SMFS data within this publication by using the thermal noise method [60]. At each given pulling velocity 1000-5000 FDCs have been performed and statistically analyzed. The most probable unbinding force was determined by construction a probability density function where the values are weighted by their reliability [47] as shown exemplarily in Figure 3A. To gain insights into the energy landscape this rupture force was measured at different pulling velocities. Combining the Boltzmann ansatz with the stochastic description of the unbinding process according to Evans theory [61] allows revealing the energy landscape of the complex.

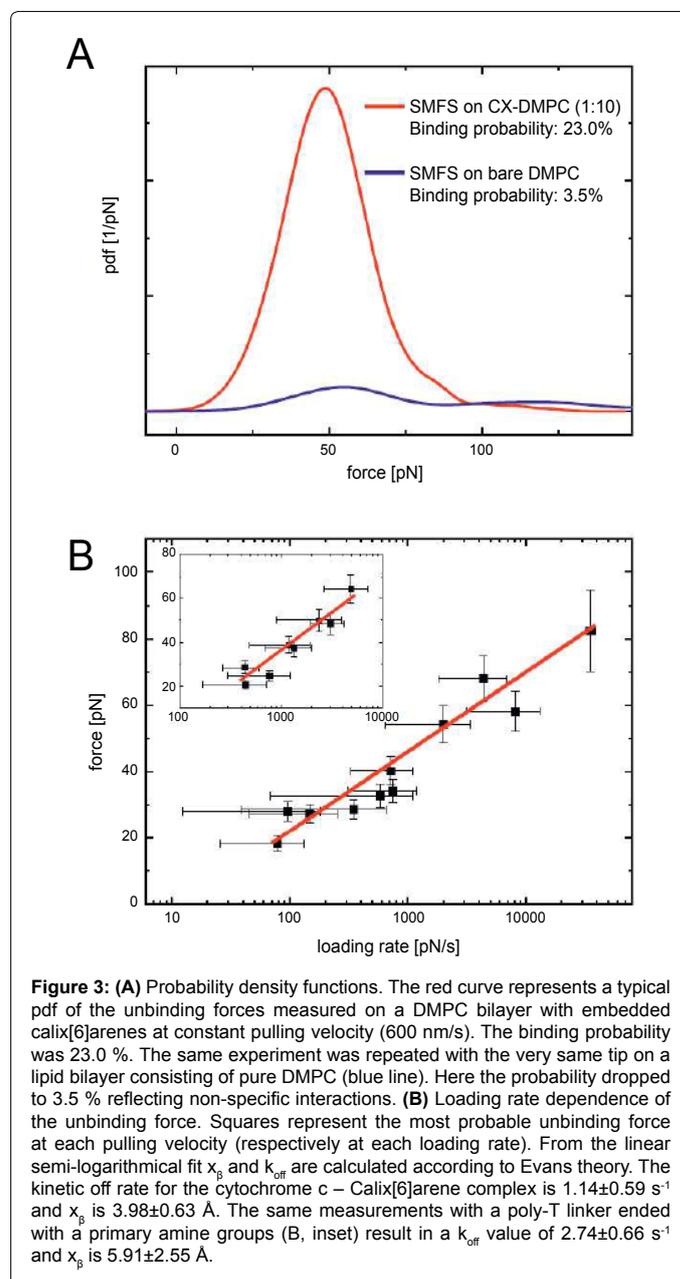
Within this study we explored the interaction of calix[6]arene and cyt c at the molecular level and compared it with the binding behavior of calix[6]arene with an isolated amine group. Thus, cyt c and an isolated amine had to be bound to the outer tip apex of an APTES [45] coated cantilever. While cyt c was coupled directly via one of its lysins using the NHS-PEG-Acetal [42] linker (Figure 2A), the isolated amino group was generated on the tip in a multi-step procedure (Figure 2B). Thus, NHS-PEG-biotin [41] was coupled to the APTES tip resulting in a biotinylated surface followed by coupling of neutravidin (via the biotin binding pocket of the tetrameric neutravidin protein). Without sterical hindrance a second biotinylated molecule can be bound a binding site opposite to the first tether. Here the artificial DNA based



linker biotin-TTTTTTTTTTTTTTTT-NH₂ was used resulting in an amino terminated distensible linker on the outer tip apex. As shown by Pincet et al. [62] the strength of a streptavidin-biotin complex incubated for a longer time period are significant higher compared to typical rupture forces in SMFS experiments allowing using the non-covalent neutravidin-biotin interaction within the linker structure. The pulling velocity in the SMFS experiments of the cyt c – CX interaction was varied from 15 – 2000 nm/s yielding values of the loading rate ranging from 80 to 20000 pN/s (Figure 3). It worth mentioning, that the phase of the DMPC lipid bilayer influences the measurements. At temperatures below TM the FDCs are similar to one illustrated in Figure 2C, whereas above this temperature the upper lipid layer gets partly pulled of and plateau-like structures (data not shown) appear in

the FDCs in addition to the stretching of the PEG linker. Nevertheless, these lipid-layer induced artefacts did not influence the rupture forces and thus can be neglected. Anyhow, to be on the save side all FDCs showing this plateaus have been neglected for the data evaluation. The average binding probability was $17.6 \pm 6.7\%$ whereby the lowest values were recorded at the highest pulling velocity (and vice versa) which can simply be explained by the lowered contact times and thereby lowered probability of complex formation.

Evaluation of the changes of the most probable unbinding force in dependence of the loading rate (Figure 3B) according to Evans theory resulted in kinetic off rate (k_{off}) of $1.14 \pm 0.59\text{ s}^{-1}$ and x_b is $3.98 \pm 0.63\text{ \AA}$. Since only one slope is evident in Figure 3B only a single barrier in the energy landscape can be assumed. Cytochrome c is known to be highly positive charged since this small protein consists of 19 lysins (in case of bovine cyt c) within its amino-acid sequence. As a result of its



six carboxylic acid groups protruding out of the DMPC lipid bilayer membrane, the embedded calix[6]arene used in this study is highly negatively charged. Nevertheless the shown interaction does not give a direct proof that the amino residues of cyt c are the main driving force for complex formation. Thus, we performed SMFS experiments using the mono-NH₂ linker biotin-TTTTTTTTTTTTTTTT-NH₂ on the same surface, on the DMPC bilayer with embedded calixarenes (ratio 1:10). Again by varying the pulling velocities the loading rate dependence of the unbinding force was measured and evaluated according to Evans theory. As a result (Figure 3B, inset) we calculated a k_{off} value of $2.74 \pm 0.66 \text{ s}^{-1}$ and x_{β} is $5.91 \pm 2.55 \text{ \AA}$. Although the dissociation appears somewhat faster compared to cyt c x_{β} does not change significantly. The binding probability for these measurements was $23.7 \pm 11.9 \%$ which is even higher but not significant different compared to cytochrome c. All measurements were performed at 19°C in the gel-phase of DMPC. To proof the specificity of the interaction (i.e. to ensure that neither the DNA-NH₂ nor cyt c interacts with the DMPC lipids itself instead of the embedded calixarenes) the measurements were repeated using the same functionalized tips but on a pure DMPC bilayer. As shown in Figure 3A the binding probability (exemplary shown at a pulling velocity of 600 nm/s) is 23.0 % (red line) for a DMPC bilayer surface with embedded calixarenes, whereas it drops down to 3.5 % when the surface is changed to pure DMPC. This proves, that the measured interactions are highly specific.

Conclusion

In this study we investigated the formation of DMPC bilayers with embedded calix[6]arenes. It could be shown that the addition of calix[6]arenes (at a ratio of 1:10) caused their embedding into the outer lipid layer. Below the DMPC bilayer transition temperature (i.e. below 23.9°C) two clearly separated height regimes are observable in the DMPC-CX sBLM, most probable corresponding to regions with and without embedded calixarenes. By adding cytochrome c to the formed layer the addressability of calix[6]arenes could be demonstrated indicated by a height increase of 0.7-1.2 nm. The specificity of the interaction, expected to be caused mainly by electrostatic adhesion was successfully proven by adding cyt c to pure DMPC resulting in no height increase. In general it was shown that mica covered with lipid bilayer and that modified by CX is a useful tool for mimicking and anchoring receptors of biomembranes. The properties of these surfaces were studied by near physiological AFM imaging and by atomic force spectroscopy methods. SMFS experiments with cyt c modified tips clearly demonstrated the addressability and allowed to determine k_{off} ($1.14 \pm 0.59 \text{ s}^{-1}$) and x_{β} ($3.98 \pm 0.63 \text{ \AA}$). To prove the assumption that this interaction is based on electrostatic interaction SMFS experiments on calixarenes embedded in DMPC bilayers using an amino-ended linker were performed and resulted again in highly specific interactions with comparable values for k_{off} ($2.74 \pm 0.66 \text{ s}^{-1}$) and x_{β} ($5.91 \pm 2.55 \text{ \AA}$). Thus, it could be demonstrated that calixarenes are perfect candidates for immobilization of highly positively charged proteins like calixarene and that this anchoring process is mainly based on electrostatic interactions. These findings are expected to play an important role in the generation and use of surface based (e.g. electrochemical) biosensors.

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