Novel surface architectures for biomimetic lipid membranes

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ABSTRACT

Many important biological processes occur at, via or across one of the various lipid membranes present in the cell. These include transport governed by transmembrane proteins constituting more than 50% of all drug targets of interest [1]. Designing and controlling the self-assembly of model membranes onto sensor substrates thus constitutes an important field of research, enabling applications in e.g. drug-screening, dynamic biointerfaces and artificial noses [2]. We present how mimics of bacterial membranes with applications in antibiotics research can be self-assembled at common biosensor interfaces and the process investigated. Results are also presented on the assembly of poly(ethyleneglycol)-cushioned lipid membranes for incorporation of large transmembrane proteins on anz substrate with negative surface charge, where the polymer cushion and lipid layers are self-assembled sequentially by use of a trigger step.

Keywords: lipid, bilayer, bacteria, biosensor, PEG

1 INTRODUCTION

The various cell membranes are crucial structural and functional components of living cells. The outer cell membrane works as an efficient electrochemical delimiter of the cell and its surroundings and simultaneously provides a scaffold for functional proteins controlling communication and transport with the outside environment. Thus, the cell membranes contain a variety of components which are associated with life-sustaining functions and a variety of medical disorders. Given the complexity of real cell membranes and the organism they are surrounding there is an ongoing search for simpler model systems where their properties and the properties of their constituents can be investigated under controlled conditions with common quantitative biosensor techniques.

Due to the development of so many quantitative surface sensitive tools there is currently a strong desire to find efficient means to pattern and control the assembly of biomembranes on surfaces, in part motivated by a lack of current detection methods that can address membrane proteins and lipid bilayer interaction with membrane active peptides [1]. In addition to often discussed proteins of interest like transporters and channels with application in drug screening there is a need for model systems to study the effect of bacteriocidal peptides and other compounds thought to kill bacteria through their interaction with and disruption of bacterial membranes.

Two important examples of biomembrane model systems are unilamellar phospholipid vesicles (liposomes) and supported lipid bilayers (SLB) [2]. In the former, a bilayer of amphiphilic phospholipid molecules forms a spherical shell, separating an "intra-cellular" liquid volume from the "extra-cellular" space, while SLBs are planar, two dimensional, extended bilayers of the same composition as vesicles, adsorbed on a suitable substrate. SLBs are preferably prepared by a method pioneered by McConnell et al. [3], in which liposomes adsorb on a suitable surface. The surface interaction induces rupture and fusion of the vesicles to a coherent planar bilayer. The method produces – when successful – solvent free fluid lipid bilayers spanning even macroscopic surface areas with few defects.

Despite the number of publications in recent years on SLB formation on solid substrates from liposomes comprising simple mixtures of eukaryote lipids like egg-posphatidylcholine (see e.g. [4]) and the in depth knowledge generated from that, there are no similar investigations on how other kinds of supported lipid membranes, based on for example bacterial lipids can be self assembled from liposomes. There is still also only limited SLB platforms available which allow insertion also of large transmembrane proteins. The latter requires for example a controlled way of forming a SLB on top of a a hydrophilic polymer cushion.

We present here an investigation of how to form SLB on substrates of interest in biosensor research from liposomes which to varying degrees of complexity mimic membranes of E. Coli bacteria. It is demonstrated how the concentration of $CaCl_2$ can be varied to tune the vesicle-surface and vesicle-vesicle interaction to achieve vesicle adsorption, rupture or other aggregated states at the biosensor interface. We also present results on the formation of poly(ethyleneglycol) (PEG) supported lipid bilayers on an easily synthesized polymer block co-polymer which can self assemble on any substrate with negative surface charge. The system is thus compatible with a large range of biosensing techniques and through the use of sequential adsorption and rupture steps not sensitive to e.g. roughness, charge or other properties of the underlying substrate.

2 MATERIALS

All lipids 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine (POPC), 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-[Phospho-rac-(1-glycerol)] (POPG), 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphoethanolamine (POPE), Phosphatidylcholine-NBD (NBD-PC), cholesterol (CH), sphingomyelin (SM), 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine (DOPC), 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine (DOPE), 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-phosphatidic acid (POPA) and E. Coli total lipid extract were purchased from Avanti Polar Lipids (USA). All samples were prepared in 10 mM HEPES and 150 mM NaCl buffer, and $CaCl_2$ added at described concentration for experiments on bacterial mimic liposomes. Unilamellar liposomes were prepared by bath sonication in a Branson ultrasonicator at 5 mg/ml of the desired lipid composition. Experiments were performed at a diluted concentration of 50 µg/ml. PLL(20kD)-g[3.5]-PEG(3.4kD)-N^+-$C_{12}H_{25}$ (PLL-g-PEG-biocide) was synthesized in house and provided by Dr. R. Konradi. PEG(8kD) was purchased from Sigma-Aldrich and used at 30% w/v. All sensor substrates were coated by magnetron sputtering with 12-50 nm thin layers of SiO_2, TiO_2 or Indium Tin Oxide (ITO).

3 METHODS

Quartz crystal microbalance with dissipation monitoring (QCM-D) experiments were conducted on a Q-Sense E4 instrument (Q-Sense, Sweden). Measurements were carried out at 50 µL/min buffer flow and 24 °C. After stabilization of the baseline in buffer liposome solution was added and the adsorption monitored for changes in resonant frequency Δf and energy dissipation ΔD as a function of time using overtones 3 to 13 (15 to 65 MHz). The mass of the adlayer (including trapped water) is roughly proportional to the change in Δf according to the Sauerbrey relation, $m = -k\Delta f$.

Fluorescence recovery after photobleaching (FRAP) was conducted with a confocal laser scanning microscope. 1-2% NBD-PC (by weight) was used as fluorescent lipids with all lipid mixtures. After vesicle adsorption on a cover glass in an open cell the vesicle solution was exchanged for pure buffer. A focused circular laser pulse was used to bleach a spot of the membrane. The fluidity of the membrane is measured by the rate of and percentage recovery of fluorescence intensity of the bleached spot. While a SLB shows near complete recovery a vesicle layer shows none.

Samples were injected using syringes into a liquid cell for Optical Waveguide Lightmode Spectroscopy (OWLS, MicroVacuum, Hungary) after a stable baseline was obtained. The adsorbed lipid mass at the interface is measured by probing changes in the refractive index within the evanescent field at the interface of the waveguide. By solving the mode equations and inserting into de Feijter's formula, $m = d_A(n_A-n_C)/(dn/dc)$, where m is the adsorbed mass, d_A is the layer thickness, n_A the refractive index of the adsorbed layer, n_C the refractive index of the medium and dn/dc the refractive index increment. The value of dn/dc is not well established for optically anisotropic lipid bilayers. Based on a survey of different values used in the literature $dn/dc = 0.1$ cm^3/g was assumed for lipid bilayers.

The three techniques employed in this work are instructively complementary for studying biomimetic adsorption processes. For an in depth discussion of the complementarity see Reimhult et al. [5].

4 RESULTS

4.1 Bacterial membrane mimics

QCM-D is a well established technique to study the process of vesicle adsorption and SLB formation due to its sensitivity to different structural conformations [5, 6]. While vesicle adsorption is monitored as a large decrease in Δf (high mass) and high ΔD (or $\Delta D/\Delta f$ ratio) SLB formation mostly shows a multi-phase behavior of initial vesicle adsorption (characterized as above), followed by vesicle rupture (decrease in mass from release of trapped water and decrease in ΔD from conversion of viscous liposomes) and a final stable SLB (Δf = 24-27 Hz and ΔD < 0.3e-6, depending on system). Partial SLB formation can also occur, which yields the same multi-phase kinetics as SLB formation, but the low Δf and ΔD values of a SLB are not reached. In this work a fourth process was also observed, which has never been fully characterized. The combined data of OWLS and QCM-D is consistent with aggregation of liposomes into a loose network on the surface, possibly after forming an underlying SLB. With the QCM-D this is monitored as a continuous increase in ΔD and mass at an increasing $\Delta D/\Delta f$ ratio and seem to occur with liposomes with high net charge in the presence of strongly associating counter-ions [7].

c($CaCl_2$)	SiO_2	TiO_2	ITO
0 mM	Vesicles	No ads.	Vesicles
0.2 mM	Vesicles	Vesicles	Vesicles
1 mM	SLB	Vesicles	Vesicles
2 mM	SLB	Vesicles	Vesicles
20 mM	Aggregate	SLB	Aggregate

Table 1: POPC:POPG (2:1 w/w) adsorption results.

With these classifications of vesicle adsorption outcomes based on QCM-D response SiO_2, TiO_2 and ITO substrates were screened for different experimental conditions in terms of $CaCl_2$ concentration and liposome composition. Further characterization was performed with FRAP and OWLS to verify that the adsorbed lipid mass and lateral fluidity agreed with the classification based on the QCM-D results.

The criteria for determining whether a SLB was formed from QCM-D measurements were a final Δf close to 26 Hz, ΔD close to 0e-6 and close to full recovery in FRAP measurements. Although fluorescence recovery was

observed for the classified SLBs, the diffusion coefficients were ~2e-9 cm^2/s regardless of probed substrate and condition, which is one order of magnitude lower than usually observed for PC SLB on glass. Tables 1 and 2 show the outcome of the screening for adsorption of POPC:POPG (2:1 w/w) and POPE:POPG (2:1 w/w) with the most optimal conditions for SLB formation in red text. The latter mixture mimics what is called E. Coli polar extract in composition.

c($CaCl_2$)	SiO_2	TiO_2	ITO
0 mM	Vesicles	SLB	Vesicles
0.2 mM	Vesicles	SLB	Aggregate
1 mM	SLB	SLB	Aggregate
2mM	Aggregate	SLB	Aggregate
20 mM	Very low and slow adsorption		

Table 2: POPE:POPG (2:1 w/w) adsorption results.

Most challenging was to produce a SLB from E. Coli total lipid extract liposomes. This lipid mixture faithfully reproduces the typical lipid composition of bacterial outer cell membranes. The results from the QCM-D measurements of vesicle rupture for 1 mM $CaCl_2$ on TiO_2 is shown in figure 1a. A comparison of this data set to the typical case for SLB formation described above seems to indicate that for E. Coli liposomes only an initial partial SLB formation (the local maximum and minimum in ΔD and Δf respectively) followed by possible aggregation is observed. Figure 1b and c show FRAP and OWLS data sets corresponding to the same experimental conditions. As can be observed our simple first analysis of the QCM-D data is contradictory to the other data sets. While QCM-D indicates continued *mass increase* and likely aggregation, the OWLS data shows typical kinetics of SLB formation (cf. [5]) and slow *mass loss* during the final phase. The FRAP recovery curve shows that the lipid layer on the surface has lateral fluidity allowing close to full recovery. The recovery is however much slower than usually observed and does not follow the expected profile for the used geometry and can thus not be accurately fitted to obtain a diffusion coefficient.

c($CaCl_2$)	SiO_2	TiO_2	ITO
2 mM EDTA	part. SLB	part. SLB	Vesicles
0 mM	part. SLB	part. SLB	Vesicles
0.2 mM	part. SLB	SLB	Vesicles
1 mM	part. SLB	SLB	Vesicles
2 mM	part. SLB	SLB	Vesicles
20 mM	Very low and slow adsorption		

Table 3: E. Coli total lipid extract adsorption results.

The results were similar for 0.2-2 mM $CaCl_2$ concentrations on TiO_2. FRAP measurements are indisputable evidence of SLBs on the surface since SLBs are the only laterally connected lipid structure and this was further verified by adding serum after adsorption and rinsing. The E. Coli SLB surface showed no adsorption of serum protein, while the bare TiO_2 sensor surface does. Thus, these were judged as SLB for compilation of the results in Table 3, despite the indications of the QCM-D data. An explanation consistent with the complementary data sets is that E. Coli SLB slightly detaches over time and likely forms undulations on the surface. This could be a consequence of the complex mixture of lipids allowing lateral phase segregation of lipids with different preferred local curvature.

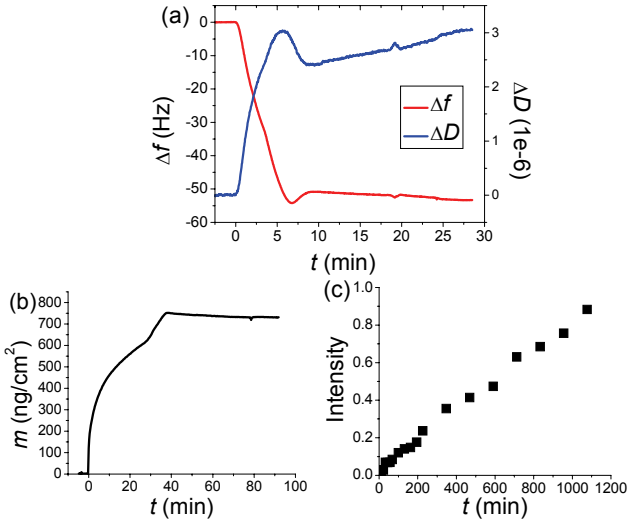

Figure 1: (a) QCM-D adsorption curve for E. Coli liposomes on TiO_2 at 1 mM $CaCl_2$. (b) OWLS adsorption curve. (c) FRAP recovery data.

4.2 PLL-g-PEG-supported lipid bilayers

TiO_2-coated QCM-D crystals, OWLS waveguides and glass slides for FRAP were exposed to PLL-g-PEG-biocide (100% functionalized with biocide) until fully covered. The PLL-g-PEG-biocide functionalized surfaces were then exposed to vesicle solutions of different lipid compositions with net negative charge. The results of the vesicle adsorption are shown in table 4. Although there are variations in liposome size between different lipid mixtures it is clear that all lipid compositions show similar uptake of highly deformed liposomes (low $\Delta D/\Delta f$) except DOPC:DOPE:SM:CH (35:30:15:20 mol/mol), which is a lipid mixture optimized for liposomes fusion [8].
Table 5 shows how the ratio of dissipation to frequency changes with percentage functionalization of biocide for one of the lipid mixtures with larger liposomes. This ratio is a measure of softness and hence deformation of the adsorbed lipid structure. It is clearly demonstrated how a higher concentration of biocide at the interface increases the deformation, while the mass uptake (Δf) increases much less. The same trend was shown for other lipid mixtures as well and it was also shown that a net negative charge of the lipid membrane did not strongly affect the total uptake of liposomes. It was thus concluded that it is the total density of hydrophobic moieties at the interface and not the density

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	POPC:POPA (98:2)	POPC:POPA (70:30)	POPC:POPS (80:20)	POPC:POPG (80:20)	Egg-PC: DOPE (65:35)	DOPC:DOPE:SM: CH (35:30:15:20)
Δf (Hz)	72	85	82	70	105	163
ΔD (1e-6)	2.0	4.8	4.8	2.6	4.9	18
$\Delta D/\Delta f$ (1/GHz)	28	56	59	37	47	110

Table 4: QCM-D results of vesicle adsorption on PLL-g-PEG-biocide(100%).

of positively charged quartenary ammonium that decides the strength of the liposome-biocide interaction. In no case, even at very low $\Delta D/\Delta f$ ratios, was spontaneous formation of a SLB on top of the PLL-g-PEG demonstrated by FRAP control measurements.

An additional step was introduced to force liposome rupture. In bulk solutions addition of PEG of low molecular weight is known to cause fusion of vesicles [8]. PEG(8kD) was added at concentrations up to 30% w/v. Addition of PEG(8kD) yielded no rupture for most of the different lipid compositions despite causing liposome fusion in bulk. The only effect was a large increase in dissipation in the QCM-D measurements. A lipid composition of DOPC:DOPE:SM:CH (35:30:15:20 mol/mol) demonstrating a high and not very deformed uptake before the addition of PEG(8kD), however showed a different behavior (figure 2). A pronounced mass decrease (increase in Δf) and a first decrease in dissipation followed by rapid increase to the original level is measured. This indicated mass loss through liposome rupture. Formation of a highly fluid SLB (D ~1e-7) was also demonstrated by FRAP, while the other lipid compositions showed no recovery and thus no connected lipid bilayers. Additional OWLS measurements also showed a dramatic lipid mass loss after addition of PEG(8kD), although the total mass is still higher than usually expected for a single SLB.

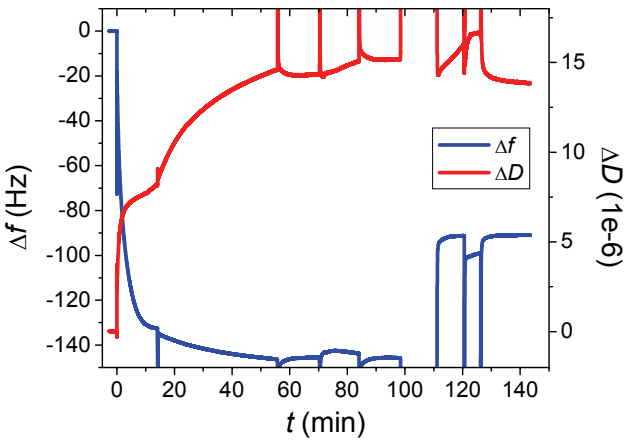

Figure 2: QCM-D measurements of DOPC:DOPE:SM:CH (35:30:15:20 mol/mol) liposome adsorption and PEG-induced rupture at t = 100 min.

5 CONCLUSIONS

We have shown that a variety of supported lipid bacterial membrane mimics can be formed on several relevant biosensor substrates by tuning the $CaCl_2$ concentration for the lipid composition and surface charge. In particular, it was shown that SLB formed from E. Coli total lipid extract displays a novel behavior, which can be explained by partial detachment from the substrate, e.g., in the form of undulations.

Furthermore, we presented preliminary measurements on a polymer supported lipid membrane platform, which can be easily self assembled on e.g. any metal oxide surface. High degree of control can be achieved over the process by performing the self assembly in three steps: polymer scaffold adsorption, vesicle adsorption and vesicle rupture through osmotic stress. Liposome deformation can be controlled by density of biocide functional groups, but SLB formation could only be achieved for one fusogenic lipid composition, which did not correlate with the lipid composition showing the most deformed vesicles.

	10% biocide	50% biocide	100% biocide
Δf (Hz)	90	190	163
ΔD (1e-6)	13.3	20.0	10.7
$\Delta D/\Delta f$ (1/GHz)	148	105	66

Table 5: QCM-D response for POPC:POPA (98:2 w/w) vesicle adsorption on PLL-g-PEG-biocide.

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