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COMMUNICATION

Liposome fusion on proteinaceous S-layer lattices triggered *via* β-diketone ligand–europium(III) complex formation

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The present study describes a method for generating solid supported lipid membranes. Liposomes containing β-diketone ligands were chemically bound to crystalline surface (S-) layer proteins which entirely covered the gold sensors. These ligands formed complexes with added europium(III) ions and triggered fusion/rupture of the liposomes resulting in planar supported lipid bilayers.

Investigations on supported lipid membranes started approximately twenty-five years ago and there is still a reliable interest in this field. In particular, so-called biomimetic supported lipid membranes are currently receiving increasing attention. From the scientific point of view, they have proven to be highly valuable for studying the properties and function of membrane-bound or transmembrane proteins as well as the investigations on membrane-mediated processes such as cell–cell interactions and biological signal transduction.[1,2] Furthermore, a broad range of surface-sensitive techniques are now available to study all the previously mentioned issues.[3]

The present communication deals with the formation and characterization of a biomimetic model lipid membrane generated on crystalline bacterial cell surface layers (S-layers). S-Layer proteins can be recrystallized on a broad spectrum of solid substrates including gold- and silicon dioxide-coated surfaces.[4] According to the formation of solid supported lipid membranes, the highly porous S-layer lattice is utilized as (1) stabilizing scaffold, (2) tethering structure to decouple the lipid membrane from the solid support, (3) chemically modifiable binding matrix for functionalization with biomolecules or other functionalities and (4) ion reservoir necessary for electrochemical measurements.[5] For the generation of artificial lipid membranes on electrodes, the S-layer protein SbpA from *Lysinibacillus sphaericus* CCM 2177 represents a convenient building block presenting surface exposed carboxyl- and amino groups on the square lattice symmetry.[6] A commonly used method in particular to build up a lipid bilayer structure is the vesicle rupture/fusion mechanism.[7–10] Several techniques using liposomes as "water-soluble" lipid-providing structures to generate a planar lipid bilayer *via* fusion on the S-layer protein SbpA have been examined and proved to be reliable. Besides utilizing electrostatic interactions between protein domains on SbpA and head groups of the adjacent phospholipids within the liposomes, the protein surface was also chemically modified resulting in enhanced amino groups or carboxylic acid groups. Both techniques did not result in a lipid bilayer entirely covering the SbpA-coated support.[11] Additional efforts in this direction were the binding of hydrophobic linker molecules to the native protein lattice[11] and the generation of SbpA with a terminal His-tag for anchoring Ni-NTA-lipids.[12] Both techniques, however, resulted in the formation of a layer composed of adsorbed intact liposomes. In addition, liposomes composed of different lipid species were generated and the fusion efficiency was verified by electrochemical impedance spectroscopy (EIS). However, in all experiments the lipid bilayer did obviously not cover the entire S-layer coated electrode. This can be seen by the too high membrane capacitance (between 8 and 20 μF cm^{-2}) and the too low membrane resistance (between 0.5 and 1 MΩ cm^2).[13]

A further approach was the chemical binding of liposomes comprised of lipid head groups carrying free amino groups (see Fig. 1A) *via* reactive ester and amide formation on the S-layer lattice. Moreover, the addition of additives like di- and trivalent ions, polyethylene glycol, *etc.*, the utilization of bio-specific binding systems like the biotin–streptavidin, *etc.*,[14–17] and the variation of parameters like temperature, ionic strength of the buffer as well as vesicle concentration have been reported in the literature to enhance the fusion ability of vesicles on surfaces.[18,19] Unfortunately, all these methods did not result in an S-layer supported lipid membrane with the desired intrinsic features. The reason for this behavior might be the intrinsic nature of the S-layer which is known to provide an anti-fouling surface for other biological molecules.[20,21]

To benefit from the unique features of the S-layer lattice as tethering structure for lipid bilayer membranes,[5] the focus of interest was put on the search for an alternative method to force the bound liposomes to form a lipid bilayer with a reasonable success rate. In this context an interesting study reported on the incorporation of an amphiphilic β-diketone ligand into phosphatidylcholine liposomes.[22] This ligand is comprised of two C_{16} alkane chains as hydrophobic anchors and two short polyethylene glycol spacers onto which the β-diketone group is bound (see Fig. 1A) which is known to form a complex with Eu^{3+}-ions (Eu(III)). Haluska *et al.* investigated the fusion behavior of giant unilamellar vesicles (GUVs) containing

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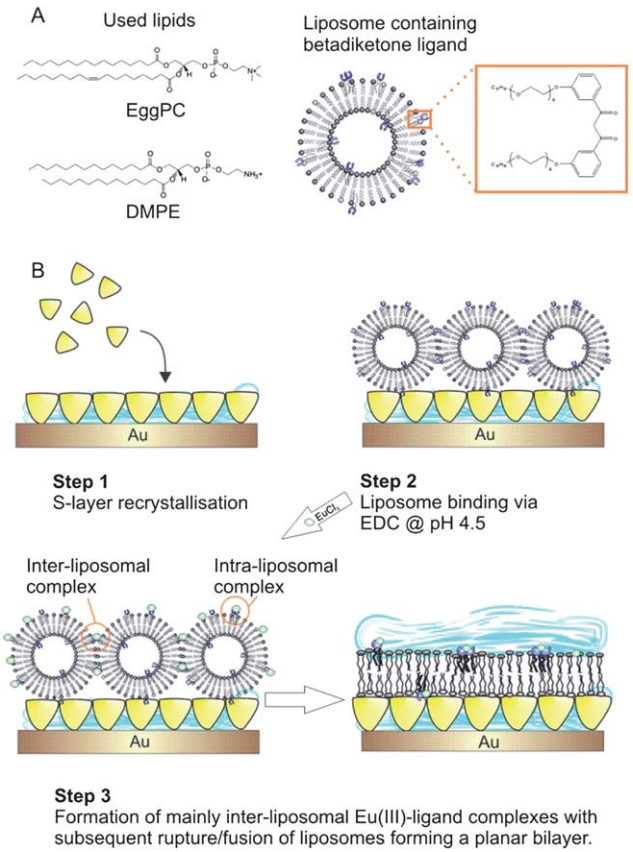

Fig. 1 Scheme of liposome-forming molecules (A) and lipid bilayer generation on an SbpA lattice (B).

a certain amount of β-diketone ligands by microscopy and patch-clamp.[23,24] The fusion starts by nonspecific binding of the Eu(III) on the outer surface of the GUVs presenting the β-diketone groups followed by specific inter-membrane conjugation between the ligands of two touching vesicles (see step 3 in Fig. 1B). The lipid bilayer of the GUVs fuses because the former desorbs Eu(III) and reduces the adhesion area resulting in a so-called fusion pore and a rearrangement of the bilayer structure. A detailed mechanism is described elsewhere.[23]

The novelty of the present study is the application of the β-diketone–Eu(III) complex triggered liposome fusion for the generation of a solid supported lipid bilayer on S-layer lattices, a surface where liposomes are not likely to fuse and rupture. In the present study, liposomes containing egg yolk phosphatidylcholine (EggPC), 1,2-dimyristoyl-*sn*-*glycero*-3-phosphoethanolamine (DMPE; mol ratio EggPC : DMPE = 4 : 1) and the β-diketone ligand (synthesized as previously reported[22]) were fabricated at room temperature by the extrusion method (see Fig. 1A). The amount of β-diketone ligand within the large unilamellar vesicles (LUVs; 200 nm in diameter) was varied between 0.04 and 1 mol% because it is known that within this concentration range GUV fusion in solution occurs.[24] Hence, to determine the optimal condition for fusion on SbpA lattices, the ζ potential of liposomes with different content of ligand as a function of $EuCl_3$ concentration was detected *via* the electrokinetic analysis technique (Zetasizer Nano ZS, Malvern Instruments Ltd, UK).

As shown on the left side of Fig. 2, the addition of Eu(III) ($EuCl_3$) to bare liposomes resulted in a ζ potential with a maximum at 1 mM Eu(III). This behavior can be explained by unspecific binding of Eu(III)

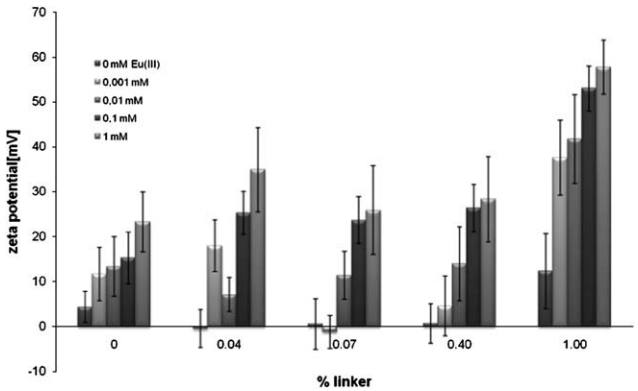

Fig. 2 Change in ζ potential upon addition of europium chloride to liposomes containing different amount of β-diketone ligands (% linker). The measurements were performed in 200 mM glucose at pH 6.

to the lipid membrane. Whenever ligand molecules were present in the lipid membrane, the ζ potential showed the tendency to increase with increasing amount of ligand content and Eu(III) concentration. Hence, it is obvious that the Eu(III) is binding in a concentration dependent manner to β-diketone ligands. The maximal adhesion and binding of Eu(III) was observed at a ligand content of 1% within the lipid bilayer. Owing to this result (Fig. 2) and due to the observation that a higher β-diketone concentration Eu(III) caused intra-liposomal complex formation,[22] a ligand concentration of 1 mol% in the LUVs was utilized in all further studies. After optimization of the liposome composition, the next step was to recrystallize the S-layer protein SbpA on glass and gold sensor surfaces. The recrystallization behavior of SbpA on these surfaces is well known and has been previously investigated by atomic force microscopy (AFM).[25,26]

For monitoring the subsequent lipid bilayer generation, the surface sensitive techniques quartz crystal microbalance with dissipation monitoring (QCM-D; E4, QSense AB, Sweden) and surface plasmon resonance spectroscopy (SPR; Biacore 2000, GE Healthcare, USA), as well as epifluorescence microscopy (Nikon Eclipse, Te 2000-S, USA) were applied. QCM-D measurements revealed not only the mass of adsorbed and hydrated (bio)material but also the energy losses during the adsorption process (dissipation). In contrast, with SPR the mass of biomolecules and binding kinetics can precisely be determined. Hence, the combination of these two techniques provides evidence for bound and coupled water to biomolecules like proteins and lipids by subtracting the mass determined by SPR from that measured by QCM-D.[26] Fluorescence microscopy constitutes a very useful imaging technique to distinguish between the generation of a lipid bilayer by fusion of fluorescently labeled LUVs (0.5 mol% Lissamine rhodamine DOPE) and just the adsorption of intact vesicles.

The recrystallization of SbpA and the planar lipid bilayer formation on SbpA is schematically shown in Fig. 1B and experimentally characterized by the above mentioned techniques in Fig. 3, respectively. SbpA was recrystallized for 3 h on the gold sensors because a plateau in mass increase was reached (see Fig. 3A and B) and as it has previously been reported that the entire surface is completely covered by SbpA after this time.[13] After SbpA recrystallization[27] (Step 1) the surface was flushed with buffer and LUVs containing DMPE were bound *via* 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) coupling onto the SbpA lattice (Step 2). A screening for optimal binding conditions of LUVs on SbpA (Step 2) was

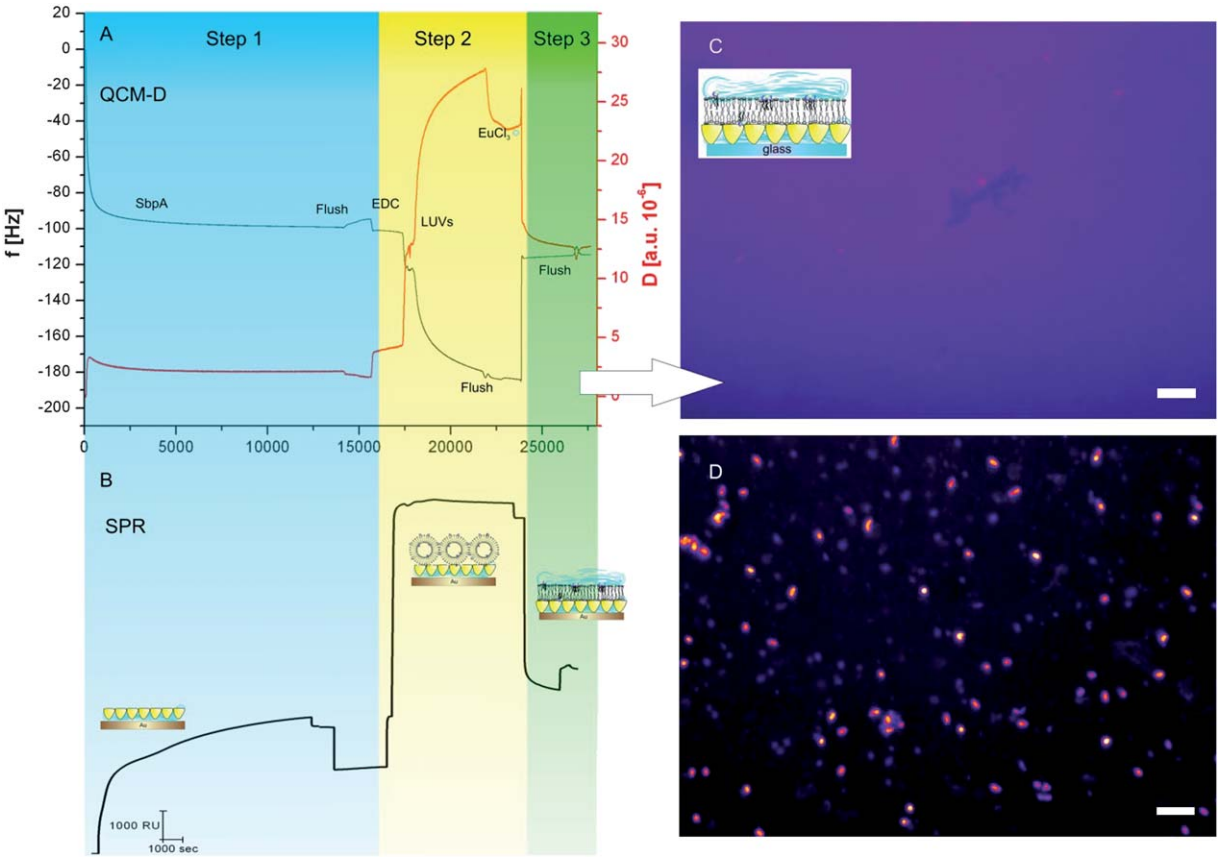

Fig. 3 Summary of the experimental results of lipid bilayer generation investigated by (A) QCM-D with gold sensors as solid support (the time in seconds is given on the x-axis; data obtained at the 7th overtone), (B) by SPR on gold surfaces, and (C) by fluorescence microscopy on glass at an exposure time of 5 s. In (D) the fluorescence microscopy image of bound LUVs without containing the β-diketone ligands treated with Eu(III) is shown. In (A) and (B), the blue area represents the recrystallization of SbpA on gold sensors (Step 1), the yellow area the subsequent binding of intact liposomes *via* EDC-activation to the S-layer lattice (Step 2), and the green area the fusion of the bound liposome by inter-liposomal β-diketone–Eu(III) complex formation to form a supported planar lipid membrane (Step 3). All reactions except EDC-activation were performed in 200 mM glucose. The bar in (C) and (D) represents 20 μm.

performed and resulted in an EDC concentration of 15 mg mL^{-1} at pH 4.5 and a LUV concentration of 0.3 or 0.5 mg mL^{-1} (LUVs approx. 200 nm in diameter) in a 200 mM sucrose/glucose gradient.† The next step was the addition of Eu(III) which caused upon binding to the β-diketone ligands inter-liposomal complex formation between two adjacent liposomes (see Fig. 3A and B).[23,24] The fusion and subsequent bilayer formation of the SbpA-bound LUVs containing β-diketone ligands (Step 3) occurred immediately after addition of the Eu(III) as evidenced by both surface sensitive techniques. The fusion mechanism (Step 3) could, however, not be elucidated in detail by the applied techniques but this result correlates with the finding on the fusion of liposomes containing β-diketone ligands in solution triggered by Eu(III).[22,24]

Most knowledge on the mechanism of liposome fusion on solid supports has been accumulated by the QCM-D technique as the combined information on the shift in frequency and dissipation evidences the behavior of vesicles on surfaces.[9,10] On silicon dioxide, the surface attached liposomes finally fused to form a lipid bilayer.[18]

It is assumed that the interaction between the liposomes themselves as well as the surface–vesicle interaction is important. In contrast, on gold surfaces adsorption of intact liposomes and no fusion were observed. In the present case, intact LUVs have been bound *via* EDC coupling on the SbpA lattice. The accompanied frequency change, Δf, reached a plateau at a value higher than 100 Hz. Moreover, the dissipation change, ΔD, of more than 20.0×10^{-6} a.u. reflected the high viscoelasticity of the vesicular layer. Immediately after addition of Eu(III), Δf increased and reached a plateau at a final value of 23 Hz and ΔD decreased to a final value of 11.5×10^{-6} a.u. (Table 1).‡ These data evidenced the fusion and rupture of LUVs containing β-diketone ligands triggered by Eu(III). Although ΔD of the presently generated lipid bilayer (BLM[1]) is higher compared to membranes on silicon dioxide, one has to keep in mind that the adjacent lipid leaflet is anchored only by head groups to the S-layer-covered surface.[5,28] Hence, the intrinsic feature of this structure (*i.e.* sparsely anchored adjacent lipid leaflet with a floating second leaflet on the top) gives rise to a fluid, viscoelastic membrane. The obtained shift in frequency for the final lipid membrane is in good agreement with the reported value of 25 Hz for a lipid bilayer.[18,19] In comparison, the epifluorescence image in Fig. 3C provides evidence that indeed a lipid bilayer is formed by fusion of the fluorescent LUVs as a homogeneous color has been observed. It has to be mentioned that the homogeneous surface area was larger than 0.5 cm^2. To prove the layered structure of the S-layer supported lipid membrane, the edge of the surface area was scanned for rifts. Indeed, in the middle of Fig. 3C a rift can be

Table 1 Summary of the QCM-D (taken at the 7th overtone) and SPR data. LUVs correspond to bound liposomes before $EuCl_3$ addition. BLM^0 and BLM^1 correspond to bilayer formation by LUVs without and with ligands, respectively. The standard deviation for each given value was calculated by 3 to 4 independent measurements

Surface	Δf/Hz	$\Delta D/10^{-6}$ a.u.	$m_{QCM\text{-}D}$/ ng cm^{-2}	m_{SPR}/ ng cm^{-2}
SbpA	86.45 ± 12.07	3.02 ± 1.66	1512.82 ± 211.26	441.92 ± 11.84
LUVs	>100	>20	>1770	>1000
BLM^0	>35	>15	>700	>400
BLM^1	22.92 ± 4.08	11.51 ± 0.73	405.68 ± 72.22	300.01 ± 7.37

seen which allows one to distinguish between the fluorescent lipid membrane and the underlying S-layer lattice. If no ligand was present in the liposomes the fusion occurred only rarely, most probably induced by osmotic stress. In that case, however, few intact liposomes were attached on the S-layer protein (BLM^0) which is nicely evidenced by fluorescence microscopy shown in Fig. 3D.

The difference between the mass of SbpA determined by QCM-D ($m_{QCM\text{-}D}$), calculated by the Sauerbrey equation,[29] and SPR (m_{SPR}) is attributed to trapped water of 70%, which is slightly higher than previously published.[30] The mass of the vesicular layer and the planar bilayer lipid membrane are also given in Table 1. Due to the low reproducibility of these unwanted structures, the mass of the adsorbed vesicle layer and BLM^0 are given by its minimal observed mass. The mass of the planar bilayer without considering trapped or coupled water was calculated to be 392 ng cm^{-2} assuming a surface area of 0.627 nm^2 and a molecular weight of 740 g mol^{-1} as mean values for the mixed phospholipid bilayer.[31,32] The hydrated mass of the planar lipid bilayer was determined to be 406 ng cm^{-2} (Table 1) at the 7th overtone frequency. These data indicated that only few water molecules are trapped or coupled to the lipid head groups (approximately four water molecules per lipid molecule). Therefore, these data also confirmed the fusion and rupture of ligand-containing liposomes to form a lipid bilayer without embedded intact liposomes. However, at this point one has to consider that the lipid bilayer is generated on a highly porous layer and hence, a considerable amount of water must be entrapped in the inter-membrane region between proteinaceous solid support and lipid membrane.[28]

This inter-membrane region may also influence the capacitance and resistance of the lipid membrane on the SbpA lattice which can be determined by electrochemical impedance spectroscopy (EIS). For the simultaneous QCM-D and EIS (CHI660c; CHI, Austin, USA) measurements an EQCM-D cell (QSense) was used with the QCM-D gold sensor as working electrode and an Ag/AgCl electrode and platinum plate as reference and counter electrode, respectively. An AC potential of 15 mV was applied at a DC bias voltage of 0 mV *versus* the reference electrode. The EIS spectra were fitted by the provided CHI software in a parallel equivalent circuit $R_{el} - R_m Z_{CPE}$; where Z_{CPE} is a constant phase element (CPE), R_{el} is the electrolyte resistance, and R_m the membrane resistance. Due to the high amount of entrapped water in the S-layer lattice one cannot assume that the membrane capacitance C_m is equally distributed over the whole surface. Therefore, the coefficient of CPE of the membrane, Q, cannot be set equal to C_m because this fit parameter is coupled to R_{el} (in this case 7.6 Ω cm^2).[33] C_m was determined by the following formula:

$$C_m = \left[Q R_{el}^{(1-\alpha)} \right]^{1/\alpha}$$

The exponent α may vary in the range from 0 (ideal resistor) to 1 (ideal capacitor). The electrolyte used in all experiments was 10 mM HEPES buffer containing 150 mM NaCl at pH 7.4. EIS spectra were determined for the S-layer surface and the generated lipid bilayer. EIS was not performed on the vesicular layer because the electrochemical behavior of the present intact liposomes would be changed by shrinking effects caused by the osmotic stress in the presence of the used electrolyte. First results revealed that C_m decreases significantly from $C_m = 22.56$ μF cm^{-2} for the SbpA lattice to $C_m = 2.72$ μF cm^{-2} after lipid bilayer generation. Furthermore, the membrane resistance R_m increases from 0.55 MΩ cm^2 to 3.84 MΩ cm^2. These values are compared to other lipid bilayers up to three times larger which can be explained by the trapped water in the inter-membrane region, as described before, resulting in a higher C_m value.[34]

Conclusions

To sum up, the present study outlined the possibility to use a β-diketone ligand for triggering liposome fusion by Eu(III) on biological surfaces where fusion processes can hardly be achieved. For liposome characterization, electrokinetic analysis was performed to gain information on the change in ζ potential dependence on the ligand content. Moreover, to investigate the fusion/rupture of liposomes on S-layer lattices, SPR and QCM-D were utilized. The combination of these two techniques gives an insight into the trapped water associated with the S-layer lattice as well as the membrane architecture. Moreover, fluorescence microscopy was used to image the different structures obtained by investigating the fusion events of S-layer bound LUVs with and without β-diketone ligands. The results evidenced fusion and/or rupture of the liposomes immediately after addition of Eu^{3+}-ions as fusion agent resulting in complex formation between two vesicles touching each other and the final formation of a planar lipid bilayer. Without ligand just partly fused and adsorbed intact vesicles were observed. A further characterization was performed by EIS which reveals a final C_m of 2.7 μF cm^{-2} and R_m of 3.8 MΩ cm^2 which is in good agreement with other tethered solid supported lipid bilayers.[35–37] In future, these model lipid membranes will be utilized for reconstitution studies on membrane-active peptides like valinomycin or gramicidin and transmembrane proteins like *e.g.* α-hemolysin or the receptor tyrosine kinase. It is anticipated that this platform will prove its suitability as a tool to investigate the function of transmembrane proteins but may have also notable potential for commercial applications as a biosensor for single molecules and DNA sequencing device. Moreover, this technique can also be adjusted to more general applications like the coating of micro- and nanoparticles with lipid bilayers for the generation of smart materials for *e.g.* nanomedicinal applications.

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Notes and references

† The size of the ligand-containing liposomes is adjusted to 200 nm for all experiments. A smaller size of the LUVs down to 50 nm revealed no significant difference in the fusion/rupture success rate. Furthermore, preparing the LUVs in a 200 mM glucose solution turned out to cause a faster attachment of the LUVs on the SbpA lattice. For comparison, LUVs filled with Milli-Q water took 6 hours longer to be bound on the activated S-layer surface compared to LUVs filled with glucose (data not shown). This behaviour is widely known from studies on GUV preparation and visualization.[38,39]

‡ The fusion behavior of vesicles containing no and 1% β-diketone ligand was analyzed on bare Au and SiO_2 sensors. While the shift in frequency (−25 Hz) and dissipation (2–3.55 × 10^{-6} a.u.) evidenced liposome fusion on SiO_2, no entire fusion could be determined on gold.

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