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Biotinylated lipid bilayer disks as model membranes for biosensor analyses

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ABSTRACT

The aim of this study was to investigate the potential of polyethylene glycol (PEG)-stabilized lipid bilayer disks as model membranes for surface plasmon resonance (SPR)-based biosensor analyses. Nanosized bilayer disks that included 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[biotinyl(polyethylene glycol)$_{2000}$] (DSPE–PEG$_{2000}$–biotin) were prepared and structurally characterized by cryo-transmission electron microscopy (cryo-TEM) imaging. The biotinylated disks were immobilized via streptavidin to three different types of sensor chips (CM3, CM4, and CM5) varying in their degree of carboxymethylation and thickness of the dextran matrix. The bilayer disks were found to interact with and bind stably to the streptavidin-coated sensor surfaces. As a first step toward the use of these bilayer disks as model membranes in SPR-based studies of membrane proteins, initial investigations were carried out with cyclooxygenases 1 and 2 (COX 1 and COX 2). Bilayer disks were preincubated with the respective protein and thereafter allowed to interact with the sensor surface. The signal resulting from the interaction was, in both cases, significantly enhanced as compared with the signal obtained when disks alone were injected over the surface. The results of the study suggest that bilayer disks constitute a new and promising type of model membranes for SPR-based biosensor studies.

1. Introduction

Biosensors using surface plasmon resonance (SPR)[1] detection have emerged as an important tool for biomolecular interaction analysis. SPR is a label-free detection method that measures changes in refractivity at a gold surface to which one of the interactants has been immobilized. The change in refractivity is registered as a change in resonance units (RU). The gold surface is typically functionalized with a layer of carboxylated dextran providing a hydrophilic and flexible matrix for immobilization of many types of molecules. Measurements are performed in real time in a continuous flow of buffer into which the analyte is injected. Analysis of analyte association with, and/or dissociation from, the surface can provide detailed kinetic information in a variety of different conditions [1–3]. In the current study, we explored the use of SPR biosensors in combination with a novel type of model membrane for the purpose of interaction analysis with membrane proteins.

Despite the understanding that membrane proteins are abundant in nature and that they serve a variety of important physiological functions, the details of their characteristics and functions are fully understood for only a few examples. This is due largely to practical problems associated with their isolation and characterization in a native environment. Because many membrane proteins are considered to be important drug targets, the lack of suitable methods for membrane protein studies also restricts drug discovery. The architecture and detection principle of SPR biosensors provide a unique platform for the immobilization of lipid membranes to which proteins can attach or be incorporated and interactions can be detected. Liposomes, continuous lipid bilayers, and monolayers have previously been used as model membranes in biosensor analyses [4–7]. However, difficulties in achieving well-defined stable surfaces that allow immobilization of sufficient concentrations of proteins limit the use of these types of surfaces. Therefore, we have explored the possibility of exploiting polyethylene glycol (PEG)-stabilized lipid bilayer disks for interaction studies of membrane proteins by use of SPR-based biosensors.

The bilayer disks used in the current study have a planar and circular shape (see Fig. 1 for a schematic illustration of the disk

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[1] *Abbreviations used:* SPR, surface plasmon resonance; RU, resonance units; PEG, polyethylene glycol; COX 1 and COX 2, cyclooxygenases 1 and 2; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DSPE–PEG$_{2000}$, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)$_{2000}$]; DSPE–PEG$_{2000}$–biotin, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[biotinyl(polyethylene glycol)$_{2000}$]; ceramide–PEG$_{2000}$, *N*-palmitoyl-sphingosine-1-[succinyl(methoxy(polyethylene glycol)$_{2000}$]; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PE, L-α-phosphatidylethanolamine; EDC, *N*-ethyl-*N*′-[(dimethylamino)propyl]carbodiimide hydrochloride; NHS, *N*-hydroxysuccinimide; DDC, dicyclohexylcarbodiimide; ^{3}H[CHE], ^{3}H-labeled cholesterylhexadecyl; OG, octyl-β-D-glucopyranoside; EDTA, ethylenediaminetetraacetic acid; MWCO, molecular weight cutoff; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; cryo-TEM, cryo-transmission electron microscopy; HPA, hydrophobic association.

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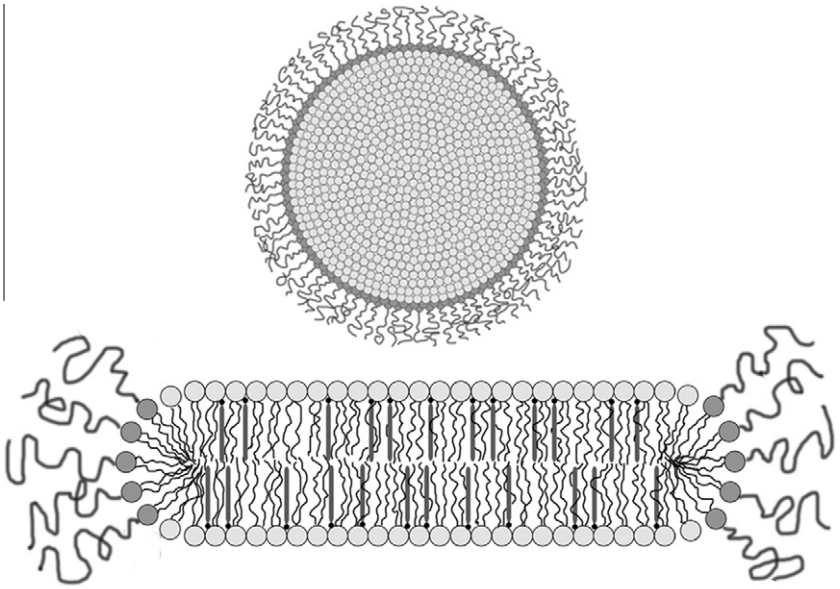

Fig. 1. Schematic pictures illustrating the structure of the lipid bilayer disks as viewed face-on (top) and in cross section (bottom). PEG–lipids, shown in dark gray, are situated preferentially at the disk rim, whereas phospholipids and cholesterol form the bulk of the disk.

structure). The disks are protected from fusion and self-closure by PEG–lipids situated preferentially at the curved disk rim [8–10]. The structure of the disks is advantageous because all of the lipids are directly accessible for interaction with potential analytes. This can be compared with, for example, the situation when using unilamellar liposomes, in which case approximately half of the lipid headgroups are directed toward the inner volume of the liposomes [9,11,12]. The long-term stability of the disks is good ($\geqslant$5 months at 8 °C), and the size of the disks can be varied over a comparably wide range [11,13]. The bilayer disks can be produced via several preparation paths, and the use of methods based on detergent depletion allows the incorporation of transmembrane proteins into the disks [11]. Moreover, the lipid composition of the disks can be chosen so as to mimic that of various biological membranes [11,12]. Previous studies have proven bilayer disks to be suitable for the incorporation of membrane proteins and to function well as model membranes in interaction studies [11,12,14].

In this initial study on the use of bilayer disks for SPR biosensor studies, we explored methods for immobilization of the disks and investigated their use as model membranes in an interaction study with cyclooxygenases 1 and 2 (COX 1 and COX 2). COX 1 and COX 2 are naturally situated peripherally in the bilayer [15], and in the current study the enzymes were allowed to interact with biotinylated bilayer disks before they were immobilized on the biosensor chips via streptavidin.

Materials and methods

Materials

1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC, >99%), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)$_{2000}$] (DSPE–PEG$_{2000}$, >99%), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[biotinyl(polyethylene glycol)$_{2000}$] (DSPE–PEG$_{2000}$–biotin), *N*-palmitoyl-sphingosine-1-[succinyl(methoxy(polyethylene glycol)$_{2000}$] (ceramide–PEG$_{2000}$), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and L-α-phosphatidylethanolamine (PE) from soybean were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Sephadex G-50 Medium was purchased from GE Healthcare (Uppsala, Sweden). *N*-Ethyl-*N*′-[(dimethylamino)propyl]carbodiimide hydrochloride (EDC), ethanolamine (1 M, pH 8.5), and *N*-hydroxysuccinimide (NHS) came from Biacore (now GE Healthcare), and streptavidin came from Calbiochem/Merck (Darmstadt, Germany). Hematin was obtained from ICN Biochemicals (Aurora, OH, USA). Ovine COX 1 from ram seminal vesicles and COX 2 from sheep placenta were supplied in 80 mM Tris–HCl (pH 8.0) with 0.1% Tween 20 and 300 μM dicyclohexylcarbodiimide (DDC) by Larodan Fine Chemicals (Malmö, Sweden). ^{3}H-labeled cholesterylhexadecyl (^{3}H[CHE]) was purchased from PerkinElmer (Waltham, MA, USA). Cholesterol (>98%), octyl-β-D-glucopyranoside (OG), and glutathione came from Sigma–Aldrich (St. Louis, MO, USA), and indomethacin came from AstraZeneca (Södertälje, Sweden). A standard buffer (buffer A) containing 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM Tris–HCl (pH 7.4) was used throughout the study apart from during streptavidin immobilization and during the study with COX 1 and COX 2. Streptavidin was dissolved in acetate buffer (10 mM, pH 5.0) and immobilized in 0.01 M Hepes and 0.15 M NaCl (pH 7.4). Studies with COX 1 and COX 2 were performed in 80 mM Tris buffer (pH 8.0) that included adrenalin (1.3 mg/ml), glutathione (0.3 mg/ml), and hematin (0.25 mg/ml) as cofactors. Chemicals not listed were of analytical grade or as stated in the given references.

Preparation of disks and liposomes

Bilayer disks were prepared by hydration or detergent depletion. In both methods, the desired lipids were first codissolved in chloroform. The chloroform was then evaporated under N_2 (g) to enable formation of a lipid film. The remaining chloroform was removed under vacuum for at least 12 h.

In the hydration method, the lipid film was dispersed in buffer to a final lipid concentration of 10 mM at 50–55 °C. The lipid suspension was subsequently freeze–thawed five times using $N_2(l)$ and a water bath (50–55 °C). Disk preparations containing ceramide–PEG$_{2000}$ were thereafter tip sonicated, while cooled in an ice bath, for 45 min using a Soniprep 150 sonicator (MSE Scientific Instruments, Crawley, UK). The sonicated samples were centrifuged at low speed (380*g*, 1 min) to remove metal particles originating from the sonicator probe.

Disks were also produced by detergent depletion as described previously [11]. In short, the lipid film was dissolved in buffer A, supplemented with OG, at 50–55 °C using an OG/lipid molar ratio

of 11:1. The solution was put in a 50–55 °C water bath for 30 min and intermittently vortexed, followed by stirring for 90 min at room temperature. OG was separated from the lipids by gel filtration on a Sephadex G-50 Medium column at 0.7 ml/min using buffer A as running buffer. The lipid fractions were concentrated to 10 mM on a Minicon B-15 membrane concentrator (Millipore, Bedford, MA, USA). Finally, the samples were dialyzed in a Spectra/Por dialysis membrane 7 (molecular weight cutoff [MWCO] = 2000) from Spectrum (Breda, Netherlands) against 500 ml of buffer A for 36 h at 8 °C with two buffer changes.

Liposomes were prepared by the hydration method as described above and subsequently extruded 31 times through a polycarbonate filter (100 nm pore size) using a LiposoFast membrane extruder from Avestin (Mannheim, Germany).

Cryo-transmission electron microscopy

A small drop of the sample was placed on an electron microscopy copper grid covered by a holey polymer film. Excess solution was thereafter removed by blotting with filter paper. The thin (10–500 nm) sample films were prepared under controlled temperature (25 °C) and humidity conditions within a custom-built environmental chamber. The specimen was vitrified by plunging the grid into liquid ethane held just above its freezing point. The grid was then transferred to a Zeiss EM 902 A transmission electron microscope (Oberkochen, Germany) for analysis. To prevent sample perturbation and the formation of ice crystals, the specimens were kept cold (below –165 °C) during both the transfer and viewing procedures. All observations were made in zero-loss bright-field mode and at an accelerating voltage of 80 kV. The technique has been described in detail elsewhere [16].

Biosensor analysis

Immobilization of disks and liposomes on sensor chips

A Biacore 2000 instrument (GE Healthcare) was used for the biosensor analyses, which were performed at 25 °C. Four types of sensor chips obtained from GE Healthcare were used; the CM series (CM3, CM4, and CM5) sensor chips, with varying degrees of carboxymethylation and dextran thickness, and the L1 chip, with lipophilic groups covalently bound to the dextran matrix.

With the L1 chip, lipophilic groups attached to the dextran matrix insert into the lipid bilayer. Therefore, the disks do not need to be functionalized (e.g., with biotinylated PEG–lipid) for immobilization on these chips. Before use, the surface of the L1 chip was rinsed with two 1-min pulses of 40 mM OG solution (OG was dissolved in water) at 25 μl/min. Immobilizations on the L1 chip were performed in triplicates

Biotinylated bilayer disks were immobilized to the CM series sensor chips functionalized with streptavidin. The streptavidin was covalently attached by amine coupling by first injecting EDC and NHS to activate the carboxyl groups on the dextran matrix. Streptavidin (60–300 μg/ml) was dissolved in 10 mM acetate buffer (pH 5.0) and then injected over the chip using Hepes buffer and a flow rate of 5 μl/min. Ethanolamine (1 M, pH 8.5) was thereafter injected to inactivate the remaining reactive NHS esters [3]. Lipid samples (1 mM), including 0.05, 1, or 4 mol% DSPE–PEG_{2000}–biotin, were injected at 5 μl/min over the streptavidin-covered surfaces for up to 20 min.

Quantification of immobilized lipid on CM5 chips

Disks composed of DSPC/cholesterol/DSPE–PEG_{2000}/DSPE–PEG_{2000}–biotin (35:40:21:4) and containing ^{3}H[CHE] (0.09 nmol ^{3}H[CHE] added to 0.32 μmol lipids) were prepared by hydration (see above). The disks were immobilized in all four flow cells of two CM5 chips. The sensor surfaces were then washed with buffer. Before elution of the immobilized disks, OG solution (40 mM) was used to clean the recovery cup and all parts of the flow system in the instrument (Biacore 2000) that had previously been in contact with the disk sample (e.g., tubing, needle). The OG solution was thereafter injected over the flow cells to solubilize and elute the immobilized lipids. The eluted lipids were collected and analyzed using a 1214 RackBeta Excel scintillation counter (PerkinElmer, Wellesley, MA, USA).

Incorporation of COX 1 and COX 2 in bilayer disks

COX 1 and COX 2 were preincubated (30 min, 37 °C) with bilayer disks (POPC/PE/cholesterol/ceramide–PEG_{2000}/DSPE–PEG_{2000}–biotin, 30:28:17:21:4 mol%) at protein (homodimer) to lipid ratios of 1:2000, 1:1000, and 1:500 (protein concentrations 3, 5, and 6 μM) in Tris buffer with cofactors (adrenalin, glutathione, and hematin). An incubation procedure (30 min, 37 °C) was previously used by MirAfzali and coworkers to incorporate COX into liposomes (DOPC/DOPS/oleic acid) [17]. The incubated samples were diluted to obtain a lipid concentration of 1 mM and thereafter immediately injected over the streptavidin-covered CM5 surface. The immobilization step was optimized to 10 min, and the flow was kept at 5 μl/min.

Results and discussion

Preparation and characterization of bilayer disks

Bilayer disks were prepared by two different methods: hydration and detergent depletion (see Materials and Methods for details). The former method has the advantage of being fast and comparably simple and, hence, was used for the preparation of disks to be employed in the development and initial evaluation of the immobilization protocols. However, incorporation of transmembrane proteins requires a modified protocol such as that based on detergent depletion described by Johansson and coworkers [11]. To prepare for future studies focused on this important class of proteins, it was essential to include disks prepared by detergent depletion in the current study.

Fig. 2A shows the aggregate structure as visualized by cryo-transmission electron microscopy (cryo-TEM) for samples containing disks composed of DSPC, cholesterol, and DSPE–PEG_{2000} (35:40:25) prepared by the hydration method. Inclusion of 0.05, 1, or 4 mol% DSPE–PEG_{2000}–biotin did not change the aggregate size or structure; both samples with and without DSPE–PEG_{2000}–biotin had an average diameter of approximately 30 nm and an appearance corresponding to that shown in Fig. 2A. When viewing the cryo-TEM micrographs, it is important to realize that the contrast of PEG is too low for the polymer to be visualized. Thus, an extra 7 nm [18] has been added to the aggregate diameter to account for the PEG layer.

Cryo-TEM was used also for the characterization of disks composed of DSPC, cholesterol, and DSPE–PEG prepared by the detergent depletion method. In this case, the preparations contained 1 mol% DSPE–PEG_{2000}–biotin. The TEM images revealed disks that, with the PEG layer included, had an average diameter of approximately 20 nm (results not shown) and, thus, were significantly smaller than those produced by the hydration method. The detergent depletion method has previously been reported to give smaller disks than the hydration method [11], and a discussion on the influence of preparation path on size and structure in lipid/PEG–lipid systems can be found in a publication by Sandström and coworkers [13].

DSPE–PEG_{2000} carries one negative charge; therefore, electrostatic interactions between DSPE–PEG_{2000} and the negatively charged dextran matrix could potentially reduce the effectiveness of immobilization. Furthermore, the negative charge of DSPE–

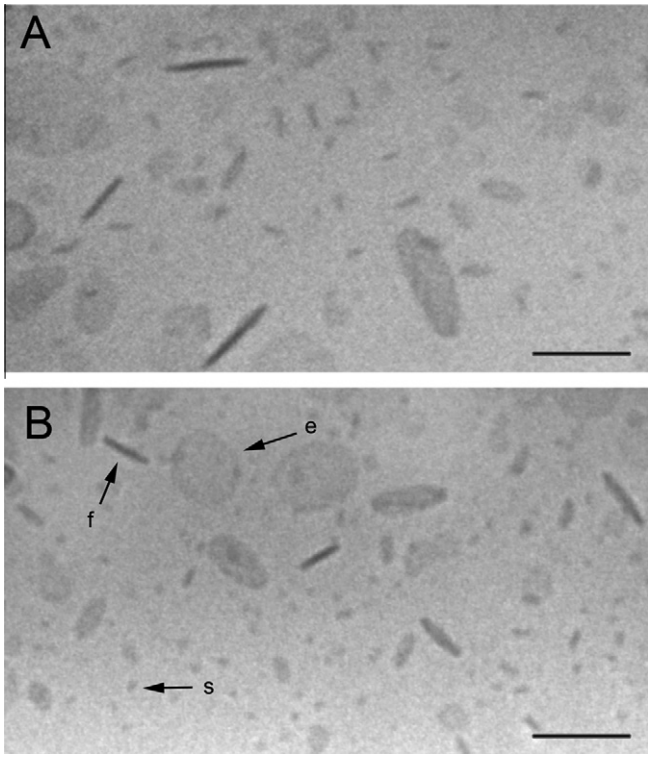

Fig. 2. Cryo-TEM images of disks stabilized by DSPE–PEG_{2000} (DSPC/cholesterol/DSPE–PEG_{2000}/DSPE–PEG_{2000}–biotin, 35:40:21:4 mol%) (A) and ceramide–PEG_{2000} (DSPC/cholesterol/ceramide–PEG_{2000}/DSPE–PEG_{2000}–biotin, 35:40:24:1 mol%) (B). Both samples were produced by the hydration method. To aid interpretation of the pictures, arrows marked "f" and "e" point out disks as viewed face-on and edge-on, respectively. The arrow marked "s" points to a small, but still likely disk-shaped, lipid particle. Lipid concentration = 10 mM. Bar = 100 nm.

PEG_{2000} could cause interference in interaction studies focused on charged analytes. Therefore, disk preparations in which DSPE–PEG_{2000} was replaced by the uncharged ceramide–PEG_{2000} were produced by the hydration method. To enable immobilization, 1 mol% DSPE–PEG_{2000}–biotin was included. Sonication of the ceramide–PEG_{2000}-containing preparations was necessary to obtain homogeneous samples dominated by bilayer disks, in accordance with previously published observations [11]. A typical example of a hydrated and thereafter sonicated sample can be seen in Fig. 2B. The micrograph suggest a somewhat higher fraction of small particles (~10 nm), but the sample structure is otherwise similar to that shown in Fig. 2A.

Bilayer disks were successfully produced also from a more physiologically relevant lipid mixture containing POPC, PE, and cholesterol to which ceramide–PEG_{2000} and DSPE–PEG_{2000}–biotin were added (POPC/PE/cholesterol/ceramide–PEG_{2000}/DSPE–PEG_{2000}–biotin, 30:28:17:21:4 mol%). The composition of the disks, which were prepared by the hydration method, was selected to optimize the conditions for studies including COX 1 and COX 2. A cryo-TEM micrograph showing the appearance of the disks after the addition of COX 2 to give a protein (homodimer)/lipid ratio of 1:500 is displayed in Fig. 3. Similar to the situation before protein addition (results not shown), the sample contains discoidal structures that appear to be well separated from each other. Thus, incubation with COX did not lead to aggregation or structural changes of the disks.

Immobilization of bilayer disks on the sensor chips

Biotinylated bilayer disks were immobilized to CM5 sensor chips via a two-step procedure that first involved the covalent immobilization of streptavidin to the dextran surface and then non-covalent attachment of disks via the biotin group. SPR sensorgrams shown in Fig. 4 verify that disks stabilized by DSPE–PEG_{2000}, as well as by ceramide–PEG_{2000}, were stably attached to the sensor surface by means of this method. The signal obtained on immobilization of disks was found to be clearly dependent on the amount of immobilized streptavidin, as illustrated in Fig. 5. The maximum signal after immobilization of streptavidin with the current procedure was 9050 ± 396 RU. On injection of the disks (DSPC/cholesterol/DSPE–PEG_{2000}/DSPE–PEG_{2000}–biotin, 35:40:21:4 mol%), the signal increased by 3210 ± 408 RU. The biotinylated lipid was essential for immobilization because disks without this functional group did not interact with the sensor surfaces (see bottom of Fig. 4).

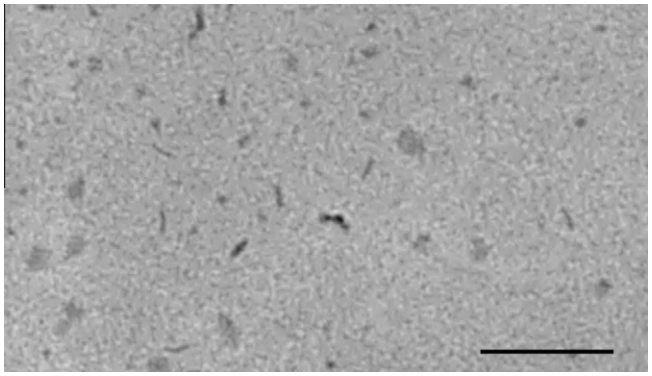

Fig. 3. Cryo-TEM image of disks (POPC/PE/cholesterol/ceramide–PEG_{2000}/DSPE–PEG_{2000}–biotin, 30:28:17:21:4 mol%) incubated with COX 2 at a protein/lipid molar ratio of 1:500 and diluted to a lipid concentration of 1 mM. Bar = 100 nm.

To correlate the signal (in RU) with the actual amount of immobilized lipid, disks containing trace amounts of ^{3}H[CHE] were immobilized on two CM5 chips. The disks were eluted by injecting OG solution over the flow cells. The amount of ^{3}H[CHE] in the collected eluate was determined by scintillation counting, and the total amount of lipid was determined from the initial molar ratios between ^{3}H-labeled and unlabeled lipids. The sums of the signals obtained when immobilizing disks on four flow cells on two different CM5 chips were 8645 and 8760 RU. As assessed from the scintillation count, the total amounts of lipid immobilized on the two chips (4 flow cells/chip) were 31.2 and 31.4 pmol. Correlating the SPR signal with the eluted lipid amount suggested that 1 RU = 3.0 fmol

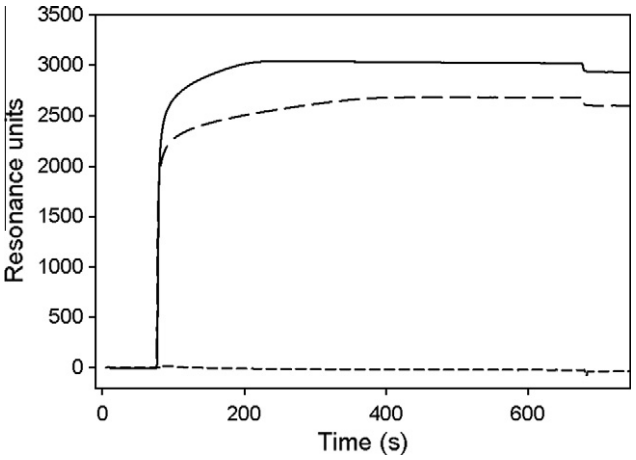

Fig. 4. Sensorgrams showing disk immobilization on streptavidin-treated CM5 sensor chips: DSPC/cholesterol/DSPE–PEG_{2000}/DSPE–PEG_{2000}–biotin (35:40:24:1 mol%) disks (– – –) and DSPC/cholesterol/ceramide–PEG_{2000}/DSPE–PEG_{2000}–biotin (35:40:24:1 mol%) disks (—). Also shown is immobilization of disks (DSPC/cholesterol/DSPE–PEG_{2000} (41:39:20 mol%) in the absence of biotinylated lipid (- - -).

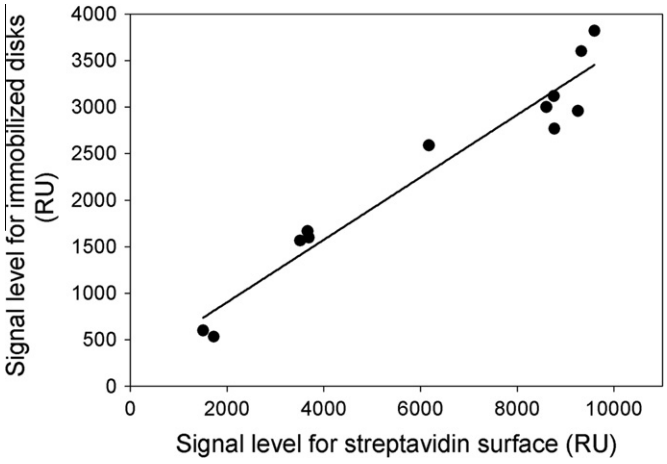

Fig. 5. Level of disk (DSPC/cholesterol/DSPE–PEG_{2000}/DSPE–PEG_{2000}–biotin, 35:40:21:4 mol%) immobilization plotted versus level of immobilized streptavidin.

lipid/mm^2 or 3.4 pg lipid/mm^2 (accounting for the apparent molar weight of the lipid mixture of 1143 g/mol). It is worth noting that this value is approximately three times higher than the 1 pg/mm^2 determined by Stenberg and coworkers [19] for proteins and also is significantly different from the 0.92 pg/mm^2 estimated by Cooper and coworkers [5] for lipid monolayers deposited on hydrophobic association (HPA) chips. Several factors likely contribute to the reduced response levels obtained with the disks. Among these is the comparably high refractive index of lipid/cholesterol mixtures [20]. The signal is, furthermore, expected to decrease when the adsorbed material, as in the case with disks immobilized to the currently used CM5 sensor chips, is located a fair distance away from the sensor surface. Still, further investigations are clearly needed to fully elucidate the reasons for the comparably low signal levels.

Based on the assumption that 1 RU = 3.0 fmol lipid/mm^2, a signal of 3210 RU (see above) corresponds to a lipid amount of 9.6 pmol/mm^2 or 11.6 pmol/flow cell given that the flow cell area is approximately 1.2 mm^2. Taking into account that the bilayer-forming lipids (DSPC/cholesterol) constitute 75 mol% of the total lipid and that the area per lipid headgroup is approximately 0.38 nm^2 [21], this amount equals a total lipid bilayer area of approximately 1 mm^2/flow cell. Importantly, the structure of the disk ensures that the complete bilayer area (including both monolayer leaflets of the bilayer) is readily accessible for interaction. It is noteworthy that the 1 mm^2 lipid area obtained in our experiments is comparable to the lipid area that at maximum could be obtained on covering the entire chip surface with a continuous lipid bilayer.

Effect of DSPE–PEG_{2000}–biotin content on sensor stability

Sensor surface stability is a critical factor for interaction analysis using time-resolved biosensor experiments. Therefore, it was of interest to determine the minimal amount of biotin-conjugated lipid required in the disks for production of stable surfaces. Hence, bilayer disks with a total amount of 25 mol% PEGylated DSPE, but with varying amounts (0.05, 1, and 4 mol%) of DSPE–PEG_{2000}–biotin, were immobilized on CM5 chips and the SPR signal was followed over time. Disks containing 1 and 4 mol% of the functionalized PEG–lipid formed surfaces that remained stable over time. On the other hand, for disks including only 0.05 mol% DSPE–PEG_{2000}–biotin, the signal was found to drop by 20% after 30 min and by as much as 85% after 12 h. The reason why the disks were washed off the surface was probably not due to the breakage of the very strong biotin–avidin bond [22] but more likely due to biotinylated PEG–lipids being pulled from the bilayer.

Stable and reproducible surfaces were obtained also when DSPE–PEG_{2000} was exchanged for the uncharged ceramide–PEG_{2000} and the disks (DSPC/cholesterol/ceramide–PEG_{2000}/DSPE–PEG_{2000}–biotin, 40:35:24:1 mol%) were immobilized onto the CM5 chip. A signal of 2971 ± 24 RU was obtained on surfaces with 10,385 ± 22 RU of streptavidin. Thus, the level of immobilization was similar to that observed for disks stabilized with DSPE–PEG_{2000} (Fig. 4), showing that the negative charge present on DSPE–PEG_{2000} does not significantly hamper the immobilization.

Disks prepared by detergent depletion

As discussed previously, the possibility to produce functionalized bilayer disks by detergent depletion allows their use as model membranes in biosensor analyses on transmembrane proteins. Therefore, in addition to the disks prepared by simple hydration, bilayer disks (DSPC/cholesterol/DSPE–PEG/DSPE–PEG_{2000}–biotin, 35:40:24:1 mol%) produced with the detergent depletion method were immobilized on CM5 chips. At low streptavidin coverage (1208 RU), the immobilized disks gave a response of 1237 RU. This corresponds to a considerably higher lipid/streptavidin signal ratio than that obtained for disks prepared by simple hydration (Table 1). On immobilization of more streptavidin (9875 RU), the response increased to 3597 RU. Thus, the lipid/streptavidin ratio was 0.36, which is similar to the ratio obtained for disks produced by hydration (Table 1). The comparatively high response found at low streptavidin levels for the small disks prepared by detergent depletion suggests that steric hindrance might affect the immobilization of the larger disks and/or that the signal is affected by the positioning of the bilayer disks in the dextran matrix (see discussion below). The lower lipid/streptavidin signal ratio obtained on increasing the amount of immobilized streptavidin could possibly also be explained by such effects.

Immobilization of disks on different sensor chips from the CM series

To investigate how the characteristics of the sensor chips affect the immobilization, disks containing DSPC/cholesterol/DSPE–PEG/

Table 1
Response levels obtained on immobilization of bilayer disks (DSPC/cholesterol/ DSPE–PEG_{2000}/DSPE–PEG_{2000}–biotin, 35:40:21:4 mol%) onto sensor chips from the CM series.

Sensor chip	Streptavidin immobilization signal (RU)	Disk immobilization signal (RU)	Disk/streptavidin immobilization ratio
CM3	2318	1646	0.71
	4160[a]	2970	0.71
CM4	1966	1055	0.54
	4003[a]	2270	0.57
CM5	–	–	0.38[b]
	1208[c]	1237[c]	1.02[c]
	9875[c]	3597[c]	0.36[c]

[a] Corresponds to maximum streptavidin immobilization level.
[b] Averaged immobilization ratio obtained from results in Fig. 4.
[c] Disks produced by detergent depletion (DSPE–EG_{2000}–iotin/DSPE-PEG_{2000}/DSPC/cholesterol, 1:24:35:40 mol%)

DSPE–PEG_{2000}–biotin (35:40:21:4 mol%) were immobilized on CM3, CM4, and CM5 chips. The CM4 chip has a lower level of carboxylation, and the CM3 chip has a thinner dextran layer (shorter chains), than the CM5 chip. When employing the CM3 and CM4 ships for immobilization, the shapes of the sensorgrams (data not shown) were very similar to those obtained when using the CM5 chip (Fig. 4). However, the disk/streptavidin ratio obtained for the three chips decreased in the following order: CM3 (0.71) > CM4 (0.57) > CM5 (0.38) (see Table 1). For each chip type, the disk/streptavidin signal ratio remained more or less constant irrespective of the amount of immobilized streptavidin (Table 1).

Assuming that the signal is unaffected by the positioning of the bilayer disks on the sensor surface, it appears that the lower level of carboxylation on the CM4 chip compared with the CM5 chip increases the binding ratio. However, as expected from the lower maximal streptavidin level, the maximum signal obtained during disk immobilization was still lower for the CM4 chip than for the CM5 chip (2270 and 3210 RU). On the other hand, the maximum responses were quite similar for the CM3 chip (2970 RU) and the CM5 chip (3210 ± 408) at high streptavidin levels. Hence, the longer dextran chains of the CM5 chip, compared with those of the CM3 chip, did not increase the apparent binding capacity, indicating that the length of the dextran chain was irrelevant. It should be noted, in this context, that a straightforward comparison of the signals obtained on the different sensor chips can be problematic because it cannot be excluded that the recorded signals decrease with the distance from the surface and, therefore, that the signals are affected by the positioning and density distribution of the disks in the matrix.

Immobilizations of disks by means of lipophilic anchoring

The possibility of using simple lipophilic anchoring (instead of streptavidin coupling) for immobilization of the bilayer disks was investigated by use of the L1 sensor chip. When disks (DSPC/cholesterol/DSPE–PEG, 35:40:25 mol%) were injected over the L1 chip, the responses obtained were only on the order of a few hundred RU. Moreover, even after prolonged injection times, no maximum in the immobilization level could be reached (see inset of Fig. 6). To find a plausible explanation for the poor immobilization, we carried out a complementary study in which the disks were replaced by liposomes.

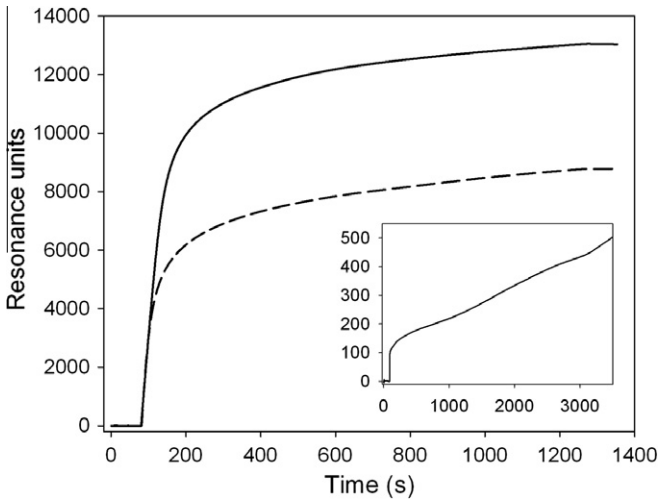

Fig. 6. Sensorgrams showing immobilization of PEGylated liposomes (DSPC/cholesterol/DSPE–PEG_{2000} (56:40:4 mol%) (– – –) and non-PEGylated liposomes (DSPC/cholesterol, 60:40 mol%) (—) to the L1 chip. The inset shows a typical sensorgram obtained on immobilization of bilayer disks (DSPC/cholesterol/DSPE–PEG_{2000}, 35:40:25 mol%).

We began our investigation by immobilizing liposomes with and without PEG–lipid onto the L1 chip (typical sensorgrams are shown in Fig. 6). DSPC/cholesterol (60:40 mol%) liposomes reached a response level of 12,708 ± 286 RU, whereas the corresponding level was 8562 ± 360 RU for DSPC/cholesterol/DSPE–PEG (56:40:4 mol%) liposomes. The immobilization levels obtained for the non-PEGylated liposomes correspond well to previously published data concerning immobilization of POPC and DOPC liposomes on the L1 chip [23]. The lower response for the PEG–lipid-containing liposomes was not surprising because the PEG extending from the surface of these liposomes sterically prevents them from packing closely and may also limit the interaction with the lipophilic anchors. In addition to reduced response levels, liposomes containing 4 mol% PEG–lipid showed signs of poor stability on the surfaces. To investigate this further, the two systems containing immobilized liposomes with and without PEG–lipid were left on standby flow (5 μl/min) overnight. By this treatment, the recorded response levels dropped to 3554 and 10801 RU, respectively. In other words, the levels dropped by 59% for the PEGylated liposomes and by only 14% for the non-PEGylated liposomes. This result offers further support to the hypothesis that the presence of PEG prevents deep penetration of the lipophilic anchor groups into the hydrophobic core of the lipid bilayer. The steric protection offered by the PEG–polymer likely explains why neither PEGylated liposomes nor PEG-stabilized bilayer disks could be stably immobilized onto the L1 chip.

Incorporation of COX 1 and COX 2 in bilayer disks

Experiments including COX 1 and COX 2 were performed to explore the possibility of using bilayer disks as lipid membrane models in SPR-based protein studies. COX 1 and COX 2 are monotopic membrane proteins, with the membrane-binding motif integrated into one leaflet of the lipid bilayer. Previous studies have demonstrated that incubation of COX 2 with preformed liposomes leads to binding of the protein to the liposome membrane [17]. In the current investigation, therefore, COX 1 or COX 2 was preincubated with bilayer disks in a similar fashion.

Disks composed of POPC/PE/cholesterol/ceramide–PEG_{2000}/DSPE–PEG_{2000}–biotin (30:28:17:21:4 mol%) were preincubated with COX 1 and COX 2 at a protein/lipid ratio of 1:500 and injected over streptavidin surfaces. This resulted in signal levels of 2273 and 2450 RU, respectively. The capture of disks alone gave signal levels of 1774 and 1870 RU, respectively. Thus, it can be estimated that 529 and 580 RU of COX 1 and COX 2, respectively, were captured via the bilayer disks. On the basis of the molecular weights of COX 1 and COX 2 (140 and 144 kDa, respectively), and by assuming that 1 RU = 1 pg/mm^2 for proteins [21], it can be calculated that approximately 4.5 fmol of COX 1 and 4.8 fmol of COX 2 molecules were immobilized per flow cell (1.2 mm^2). Fig. 7 shows a typical experiment with COX 2, and similar sensorgrams were obtained when disks preincubated with COX 1 were injected (not shown).

Due to the general detection principle of SPR, a number of control experiments are required to exclude the possibility that the signals are due to artifacts. One concern is that the signal differences are a result of protein-induced changes in the size or morphology of the disks. However, mixing of disks with protein at a protein/lipid ratio of 1:500 did not affect the size or structure of the disks as visualized by cryo-TEM (Fig. 2). Therefore, it can be assumed that signal differences reflect a difference in disk mass rather than structural changes. Moreover, the possibility that free COX from the preincubation mixture interacted directly with streptavidin was investigated by control injections of COX alone (i.e., in the absence of disks) to surfaces having low or high streptavidin immobilization levels. The signals were found to be independent on the streptavidin levels (results not shown).

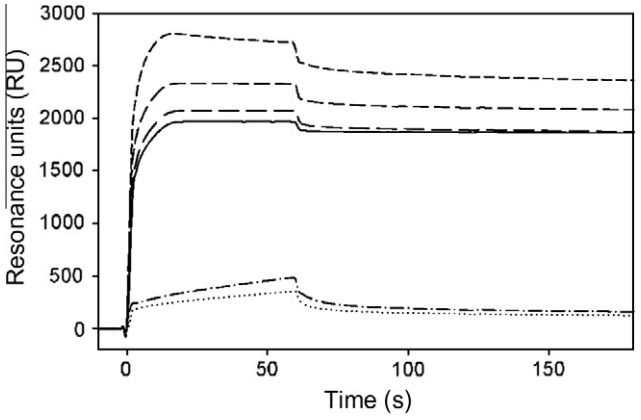

Fig. 7. Sensorgrams showing immobilization on the CM5 chip of pure bilayer disks (—); disks preincubated with COX 2 at COX/lipid molar ratios of 1:2000 (— — —), 1:1000 (– – –), and 1:500 (- - -); and the response on injection of pure COX 2 solution with protein concentrations of 2 μM (. . .) and 4 μM (– –). The immobilizations were performed on flow cells with similar levels of streptavidin (8839, 8898, 8495, 8276, 8770, and 8345 RU, respectively). Bilayer disks were composed of POPC/PE/cholesterol/ceramide–PEG_{2000}/DSPE–PEG_{2000}–biotin (30:28:17:21:4 mol%).

Furthermore, when pure COX at two different concentrations (2 and 4 μM protein) was injected into surfaces with high and comparable streptavidin levels, the recorded signals were nearly identical (Fig. 7). Taken together, these results suggest that COX had no detectable affinity for streptavidin. Furthermore, nonspecific interactions with the dextran matrix could be excluded based on control experiments using reference surfaces without immobilized streptavidin.

The above-described initial investigations show that it is possible to form stable sensor surfaces built from proteodisks. This finding allows the possibility of employing bilayer disks for immobilization of membrane proteins and studying their interaction with, for example, low-molecular-weight ligands by SPR biosensor technology.

Conclusion

This investigation has shown that nanosized bilayer disks provided with biotinylated PEG–lipids can be stably attached to streptavidin-treated SPR sensor surfaces. The structure of the disks ensures, in contrast to the case with liposomes, that the entire lipid area is immediately available for interaction with potential analytes. Disks carrying the membrane proteins COX 1 and COX 2 were also successfully immobilized on the sensor surfaces. Taken together, the data from this study suggest that the bilayer disks have high potential as model membranes for interaction studies based on biosensor technology.

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