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Supramolecular Chemistry

Supramolecular Protein Immobilization on Lipid Bilayers


Abstract: Protein immobilization on surfaces, and on lipid bilayers specifically, has great potential in biomolecular and biotechnological research. Of current special interest is the immobilization of proteins using supramolecular noncovalent interactions. This allows for a reversible immobilization and obviates the use of harsh ligation conditions that could denature fragile proteins. In the work presented here, reversible supramolecular immobilization of proteins on lipid bilayer surfaces was achieved by using the host–guest interaction of the macrocyclic molecule cucurbit[8]uril. A fluorescent protein was successfully immobilized on the lipid bilayer by making use of the property of cucurbit[8]uril to host together a methylviologen and the indole of a tryptophan positioned on the N-terminal of the protein. The supramolecular complex was anchored to the bilayer through a cholesterol moiety that was attached to the methylviologen tethered with a small polyethylene glycol spacer. Protein immobilization studies using a quartz crystal microbalance (QCM) showed the assembly of the supramolecular complexes on the bilayer. Specific immobilization through the protein N-terminus is more efficient than through protein side-chain events. Reversible surface release of the proteins could be achieved by washing with cucurbit[8]uril or buffer alone. The described system shows the potential of supramolecular assembly of proteins and provides a method for site-specific protein immobilization under mild conditions in a reversible manner.

Introduction

Surface-immobilized proteins have great potential in biotechnological research. By developing strategies for the controlled immobilization of proteins on surfaces, biosensors can be made for use in, for example, biomarker detection and drug discovery.[1] These protein-covered surfaces are also of great interest for the development of cell growth in or on biomaterials. Such biomaterials can also be applied for tissue engineering and the development of medical implants.[2, 3] One of the main challenges for protein immobilization strategies is to find chemical methodologies that allow for an efficient immobilization of proteins while ensuring that their functionality is retained. Moreover, there is interest in molecular strategies that allow for the immobilization of the proteins through singular and defined molecular handles, rather than, for example, approaches that are based on random amide bond immobilization.[4]

Lipid bilayers are a special type of surface that are relevant for protein immobilization. In nature, the cellular bilayer functions as a complex boundary that confines the cellular interior and communicates with the environment through the display of proteins on the inside and outside. To investigate these intriguing self-assembling structures, model systems have been developed to understand the mechanisms of biological membranes on a molecular level.[5] An interesting topic here is the search for techniques that control the introduction of proteins on these bilayer surfaces. This typically requires the introduction of hydrophobic domains or elements that have high affinity for the lipid bilayers, into the proteins of interest. As a result, many covalent chemical modification strategies have been developed to synthesize membrane-anchored proteins.[6–14] Although successful, synthetic access to these protein constructs is typically troublesome and only amenable to a limited set of proteins.

Strategies to immobilize proteins on lipid bilayer membranes through a supramolecular chemistry based approach might provide a novel method with which to overcome the challenges described above. By using supramolecular chemistry, multicomponent complexes can be engineered by making use of noncovalent interactions between molecules.[15, 16] Specifically, for the immobilization of proteins on lipid bilayers,
supramolecular chemistry offers the potential advantage that there is no need for modification of the proteins through potentially cumbersome chemical protein lipidation methods. Additionally, the use of noncovalent interactions also provides a way to ensure that the immobilization occurs site-specifically and under milder conditions than required for many covalent immobilization strategies. Finally, of course, supramolecular protein immobilization would also enable reversible release of the proteins from the lipid bilayer, for example, upon addition of noncovalent competitor molecules.11

The use of supramolecular chemistry for oriented protein immobilization has been reported before. One well-described immobilization strategy makes use of the strong interaction between the biotin molecule and streptavidin. Streptavidin is able to bind simultaneously four biotin molecules, making it possible to perform protein immobilization in a stacked fashion whereby first biotin is covalently attached to the surface, then a layer of streptavidin is immobilized, and finally biotinylated proteins are added.17–21 To overcome the need for protein biotinylation, an alternative immobilization strategy is the use of a genetically encoded hexahistidine tag (His6-tag). In the presence of divalent metal ions such as Ni2+, the His6-tag forms a hexagonal complex with covalently immobilized nitri-lotriacetic acid (NTA). By introducing the His6-tag at specific positions in the protein, it can be oriented specifically on the surface without loss of protein function. This immobilization can be reversed by the addition of ethylenediaminetetraacetic acid (EDTA) or imidazole.22–26 Another reported supramolecular immobilization strategy is the use of complementary DNA strands that are fused to the protein and immobilized on the surface.27–30 More recently, synthetic supramolecular ligand–receptor pairs have been used for protein immobilization. This strategy makes use of macrocyclic host molecules, such as cyclodextrin and cucurbit[n]uril, that can accommodate different ligands. By incorporation of these synthetic receptors on a solid support, proteins containing the corresponding ligand can be immobilized.20,26,31–39 There are, however, only very limited examples of the use of supramolecular chemistry for the immobilization of proteins on lipid bilayers. The potential of a supramolecular approach for protein immobilization on lipid membranes is most impressively illustrated by the membrane incorporation of nitri-lotriacetic acids that recruit His6-tagged proteins upon addition of nickel.40,41

To address the challenges discussed above and to provide this important field of research with novel supramolecular concepts for protein immobilization on lipid bilayers, host–guest interactions mediated by cucurbit[8]uril provide an attractive strategy.42 This donut-shaped molecule can bind a range of host molecules in its hydrophobic pocket. The ability to bind two different host molecules provides cucurbit[8]uril with the potential to link different molecular elements. This feature has already seen ample application, for example in the synthesis of polymers43,44 and vesicles45 redox switches,46,47 protein and peptide assemblies,48–51 and to immobilize proteins and bacteria on solid supports.46,52 Interestingly, cucurbit[8]uril can simultaneously host electron-deficient methyliologen and the electron-rich indole of the tryptophan amino acid. The form-

formation of the ternary complex is a two-step process in which the methyliologen binds first, followed by binding of the indole. Both binding steps have individual association constants of $K_a \approx 10^5 \text{ M}^{-1}$.[49,53–55] The binding of the tryptophan to the methyliologen-cucurbit[8]uril complex is further stabilized by cation-dipole interactions of the N-terminal amine and the oxygen atoms of the cucurbit[8]uril rim.[49,56] Here, the potential of cucurbit[8]uril for immobilization of proteins on a lipid bilayer is explored (Scheme 1). As a model protein for immobilization on lipid bilayers, yellow fluorescent protein (YFP) was used. The YFP protein was generated with an N-terminal WGG peptide motif that was separated from the folded β-barrel with a 16 amino acid long flexible linker. The use of the natural amino acid tryptophan at the N-terminus offers the advantage that the protein does not need any synthetic modifications. The electron-deficient methyliologen was anchored to a cholesterol moiety to enable efficient bilayer incorporation. First, supramolecular complex formation in solution was studied and, subsequently, the scope and limitations of this supramolecular system for protein immobilization on lipid bilayers were studied using quartz crystal microbalance (QCM) studies.

Results and Discussion

Protein expression

YFP was used as a model protein for immobilization of proteins on lipid bilayers through the cucurbit[8]uril-methyliologen complex. The YFP with an N-terminal WGG peptide motif was produced by making use of intein splicing technology.[57,58] For this, the WGG-YFP construct was genetically fused at its N-
terminus to an intein splicing domain, which was fused to a chitin binding domain (CBD-intein) and expressed in E. coli bacterial cells. After cell lysis, the expressed fusion construct was loaded on a chitin bead column. Lowering the pH from 8.5 to 7 resulted in intein splicing and release of the target protein. After overnight incubation at room temperature, the WGG-YFP was eluted from the column. As small amount of co-eluted spliced CBD-intein construct was removed by using anion chromatography. The same intein splicing strategy was used to obtain the control protein MGG-YFP. The purity and correct mass of the resulting YFP proteins were verified by using SDS PAGE and QTof-MS analysis (Figure S1 in the Supporting Information).

Supramolecular complex formation in solution

The binding of an N-terminal tryptophan to a cucurbit[8]-uril-methylviologen complex in solution has already been described and quantified by using the short WGG tripeptide. However, the effect of an appended protein on the binding affinity has not been reported. Therefore, isothermal titration calorimetry (ITC) experiments with a WGG tripeptide and a YFP derivative with an N-terminal WGG motif regarding their binding characteristics to the cucurbit[8]uril-methylviologen complex were performed. As a control, the WGG sequence was replaced with the MGG tripeptide sequence. The ITC experiments with the peptides and proteins were performed in 10 mM sodium phosphate buffer at pH 7.0. Cucurbit[8]uril and methylviologen were mixed (both 100 mM) and the binding partners were titrated to the complex. The WGG showed the same binding affinity as that reported by Urbach and co-workers (Table 1 and Figure S2 in the Supporting Information).

The ITC titration revealed an enthalpically driven ($\Delta H > |T\Delta S|$) and entropically unfavorable ($-T\Delta S > 0$) binding event. No binding was observed for the MGG tripeptide, showing the inability of the methionine to form a ternary complex with cucurbit[8]uril-methylviologen and the key importance of the N-terminal tryptophan.

The titrations of the proteins (Figure 1) revealed an association constant of $K_a = 2.3(\pm 0.3) \times 10^4$ M$^{-1}$ for WGG-YFP. This affinity is comparable to that of the simple WGG tripeptide (Table 1). However, a weaker enthalpic and more favorable entropic component were observed compared with those of the WGG tripeptide. The protein component thus leads to a change in the enthalpy and entropy contributions, probably resulting from a modulated binding mode. Although no binding was found for the MGG peptide, the MGG-YFP protein featured an appreciable association constant with the cucurbit[8]uril-methylviologen complex ($K_a = 1.2(\pm 0.4) \times 10^5$ M$^{-1}$).
Compared with WGG-YFP, the binding of MGG-YFP was less enthalpically driven and more entropically favored. Given that binding of the MGG-YFP complex does not involve the MGG N-terminus, most likely in-chain aromatic amino acid side-chains are responsible. It has been described that not only tryptophan, but also the aromatic amino acids phenylalanine and tyrosine can bind to the cucurbit[8]uril-methylviologen complex.\(^{[59-62]}\) It is thus most likely that MGG-YFP, and potentially in part also WGG-YFP, interact with the cucurbit[8]uril-methylviologen complex through some of its other aromatic amino acids. The absence of stabilizing electrostatic interactions of an N-terminal amine with the rim of the cucurbit[8]uril mean that the binding of in-chain aromatic amino acids to the cucurbit[8]uril host is typically lower than that of the N-terminal positioned aromatic amino acid.\(^{[49]}\) Analysis of the amino acid sequence and crystal structure of YFP (pdb code: 3V3D) reveals the presence of four phenylalanine and seven tyrosine groups at the surface of the protein that are potentially accessible for binding to the cucurbit[8]uril-methylviologen complex.\(^{[64]}\)

Supramolecular protein immobilization on a lipid bilayer

The formation of the cucurbit[8]uril-mediated supramolecular complexes on a lipid bilayer, and hence immobilization, was studied with a quartz crystal microbalance with dissipation monitoring (QCM-D). To anchor the supramolecular complex on the bilayer, a membrane-anchored methylviologen derivative was designed. For this, the methylviologen was attached to a cholesterol moiety (Scheme 1). A small, discrete polyethylene glycol (PEG) spacer of four ethylene glycol units was introduced between the methylviologen and cholesterol, which was envisioned to allow the methylviologen to be exposed on the outside of the membrane and thus be accessible for binding. A comparable anchor design was described by Ravoo et al. showing the suitability of this molecule for membrane incorporation and accessibility for cucurbit[8]uril binding.\(^{[54]}\)

First, potential aspecific binding events of the supramolecular building blocks and proteins towards a bare dioleoylphosphatidylcholine (DOPC) bilayer were investigated (Figure S3 in the Supporting Information). Flowing DOPC vesicles over a silica-coated quartz crystal resulted in the absorption of the vesicles and formation of a supported lipid bilayer, as evidenced by a decrease in frequency \(\Delta f\) and increase in dissipation \(\Delta D\). The frequency and dissipation leveled off at the reported values of \(\Delta f - 25\) Hz and \(\Delta D \leq 0.5 \times 10^{-6}\).\(^{[65,66]}\) Throughout this work, the changes in frequency and dissipation of the 7th overtone are depicted. This overtone showed a good trade-off between signal stability and sensitivity. After formation of the DOPC bilayer, different amounts of WGG-YFP protein were flowed over the sensor. Buffer washing was performed after every step. Fluorescent protein concentrations up to 2 \(\mu M\), both in the presence and in the absence of equimolar cucurbit[8]uril, did not result in any adsorption on the bare bilayer. Similarly, cucurbit[8]uril at high concentrations (75 \(\mu M\)) did not show any affinity towards the DOPC bilayer. These results show that there is no unspecific interaction of the protein and cucurbit[8]uril with the lipid bilayer, which bodes well as a surface for the selective immobilization of proteins.

To immobilize the proteins through host–guest interactions, first the methylviologen anchor was incorporated into the bilayer. After DOPC bilayer formation, the methylviologen anchor was flowed over the surface. An increase in the amount of membrane incorporation of the anchor was observed when it was applied to the bilayer in the presence of cucurbit[8]uril (Figure S4 in the Supporting Information). The enhanced membrane incorporation in the presence of cucurbit[8]uril was explained by a reduced solvent solubility of the anchor because of shielding of the positive charge of the methylviologen. To maintain a stable methylviologen-cucurbit[8]uril complex at the bilayer it is critically important to supply cucurbit[8]uril to the running buffer. As an example, after incorporation of the anchor in the presence of 50 \(\mu M\) cucurbit[8]uril (Figure 2, step II), different cucurbit[8]uril concentrations were used in

Figure 2. A) Schematic overview of the supramolecular protein immobilization experiments. After I) formation of the lipid bilayer, II) the anchor was administered in the presence of cucurbit[8]uril (Q8). III) Nonincorporated anchor was removed by washing with cucurbit[8]uril. IV) Addition of YFP results in the formation of the supramolecular complex. B) QCM sensogram showing the influence of cucurbit[8]uril for WGG-YFP binding on the bilayer. The top graph shows the change in frequency (\(\Delta f\)) and the bottom graph shows the change in dissipation (\(\Delta D\)). The lines are event marks showing the start and duration of different steps. At solid event marks, washing with buffer was performed. Dotted event marks represent the following: After formation of the bilayer (I), the anchor (5 \(\mu M\)) was incorporated in the presence of cucurbit[8]uril (50 \(\mu M\)). III) To evaluate the influence of the cucurbit[8]uril, the surface was washed with different cucurbit[8]uril concentrations (0, 2, and 20 \(\mu M\)). IV) The WGG-YFP protein (2 \(\mu M\)) was administered in the presence of the corresponding cucurbit[8]uril concentration.

the running buffer (0, 2, or 20 μM cucurbit[8]uril), resulting in either a stably maintained frequency signal (20 μM) or a decrease to a lower, stable value (Figure 2, step III). These different frequency values show that the cucurbit[8]uril loading at each surface is different, resulting from the different amounts of cucurbit[8]uril in solution.

Efficient protein immobilization on the lipid bilayer was achieved by flowing the WGG-YFP protein over the surface featuring the methylviologen anchor, in the presence of cucurbit[8]uril (Figure S5 in the Supporting Information and Figure 2, step IV). The WGG-YFP protein, together with cucurbit[8]uril at different concentrations, was flowed over the bilayer, resulting in a decrease in the frequency and an increase in dissipation. The extent of immobilization of WGG-YFP on the bilayer varied with the amount of cucurbit[8]uril in a concentration-dependent manner. In the presence of 20 μM cucurbit[8]uril, a change in frequency of Δf ≈ 19 Hz was observed, whereas at a ten-fold lower cucurbit[8]uril concentration a concomitant decrease of the frequency change of Δf ≈ 1.5 Hz was observed. In the absence of cucurbit[8]uril, no change in dissipation or frequency was observed, confirming the requirement for all three supramolecular elements. Similarly, this observation reveals that there is no unspecific binding of the protein to the methylviologen anchor.

The supramolecular protein immobilization was highly effective down to submicromolar concentrations. Four different WGG-YFP concentrations (0.5, 1, 2, and 4 μM) were consecutively flowed over the methylviologen displaying bilayer in the presence of cucurbit[8]uril (Figure 3, starting from step IV). Every step in concentration increase resulted in an additional decrease in frequency (Δf ≈ 11, 3, 4, and 5 Hz, respectively), reflecting increased levels of protein immobilization. Flowing of buffer at the end of the protein titration series resulted in the complete disassembly of the supramolecular complex and removal of all protein from the bilayer. These results show that the amount of protein immobilized at the surface can thus be tuned in a stepwise and reversible manner. Assuming a linear relation between frequency change and molecular mass, it was estimated that 23% of the anchors were occupied with proteins, finally. However, care needs to be taken because the change in frequency might not be linearly correlated to the mass, because of changes to the surface of the bilayer caused by the attraction of additional water due to the hydrophilicity of the large protein.

The efficiency and reversibility of the supramolecular protein immobilization as well as a comparison of WGG mediated versus random side-chain mediated protein immobilization was investigated by comparing the WGG-YFP with the MGG-YFP. Experiments were performed in which the proteins were repeatedly administered and washed off the surface (Figure 4). After formation of the bilayer and incorporation of the anchor (Figure 4, steps I–III), WGG-YFP (step IV, black line) or MGG-YFP (step IV, gray line) were flowed over the bilayer at the same concentration. This resulted in a strong frequency change of Δf of approximately 25 Hz for the WGG-YFP and a weaker change of approximately 7 Hz for MGG-YFP. The weaker binding affinity of the MGG-YFP for the methylviologen cucurbit-

![Figure 3. QCM sensogram showing the effect of increased amounts of WGG-YFP protein. The top graph shows the change in frequency (Δf) and the bottom graph shows the change in dissipation (ΔD). The lines are event marks showing the start and duration of different steps. At solid event marks, washing with buffer was performed. Dotted event marks represent the following: After I) formation of the lipid bilayer, II) the anchor (5 μM) with cucurbit[8]uril (50 μM) was incorporated. III) After removing nonincorporated anchor by washing with cucurbit[8]uril (50 μM), IV) increasing amounts of WGG-YFP were added. In four steps (IVa–d), four different WGG-YFP concentrations (0.5, 1, 2, and 4 μM) were administered in the presence of cucurbit[8]uril (50 μM).](image)
binding cycle might thus result from a partial extraction of the methylviologen anchor during the washing steps.

**Conclusion**

We have demonstrated a new supramolecular strategy for the immobilization of proteins on lipid bilayers. The supramolecular complex is composed of a bilayer-incorporated methylviologen anchor, cucurbit[8]uril, and a WGG-tagged protein. Quartz crystal microbalance studies revealed the controlled assembly and disassembly of the complexes on a DOPC bilayer at concentrations as low as 0.5 μm protein. The formation of the complex critically depends on the presence of cucurbit[8]uril during formation and stabilization of the protein adhered bilayer. This feature allows for a reversible disassembly of the supramolecular complex and protein immobilization through simple buffer washing steps, which is a concept that is not easily achieved by using the more classical membrane insertion approaches.[6–13]

The ITC and QCM experiments also reveal the possibility of the formation of a protein-cucurbit[8]uril-methylviologen complex in a WGG-independent manner. Whereas the MGG peptide itself does not form a complex with the cucurbit[8]uril-methylviologen system, a YFP protein with the N-terminal tag does show complexation, albeit with lower affinity and resulting lowered level of protein immobilization. Cucurbit[8]uril alone (in the absence of methylviologen) does not significantly bind to aromatic amino acids of YFP that are not located at the terminal.[67] This indicates that for the current system, the presence of the methylviologen is crucial for protein binding, most probably through a charge-transfer interaction with other aromatic YFP side-chains. This result brings forward the possibility of tuning cucurbit[8]uril–protein interactions by making use of charge-transfer complex formation for diverse applications in protein immobilization and materials sciences.[49,55,59]

The introduction of an N-terminal WGG motif significantly improves the extent of supramolecular protein immobilization on the lipid bilayer. This singular and defined small molecular handle opens up a facile general method for the attachment of proteins to bilayers and other supramolecular surfaces, without the need for cumbersome covalent protein modifications. The studies reported here provide first insights into the efficiency and selectivity of this cucurbit[8]uril-mediated process. The system provides a method for site-specific immobilization under mild conditions in a reversible manner. These insights and results can be used to further tune the interaction strength and selectivity of the supramolecular motif itself and for specific proteins, thus establishing a novel, reversible protein immobilization platform.

**Experimental Section**

**WGG-YFP and MGG-YFP expression and purification**

YFP protein constructs featuring an N-terminal tryptophan or methionine were obtained by making use of intein splicing. E. coli BL21(DE3) (Novagen) bacteria were transfected with the fluorescent protein in a pTWIN vector were cultured in 2 L LB medium containing 100 μg/mL ampicillin at 37 °C and 180 rpm until an OD_{600} of approximately 0.7. Subsequently, protein expression was induced by adding isopropyl-β-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. Cells were incubated for 7 h at 25 °C and 180 rpm before being harvested by centrifugation (8000 g for 10 min). Bacterial cells were lysed by resuspending the pellet in Bugbuster Protein Extraction Reagent supplemented with benzonase (Novagen) and incubated for 1 h at RT. The insoluble fraction was removed by centrifugation (40,000 g for 30 min). The soluble fraction was purified by applying it to a column filled with chitin beads (New England Biolabs) by gravity flow. The column was washed with 10 column volumes of buffer (20 mM sodium phosphate, 150 mM sodium chloride, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7). The on-column intein splicing was incubated overnight at RT by closing the column. Cleaved proteins were eluted from the column by washing with the same buffer. Subsequently, the proteins were purified on strong ion exchange spin columns (Pierce Thermo Scientific). Prior to loading the protein on the columns, the buffer was changed to buffer A (20 mM sodium phosphate, 50 mM sodium chloride, pH 8) by filtration (Millipore centrifugal filter device, MWCO 10,000). Purified proteins were eluted with increasing amounts of sodium chloride in buffer A. Both proteins eluted at 78.5 mM sodium chloride. Buffer was changed to PBS buffer using a PD-10 desalting column (GE Healthcare). Proteins were concentrated using an Amicon Ultra centrifugal filter device (MWCO: 10 kDa) (Millipore). Finally, approximately

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Figure 4. QCM sensogram showing the stepwise assembly of the supramolecular complex. The top graph shows the change in frequency (Δf) and the bottom graph the change in dissipation (ΔD). The lines are event marks showing the start and duration of different steps. At solid event marks, washing with buffer was performed. Dotted event marks represent the following: I) After formation of the bilayer and II) incorporation of the anchor (5 μM) with cucurbit[8]uril (50 μM), IV) WGG-YFP (2 μM; black line) or MGG-YFP (2 μM; gray line) in the presence of cucurbit[8]uril (50 μM) were administered to the bilayer. Afterwards, V) the protein was removed from the surface by washing with cucurbit[8]uril (50 μM). Subsequently, VI) on both sensors new WGG-YFP (2 μM) with cucurbit[8]uril (50 μM) was loaded. This cycle was repeated once again (steps VII and VIII).
The WGG-YFP and MGG-YFP proteins were present in PBS buffer after purification. Buffer was changed to 10 mM sodium phosphate buffer (pH 7.0) by making use of Centri·Pure Mini desalting columns Z-25 (emp Biotech GmbH) according to manufacturer’s protocol. The fluorescent proteins were concentrated with an Amicon Ultra centrifugal filter device (MWCO: 10 kDa) (Millipore).

Quartz crystal microbalance

Silica-coated (50 nm) QCM-D sensors (QSX303) from LOT-QTum were oxidized with UV/ozone for 30 min (UVV UV-Ozone photoreactor PR-100) and subsequently washed with EtOH and dried in a stream of nitrogen. QCM-D measurements were performed with a Q-Sense E4 4-channel quartz crystal microbalance with a peristaltic pump (Biolin Scientific). All experiments were performed in 0.5X PBS buffer with a flow rate of 100 µL min⁻¹ at 22 °C. The lipid bilayer was formed by flowing 100 nm 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Avanti Polar Lipids) vesicles over the QCM-D sensor. Lipids were made as follows: DOPC was dissolved in chloroform and dried under nitrogen flow. Subsequently the DOPC was resuspended in milliQ water to a final concentration of 1 mg mL⁻¹. To get a size of 100 nm, the vesicles were extruded 11 times (pore size 100 nm). Prior to flowing the vesicles over the QCM-D sensor, the vesicles were diluted 1:1 with PBS to enhance the extent of vesicle rupture on the sensors. After a stable bilayer was formed, the different experiments were performed as discussed in the results section.

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