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Labeled Protein Recognition at a Membrane Bilayer Interface by Embedded Synthetic Receptors

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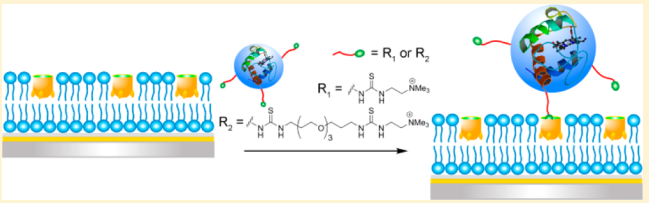
Supporting Information

ABSTRACT: Self-folding deep cavitands embedded in a supported lipid bilayer are capable of recognizing suitably labeled proteins at the bilayer interface. The addition of a choline derived binding "handle" to a number of different proteins allows their selective noncovalent recognition, with association constants on the order of 10^5 M^{-1}. The proteins are displayed at the water:bilayer interface, and a single binding handle allows recognition of the large, charged protein by a small molecule synthetic receptor via complementary shape and charge interactions.



INTRODUCTION

Noncovalent molecular recognition processes at cell membrane interfaces govern the influx of genetic material, as well as controlling intercellular communication and metabolic regulation.[1] Selective recognition of biomacromolecules is essential for these processes, and the application of synthetic receptors toward biomacromolecule recognition has become a focus of recent studies.[2,3] Synthetic cavity-containing molecules such as cyclophanes,[4,5] calixarenes,[6,7] cyclodextrins[8−10] and cucurbituril derivatives[11] have been employed as hosts for histones, drug candidates, and other protein:substrate complexes. The usual medium for their study is pure water, as the environment can be controlled for maximal binding efficiency. Study in membrane environments is far less common, even though that is far more relevant to biological recognition processes. Targeted molecular recognition at biomimetic membrane bilayers with synthetic receptors is far more challenging than in free solution: the hydrophobic effect cannot be exploited to induce binding, and incorporation of water-soluble receptors in membranes is often unsuccessful. Most bilayer-based analyses studies employ *covalent* attachment of a known biological recognition motif (rather than a synthetic cavity) to a lipid or steroid derivative.[12,13] Recognition studies in living cells are the ideal target, and some work has been done in natural systems,[14] but biomimetic membrane systems are a valuable surrogate for complex cellular environments. Supported lipid bilayers (SLB) are an excellent mimic of natural cell membranes, so they provide a robust platform to study biomimetic molecular recognition.[15,16] Real-time label free analysis is possible via noninvasive methods such as surface plasmon resonance (SPR) spectroscopy of the bilayer when supported on a calcinated nanoglassified gold surface in a flowcell.[17]

Employing synthetic receptors for macromolecule recognition in lipid membranes requires that the hosts must both self-incorporate into a bilayer and recognize specific targets. Self-folding deep cavitands such as **1**[18,19] are an excellent example of the first requirement: they display a hydrophobic body and a charged terminus, allowing incorporation into biomimetic or natural lipid aggregates such as micelles,[20,21] bilayer vesicles or supported lipid bilayers.[22] Excellent target selectivity for substituted trimethylammonium (R-NMe_3^+) groups is possible through size, shape and charge complementarity with the host cavity. Even though R-NMe_3^+-containing species such as choline are water-soluble, they can be bound in the cavity with strong (>10^4 M^{-1}) affinities due to favorable cation−π interactions with the electron rich aromatic surface that surrounds the cavity.[19] One R-NMe_3^+ group fully occupies the cavity, with the remainder of the molecule protruding above the host into the external solvent.[22] This introduces the possibility of binding large biomacromolecules such as proteins or oligonucleotides by the host cavitand, as long as the guest displays a suitable binding anchor. Unfortunately, most proteins do not naturally possess R-NMe_3^+ groups, and so target recognition with deep cavitands has (to date) been limited to small molecules. Limited protein recognition is possible by binding a suitable small molecule epitope at the bilayer interface: NeutrAvidin was immobilized by display of a biotinylated guest molecule,[22] but this strategy has a significant limitation: the guest must be independently synthesized and only a very few biomacromolecule targets are accessible.

Large targets such as proteins are challenging targets for recognition: they are significantly larger than cavitand **1** and are generally highly hydrophilic, so the affinity of the binding handle for the host must be high to allow macromolecule immobilization at a bilayer interface. Here we describe a mild,

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in situ labeling procedure that confers a recognition element to variably sized target proteins before introduction to a supported lipid bilayer:cavitand surface. The synthetic hosts recognize the labeled proteins at the bilayer interface, and strong binding affinities are observed, even for large, hydrophilic biomacromolecules.

EXPERIMENTAL SECTION

Mass spectra were recorded by electrospray ionization on an LTQ-XL linear ion trap mass spectrometer (Thermo Scientific, San Jose, CA). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine was purchased from Avanti Polar Lipids. All other materials were obtained from Aldrich Chemical Co., St. Louis, MO, or TCI, Tokyo, Japan and were used as received. Cavitands **1** and **2** were synthesized according to literature procedures.[18] Calcinated chips and the supported lipid bilayer surfaces were prepared according to literature procedures.[22] Full synthetic procedures for new molecules and adapted synthetic procedures can be found in the Supporting Information.

Protein Derivatization. Isothiocyanates **4** or **6** were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 1 M. Protein was dissolved in 1 mL of 0.1 M phosphate buffered saline (PBS; pH 7.4) to a final concentration of 80 μM, and isothiocyanate solution was added to a final concentration of 10 mM. The mixture was incubated at 37 °C overnight. The unreacted isothiocyanate was removed from the derivatized protein by an amicon ultra-0.5 mL centrifugal filter (Millipore, Billerica, MA). The concentration of derivatized protein was determined by Bradford protein assay (Bio-Rad, Hercules, CA), then adjusted to a concentration of 15 μM by the addition of 0.1 PBS buffer (pH 7.4).

Derivatized Protein Binding Measurement. The calcinated gold substrate was first rinsed with ethanol and nanopure water and after drying under gentle stream of nitrogen gas was then clamped down by a flow cell on a high-refractive index prism for SPR measurement. Phosphatidylcholine (PC) vesicles (1 mg/mL) in 20 mM PBS (150 mM NaCl, pH 7.4) were injected through a flow-injection system and incubated for 1 h to allow vesicle fusion on the hydrophilic calcinated gold surface (see Supporting Information), forming a smooth bilayer membrane. After 10 min of rinsing to remove excess vesicle from the surface, 2 mg/mL cavitand **1** in 10% DMSO solution was subsequently injected and incubated for 30 min. The surface was extensively rinsed with nanopure water, followed by incubation with 15 μM derivatized protein for 30 min. Control experiments were performed under identical conditions in the absence of cavitand **1**. Derivatized protein binding measurement using neutral cavitand **2** was performed by the injection of PC vesicles preincorporated with cavitand **2**.

ESI MS Sample Preparation. The solvents of derivatized proteins were exchanged to water by an Amicon ultra-0.5 mL centrifugal filter (Millipore, Billerica, MA). Protein samples in 0.1 M PBS buffer were transferred to the filter and centrifuged at 14,000 rpm for 15 min. The concentrated proteins were reconstituted to the original sample volume with H_2O. This process was repeated three times, and the concentration of the protein was adjusted to 20 μM. To remove the residual salts from the protein samples, 0.1% trifluoroacetic acid (TFA) was added to the protein samples in H_2O and the sample was loaded to the protein macrotrap kit 1 (TH1/25111/02, Machrom Bioresources Inc.). The samples were washed with 2:98:0.1 MeCN:H_2O:TFA mixture 5 times and eluted with 90:10:0.1 MeCN:H_2O:TFA mixture.

Binding Analysis. Saturation binding mode (eq 1)[22] was applied to determine the equilibrium dissociation constant (K_d) value for the interaction between cavitand **1** and labeled protein guests. Increasing concentrations of labeled protein guests (3 μM to 15 μM) were injected over the cavitand **1**:membrane complex, and the minimum angle shift was recorded:

$$AB_{eq} = AB_{max}(1/(1 + K_d/[A])) \quad (1)$$

where AB_{eq} is the average of response signal at equilibrium and AB_{max} is the maximum response that can be obtained for guest binding and [A] is the concentration of guest **6** injection. AB_{max}/AB_{eq} was plotted against 1/[A], and the slope is equal to K_d value. K_a, the equilibrium association constant, can be determined by the reciprocal value of K_d.

RESULTS AND DISCUSSION

The experimental setup for the recognition process is illustrated in Figure 1: the supported lipid bilayer system was constructed

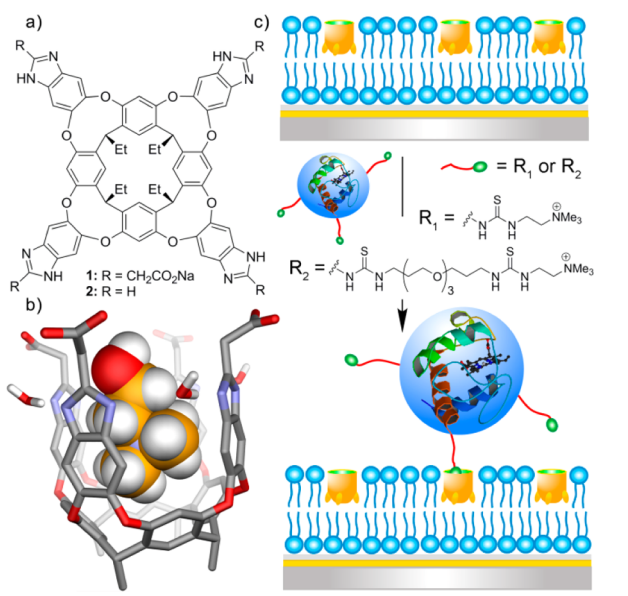

Figure 1. (a) Deep cavitand hosts; (b) minimized structure of the cavitand **1**:choline complex (SPARTAN, AM1 force field); (c) a representation of the recognition process with labeled cyt *c*.

in a flowcell apparatus by established methods.[17,22] 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) vesicles were exposed to a calcinated nanoglassified gold surface onto which they adhere, forming a fluid supported lipid bilayer. Two cavitands were employed for the recognition process: negatively charged cavitand **1** and neutral cavitand **2**. Tetracarboxylate cavitand **1** is water-soluble and can be incorporated into the membrane by simple injection after bilayer assembly.[22] The cavitand occupies the upper leaflet of the bilayer, and is oriented with the charged carboxylate groups at the rim (and as such, the open cavity) positioned toward the external surface. Charge matching interactions likely position the carboxylates close to the positive headgroups of the POPC lipids at the bilayer interface, although this has not yet been definitively proven.

Recognition of the protein targets requires the introduction of an R-NMe_3^+ binding handle, which can be provided by a suitable labeling agent. The agent must possess both a R-NMe_3^+ binding handle *and* a reactive functionality that is both insensitive to hydrolysis and capable of reaction with suitable side chains. Most known labeling agents exploit electrophilic functions that can react with nucleophilic lysine side chains on the target proteins. Isothiocyanates are well-precedented as labeling agents for biomacromolecules, showing limited hydrolysis at pH 8 and good selectivity for lysine tagging, so we focused on the synthesis of a R-NMe_3^+-containing isothiocyanate labeling agent. As steric hindrance could be a determining factor in guest affinity, two labeling agents of different lengths were synthesized, as shown in Figure 2. *unsym-*

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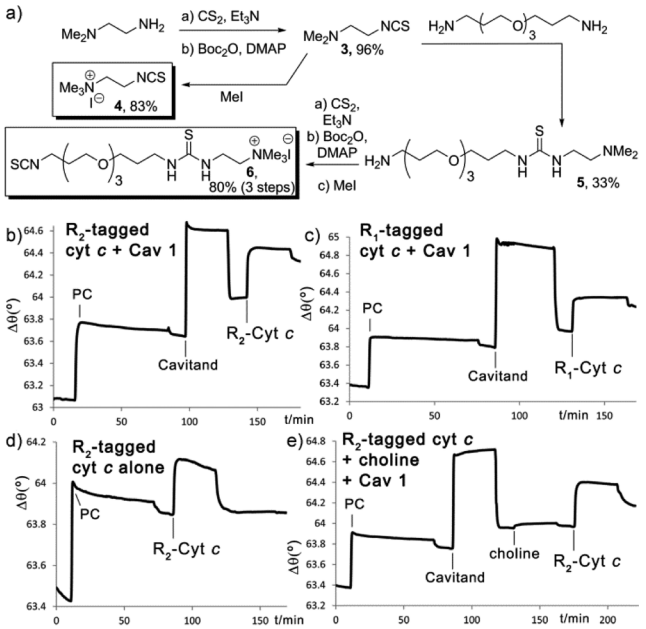

Figure 2. (a) Synthesis of trimethylammonium "tag" molecules **4** and **6**. (b–e) SPR sensorgrams of labeled cytochrome *c* immobilization by cavitand **1**: (b) 15 μM **R$_2$-cyt *c***; (c) 15 μM **R$_1$-cyt *c***; (d) control experiment in the absence of cavitand **1**; (e) control experiment showing the expulsion of choline from the host **1** by **R$_2$-cyt *c***.

Dimethylethylenediamine was converted to the corresponding isothiocyanate **3** via literature methods in 96% yield.[23] This core molecule could either be quaternized with methyl iodide to yield the short trimethylammonium tag molecule **4**, or further extended by reaction with 4,7,10-trioxa-1,13-tridecanediamine, giving **5** in 33% yield. Isothiocyanate formation followed by quaternization smoothly gave the water-soluble extended tag molecule **6**.

The chosen initial test substrate was bovine heart cytochrome *c* (cyt *c*), a well-characterized 12.4 kDa protein. As increased hydrophilicity is well-precedented to reduce the binding affinity of guest molecules for cavitand **1**,[24] a small, weakly charged protein should provide the most probable candidate for success. Cyt *c* was converted to the short and long chain "tagged" derivatives (**R$_1$/R$_2$-cyt *c***, respectively) via standard methods.[25] Cyt *c* (1 mg/mL) was incubated overnight at 37 °C with either **4** or **6** (10 mM) in 0.1 M PBS buffer (pH 7.4). Any unreacted labeling agent **4** or **6** (and their hydrolysis byproducts) were removed from the **R$_1$/R$_2$-cyt *c*** sample on a centrifugal filter. The concentration of **R$_1$/R$_2$-cyt *c*** was determined by Bradford assay,[26] then adjusted to a concentration of 15 μM by the addition of 0.1 M PBS buffer.

Preformed POPC vesicles in 20 mM PBS buffer were injected into the flowcell, and after washing, a 1.6 mM 10% DMSO aqueous solution of cavitand **1** was injected and allowed to incubate for 30 min. The unincorporated excess was washed away and a solution of the tagged proteins **R$_1$/R$_2$-cyt *c*** was injected, exposing the labeled protein to the cavitand **1**:POPC bilayer construct. Control experiments were performed in the absence of cavitand, and monitored in real time by SPR spectroscopy. The SPR sensorgrams are shown in Figure 2, and illustrate that the membrane-embedded cavitand is indeed capable of recognizing suitably derivatized proteins (Figure 2b,c). Upon injection of a 15 μM solution of **R$_1$-cyt *c*** to the POPC–**1** membrane, a change in resonance angle ($\Delta\theta_{cav\ 1}$) of 0.22° was observed. This was retained for >30 min, even after washing, indicating the derivatized **R$_1$-cyt *c*** was immobilized at the bilayer surface. The longer chain **R$_2$** was even more effective: $\Delta\theta_{cav\ 1}$ = 0.40° was observed upon injection of **R$_2$-cyt *c*** to the system. An illustration of the strength of the target binding is shown in Figure 2e: if the cavitand is "prefilled" with 2 mM choline chloride before injection of **R$_2$-cyt *c***, recognition of the protein is still possible. The affinity of **R$_2$-cyt *c*** is sufficient to displace the small guest, and immobilization of **R$_2$-cyt *c*** still occurs. Neither the derivatized proteins **R$_2$-cyt *c*** nor native cyt *c* itself have any affinity for the POPC bilayer in the absence of cavitand (Figure 2d, SI). No nonspecific interactions are present between the protein and membrane: cyt *c* is positively charged, small, and quite hydrophilic, so there are no attractive forces between it and the zwitterionic POPC bilayer. Derivatization of the protein with **4** or **6** does not change the overall charge, as lysine $-NH_3^+$ groups are converted to R-NMe_3^+ groups.

The POPC–**1** system can recognize other labeled proteins, as shown in Table 1. Equine myoglobin and bovine hemoglobin proteins can be labeled with the long chain tag **6** and immobilized at the bilayer interface through recognition of the tags by cavitand **1**. There was no noticeable affinity of the labeled proteins for the bilayer in the absence of cavitand (Table 2). **R$_2$-hemoglobin** did show a small amount of nonspecific binding with the bilayer itself ($\Delta\theta_{ctrl}$ = 0.03°), but this was far outweighed by the affinity in the presence of cavitand **1** ($\Delta\theta_{cav\ 1}$ = 0.43°). All three of these proteins are of roughly the same size (assuming that the derivatized **R$_2$-hemoglobin** dissociates into its constituent monomer units), and show similar responses in the SPR sensorgram.

Table 1. Binding Affinities for R$_2$-Labeled Proteins at the Cavitand 1–POPC Bilayer Interface[a]

R$_2$–Protein	cyt *c*	myoglobin	hemoglobin
M_W (kDa)	12.4	18	15.2 (α)/16 (β)
K_a (**1**), M^{-1}	(9.01 ± 2.55) × 10^5	(9.09 ± 1.07) × 10^5	(1.11 ± 0.07) × 10^5
K_d (**1**), μM	1.11 ± 0.31	1.10 ± 0.13	9.03 ± 0.55

[a]injected [**1**] = 1.6 mM; [**R$_2$**-Protein] = 15 μM, 100 mM PBS buffer.

Table 2. Comparison of the Recognition Properties of Different Cavitands

R$_2$–protein	M_W (kDa)	$\Delta\theta_{cav1}$ (deg)[a]	$\Delta\theta_{cav2}$ (deg)[b]	$\Delta\theta_{ctrl}$ (deg)[c]
cyt c	12.4	0.40	0.29	<0.01
myoglobin	18	0.33	0.05	<0.01
hemoglobin	15.2 (α) 16 (β)	0.43	0.25	0.03

[a]$\Delta\theta_{cav\ 1}$(deg) = resonance angle change upon target binding in the presence of cavitand **1**. [b]$\Delta\theta_{cav\ 2}$(deg) = resonance angle change upon target binding in the POPC: 2% cavitand **2** bilayer. [c]$\Delta\theta_{ctrl}$(deg) = resonance angle change upon target addition to clean POPC bilayer; injected [**1**] = 1.6 mM; [**R$_2$**–protein] = 15 μM, 100 mM PBS buffer.

More quantitative binding data on the recognition of proteins labeled with the long chain **R$_2$** tag was obtained via SPR analysis in saturation binding mode at varying concentrations of protein using an established protocol.[22] The assumption was made that the binding was monovalent (1:1 cavitand:protein ratio), and good fits were obtained using this method. The labeled proteins showed relatively strong

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association with the cavitand, as shown in Table 1. The size of the protein target did not have a large effect on the observed K_a: all three proteins labeled with the long chain linker (**R_2-cyt *c***, **R_2-myoglobin**, and **R_2-hemoglobin**) showed affinities on the order of 10^5 M^{-1} under the analysis conditions. These strong binding constants are slightly higher than those observed previously for the binding of choline and acetylcholine with cavitand **1** in pure water,[18,19] although they are within an order of magnitude. The ability of **R_2-cyt *c*** to expunge bound choline from the cavity (Figure 2e) is a qualitative indication of relative binding affinity, and is consistent with the measured K_a values. The strong binding is gratifying, but slightly unexpected: binding affinities of hydrophilic species by deep cavitand **1** are generally lower than small, hydrophobic guests, so one might expect the labeled protein targets to show lower affinities. There are a number of possibilities why the binding is so effective, the most tantalizing of which is multivalent protein:host interactions. Cavitand **1** is far smaller (1.4 kDa) than the protein targets, and an excess of **4**/**6** is used in the labeling procedure, so multiple labels could be present on each protein.

Analyzing the number of labels added to each protein is challenging, as the labels have no chromophore or electroactive group[27] that would allow for simple quantitation. Mass spectrometry allows for tentative analysis, however. The smallest protein, **R_2-cyt *c*** was successfully analyzed by electrospray ionization (ESI). The ESI spectra for unmodified cyt *c* and **R_2-cyt *c*** are shown in Figure 3a,b, respectively.

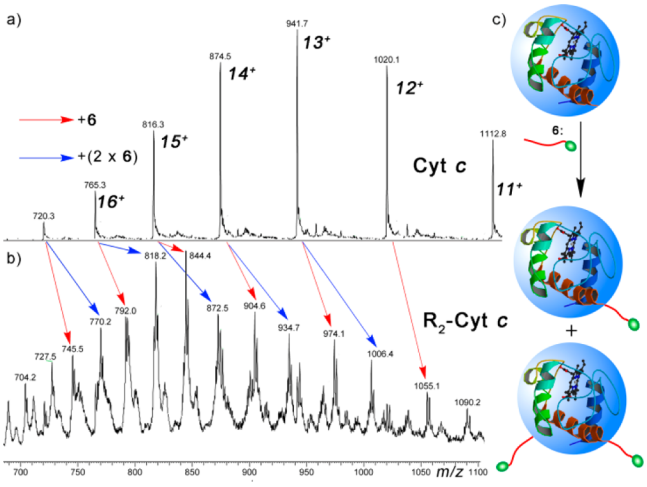

Figure 3. ESI-MS analysis of labeled **R_2-cyt *c***: (a) unmodified bovine cyt *c*; (b) **R_2-cyt c**; (c) representative labeling procedure.

Following modification, several species are present, with the singly modified and doubly modified species being most abundant. Evidence for some small amount of higher order modifications and methylation/adduct formation is also present. The typical ESI charge state distribution (Figure 3a) overlaps with the distributions created by these modifications, producing a complex spectrum. It is notable that only "successful" labeling will lead to recognition: other outcomes such as protein methylation and concomitant demethylation of **6** will not provide species capable of binding inside cavitand **1**; dimethylamino groups are well-precedented to have minimal affinity for **1**.[19] While some minor byproducts from the labeling experiment are evidently present, they are SPR silent.

This ESI mass spectrometry (ESI-MS) analysis shows that the efficiency of the labeling experiment is not particularly high, and the targets only possess a small number of binding handles each. The presence of only one or two R_2 labels on **R_2-cyt *c*** and the 1:1 fit of the binding affinity calculations strongly suggests that the binding is monovalent, and only one cavitand is involved in each recognition event. Each protein shows similar binding affinity, so this suggests the interactions of the three proteins with cavitand **1** are similar. The proteins tested here, **R_2-cyt *c***, **R_2-myoglobin**, and monomeric **R_2-hemoglobin** are only 10 to 15-fold larger than the cavitand itself, and so interaction of one labeled protein with two or more cavitands is extremely unlikely. It should be noted that we cannot completely rule out multivalent interactions (and are actively pursuing species that are capable of such), but they appear not to be dominant in this system.

Another possible explanation for the strong binding is that the proteins display charge-based affinity for the cavitand-impregnated bilayer that does not rely on host:guest interaction with the cavitand. Cavitand **1** is negatively charged, and **R_2-cyt *c*** is positively charged under the analysis conditions, so it is conceivable that nonspecific charge-based interactions with the CO_2^- groups in **1** could be a contributing factor. To illustrate that protein binding is due to *cavity-based* molecular recognition rather than charge-based interactions, neutral cavitand **2**[28] was employed. This cavitand displays an identically sized cavity to **1**, but has no charged groups at the rim. Neutral cavitand **2** is only sparingly soluble in water, so it must be preincorporated into the POPC vesicles before formation of the SLB. Different concentrations of cavitand **2** (0.5−5%) were mixed with POPC lipids in $CHCl_3$, then the resulting vesicles were exposed to the calcinated surface as previously. The bilayer formation was subsequently monitored by SPR. At cavitand concentrations of 2% or lower, a robust stable supported lipid bilayer was formed. If vesicles containing >2% cavitand **2** were injected, successful adhesion did not occur. To maximize target recognition, all tests were performed with a bilayer created from vesicles containing 2% cavitand **2**. If the (reasonable) assumption is made that cavitand **2** is evenly dispersed in the upper and lower leaflets of the SLB, the upper POPC leaflet consists of 1% cavitand **2**.

As can be seen in Figure 4c, this preformed POPC-**2** SLB is also fully functional, and strong binding of **R_2-cyt *c*** (although slightly weaker than that of cavitand **1**) occurs. The other tested protein derivatives were also immobilized by cavitand **2**. Both **R_2-myoglobin** and **R_2-hemoglobin** showed affinity for the

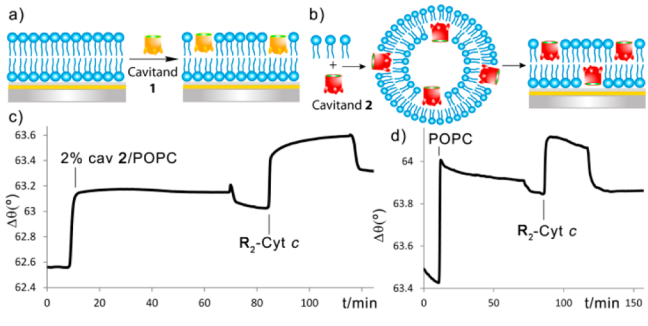

Figure 4. (a) Flowcell incorporation of water-soluble cavitand **1**; (b) 2% cavitand **2**/POPC bilayer formation; SPR sensorgrams of (c) **R_2-cyt *c*** immobilization by the POPC/cavitand **2** bilayer system and (d) **R_2-cyt *c*** on a clean POPC bilayer.

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bilayer:cavitand **2** construct, although the SPR responses were slightly lower than those for cavitand **1**. It is notable that the lack of charged, hydrophilic species at the cavitand rim has little effect on its orientation in the upper leaflet of the bilayer: as recognition still occurs, the open end of a significant proportion of cavitand **2** molecules must point to the exterior solvent. The benzimidazole groups at the rim, while only slightly more polar than the base, are still capable of providing some orientational preference in the upper leaflet. That the presence of a cavity with no negative charge is sufficient to control binding of the tested $\mathbf{R_2}$–protein conjugates indicates that this is truly a shaped-based recognition phenomenon, and the appended R-NMe_3^+ binding handles are indeed bound inside the cavitand. Without the cavitand present, no immobilization of the protein occurs.

CONCLUSIONS

In conclusion, we have demonstrated an in situ labeling/recognition method for large biomacromolecules by self-folding deep cavitands in a supported lipid bilayer. Addition of an R-NMe_3^+-containing isothiocyanate label to different proteins confers affinity for membrane-embedded deep cavitands in supported lipid bilayers. These binding interactions show micromolar dissociation constants, and MS and SPR analysis suggest monovalent binding stoichiometry between proteins and the hosts. This strategy has the potential to allow the recognition of a variety of biomacromolecules at membrane bilayers. Further studies, including more detailed analysis of the binding mechanism and the applications toward multivalent target recognition are currently underway.

ASSOCIATED CONTENT

Supporting Information

Experimental details, binding analysis, and new molecule characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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