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Phase-segregated membrane model assessed by a combined SPR-AFM approach

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ABSTRACT

Model biomembranes can provide valuable insights into the properties of complex biological membranes. Among several techniques, Surface Plasmon Resonance (SPR) provides a label-free analysis of the interactions of bioactive molecules with biomembranes with an experimental setup that allows mimicking biological environments. Nevertheless, protocols that enable the preparation of stable supported membrane systems with reproducible structural and functional properties on the biosensor chip are still needed. In this work, we present a simple protocol to modify SPR substrates that allows the formation of a phase-segregated supported lipid bilayer (SLB). SLBs are formed by fusion of lipid vesicles of pure phospholipids (DMPC, DPPC and DOPC) and of a ternary mixture (DOPC/16:0 SM/Cho in 2:1:1 molar ratio) on a SPR gold sensor chip covered with a dithiothreitol monolayer. The formation of a SLB on the SPR sensing surface in a reproducible way was assessed by the combined use of the SPR technique with AFM. The interaction of a cholesterol-extracting drug with SLBs was studied as a model of membrane-lipophilic biomolecule interaction. The proposed strategy allowed us to obtain a membrane model where phase coexistence is present and where Cho depletion from ternary mixtures was comparable to the extraction results reported for human erythrocytes.

1. Introduction

Cell membranes are the single barrier separating the intracellular environment from the extracellular space, in which several kinds of biological functions take place, namely, mechanical, electrical, signaling and transport functions. It is nowadays recognized that the investigation of the nanoscopic organization of membranes that controls their mechanical properties is meaningful not only to clarify most biological events but also to utilize them in advanced applications [1–3], such as drug screening [4,5], biosensors [6] and biomimetic separation [7]. However, biological cell membranes are very complex systems, and consequently the investigation of their structure and functions is not straightforward. Therefore, model biomembranes can provide valuable insights into the properties of complex biological membranes [8]. The lipid bilayer composition of these artificial membranes can be easily controlled by the preparation conditions. Several techniques were developed for analyzing membrane model systems at both the microscopic and the molecular levels. Among them, Surface Plasmon Resonance (SPR) has evolved into an exciting technique in the label-free analysis of biomolecular interactions, allowing high-throughput screening of the structural and compositional factors that mediate the binding of bioactive molecules to the membrane [9]. The SPR experimental setup allows mimicking the biological environment where the interaction process under study takes place. Nevertheless, protocols that enable the preparation of stable supported membrane systems with reproducible structural and physicochemical properties on the biosensor chip are still needed.

The self-assembly of supported lipid bilayers (SLBs) from vesicles in solution is the most versatile, simple and reproducible method to prepare continuous and almost defect-free fluid bilayers [10,11]. Several factors influence the results such as the surface properties, the lipid composition, size and solution composition of the vesicles, the osmotic pressure and the temperature [12]. However, liposomes interact weakly and do not rupture on unmodified gold surfaces, except for the case of Au (111) substrates [13,14]. Indeed, the mechanism of vesicle spreading on Au (111) was described in depth by Pawlowski et al. [15] On the contrary, SPR substrates are polycrystalline and require surface modification to enable vesicle adsorption and eventual fusion [3,11].

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Highly hydrophilic films or adlayers, such as SiO_2 [11,16], self-assembled monolayers [3,17] and polymer cushions [11,16,18], are frequently employed to modify gold substrates to achieve free standing bilayers. If this direct strategy fails, due to the inherent system properties, lipophilic anchors [19,20] can be added to enhance the adhesion or derivatized molecules such as thiophospholipids [18,21,22] or specific thiolated linkers (avidin/biotin, [23,24] His-Tag, and nucleotides [25]) can be employed. Another resource consists of employing detergents [26], PEG solution [23] or fusion peptides [10,27], adding washing steps to avoid remains of these agents. It is evident that increasing the chip complexity not only demands more preparation time and the use of expensive reagents but drifts the model further away from the native membrane limiting its versatility. Among the questions that could arise are whether the SPR substrate allows vesicle fusion or only their adsorption, or a mixture of both [16]; if there is a good coverage or if the compound under study is interacting with the surface [28] and whether there is a way to control and characterize the mechanical properties of the bilayer. Regarding this last point, it is noteworthy that the composition and the lateral organization, i.e. the presence of segregated domains or "lipid raft-like domains", in the lipid bilayer interface play a critical role in studies of ligand-receptor interactions on membranes. The effective exposure of ligand moieties [29], the secondary structure of peptides [30], the interaction with nanoparticles [31] and the triggering of cell responses [32], among others, have been reported to be influenced by phase-segregation studies with SLBs.

In this work, we present a simple protocol to modify SPR gold substrates that allows the formation of SLBs of a ternary lipid mixture (DOPC/SM 16:0/Cho) that exhibits phase coexistence, i.e. a liquid-ordered (L_o) phase enriched in sphingomyelin (SM) and cholesterol (Cho) which is segregated from the liquid-disordered (L_d) phase composed mainly of DOPC [33,34]. SLBs were formed by vesicle fusion on dithiothreitol (DTT) self-assembled monolayers (SAMs) on Au following previous procedures [35,36]. The presence of different lipid phases was characterized by means of atomic force microscopy (AFM), a powerful technique that enables imaging in a liquid environment [37]. Furthermore, force spectroscopy (FS) gives information about the nanomechanical properties and distribution of lipid phases in SLBs [38–40]. This technique is of particular importance since the evidence of the lipid bilayer formation cannot be directly demonstrated in a polycrystalline substrate [21].

Here, we present the first use of a combined SPR-AFM approach to prepare and characterize membrane-mimetic surfaces with lipid raft-like domains, and we demonstrate the ability of AFM to determine the topological and nanomechanical properties of the membrane on a SPR sensor chip. These lipid phases formed on the polycrystalline gold were comparable to the ones obtained on a flat mica substrate. The combination of these techniques allowed us to corroborate the adequate formation of a SLB as a model of membrane-lipophilic biomolecule interactions for SPR studies. The PC/SM/Cho lipid mixture, that mimics the outer leaflet of human erythrocytes, was exposed to methyl-β-cyclodextrin (MβCD), a Cho-extracting drug. Cho depletion from ternary mixtures was comparable to the extraction results reported for human red blood cells.

2. Material and methods

2.1. Reagents and materials

1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), N-palmitoyl-D-erythro-sphingosylphosphorylcholine (16:0 SM) and cholesterol (Cho) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). DL-Dithiothreitol (DTT), methyl-β-cyclodextrin (MβCD), N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES) and other reagents, all analytical-grade, were purchased from Sigma Aldrich (St. Louis, MO, USA) and Tris base from J. T. Baker (Center Valley, PA, USA). Chloroform and methanol, HPLC-grade, were purchased from Merck (Darmstadt, Germany). The ultrapure MilliQ water (MerckMillipore, Burlington, WI, USA) used for all the solutions and experiments had a resistivity of 18.2 MΩ cm at 23 °C.

Gold evaporated (~50 nm) on glass substrates (SPR102-AU) were obtained from Bionavis (Tampere, Finland). Muscovite mica grade V-1 was purchased from SPI Supplies (West Chester, PA, USA).

2.2. Vesicle preparation

Multilamellar vesicles (MLVs) were prepared from synthetic pure lipids (DMPC, DPPC and DOPC) dissolved in chloroform. The ternary mixture (TM) was prepared by mixing appropriate amounts of DOPC, 16:0 SM and Cho (2:1:1 molar ratio, respectively) and then dissolved in chloroform/methanol (2:1, v/v). Each sample was dried by evaporating the solvent under a stream of N_2 and placed at high vacuum for 2 h in a glass chamber connected to a vacuum pump. The samples were hydrated in PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl pH 7.0), Tris (20 mM Tris base, 150 mM NaCl pH 7.4) or HEPES (25 mM HEPES, 150 mM NaCl pH 7.4) buffers and stirred to facilitate dispersion. After completing lipid detachment from the bottom of the test tube, MLVs were introduced in a bath sonicator (TB04TA, Testlab, Argentina) and kept at 65 °C (for DPPC and TM) for 1 h. In this way, small unilamellar vesicles (SUVs) were generated.

2.3. DTT-gold substrate preparation

SPR gold substrates were washed with hot (~70 °C) ammonia-peroxide solution (30% NH_3: 100 vol H_2O_2:H_2O 1:1:2) for a couple of minutes, rinsed with ultrapure water and absolute ethanol and dried with N_2. Immediately, the substrates were immersed in ethanolic 50 μM DTT solution for 30 min in the absence of light (covered with aluminum foil). As previously reported [35], this procedure was optimized to achieve a fully covered gold surface with only one monolayer of DTT. After thoroughly rinsing with absolute ethanol, substrates were dried with a stream of N_2.

2.4. SPR measurements

SPR measurements were performed in Kretschmann configuration using a BioNavis Navi™ 200 (MP-SPR) device (Tampere, Finland) equipped with two independent lasers (670 and 785 nm) in a dual-channel system. Measurements were made in angular-scan mode (40–78 degrees), recording SPR curves every 3.5 s at a constant temperature of 23 °C.

Ex- situ prepared DTT-gold substrates were placed in the flow chamber and washed with high buffer flux (500 μL/min) and 1% Triton X-100 aqueous solution (1 min at 50 μL/min). Vesicle suspensions (0.1–1 mg/mL) were injected at 10 μL/min with times ranging from 15 to 40 min. Unbound vesicles were washed with high buffer flux and 1-min injection of 100 mM NaOH solution at 50 μL/min. The amount of immobilized lipid was recorded after 10 min of signal stabilization, the angular shifts reported are the mean value of at least 5 independent experiments. DTT-gold surfaces were regenerated with two consecutive 1-min injections of 1% Triton X-100 aqueous solution at 50 μL/min before a fresh vesicle suspension was injected.

MβCD binding measurements were performed using 30-min injection of MβCD solution (0.25, 0.5, 1 and 3 mM) in Tris buffer for the ternary mixtures. For each MβCD solution assessed, a fresh lipid sample was prepared and the amount of lipid immobilized was normalized to 1.0, in order to compare the amount of extracted cholesterol. Control experiments with DMPC were performed and as already reported [41] no material was extracted (see *Supporting information* Fig S1).