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Tethered proteoliposomes containing human ABC transporter MRP3: New perspectives for biosensor application based on transmembrane proteins

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ABSTRACT

While transmembrane proteins and transporters comprise one of the largest protein families, their use in biosensors like biochips or lab-on-a-chip devices has so far been limited by their demanding requirements of a stable and compartmentalized lipid environment. A possible remedy lies in the tethering of proteoliposomes containing the reconstituted transmembrane protein to the biosensoric surface. As a proof of concept, we reconstituted the human ABC transporter MRP3 into biotinylated proteoliposomes and tethered those to a gold surface coated with streptavidin on a biotinylated self-assembled thiol monolayer. The tethering process was investigated by quartz crystal microbalance with dissipation monitoring. The final assembly of tethered proteoliposomes exhibited biological activity in terms of drug-stimulated ATP hydrolysis and substrate translocation. The presented facile immobilization approach can be easily extended to other transmembrane proteins as it does not require any modification of the protein and will open up transmembrane proteins for future application in biosensors.

1. Introduction

Biosensors based on soluble proteins are an established technique with widespread analytical and biomedical applications ranging from quantification of small molecules in complex matrices over high-throughput ligand and inhibitor screening to fundamental kinetic and thermodynamic studies on the protein itself [1]. However, examples of transmembrane proteins used in biosensors (f.e. [2,3]) are still rare because they require a lipid or lipid-like environment, carefully optimized for both supporting the native protein conformation and long-term stability of the biosensor. Transport proteins, particularly ABC transporters, pose even more difficulties because a compartmentalized lipid bilayer environment of low permeability is necessary to facilitate transport. However, their role in metabolic barriers, excretion of toxins and multidrug resistance of cancer cells [4] would imply numerous biosensoric applications if these difficulties could be overcome.

The simplest conceivable model for coupling a transport protein to a biosensoric surface while simultaneously providing stable compartmentalization, is the tethering of proteoliposomes via the biotin–streptavidin interaction which provides irreversible immobilization with a binding constant similar to covalent attachment [5,6]. The most common biosensor surfaces silicon/silica and gold can be readily coated with streptavidin via a biotinylated SAM of either silanes (silicon/silica, [7]) or thiols (gold, [8]). Proteoliposomes containing the membrane protein and doped with the biotinylated lipid DCB can then be tethered to such a surface. Although similar models are well-characterized for liposomes [9,10], they have not yet been extended to actual proteoliposomes containing an active transmembrane transporter.

We therefore investigated the tethering of biotinylated proteoliposomes containing the human ABC transporter MRP3 to a streptavidin-functionalized gold surface (as depicted in Fig. 1) and checked the biological activity of such an assembly in terms of ATP hydrolysis and substrate translocation. MRP3 is supposedly involved in multidrug resistance of cancer cells and features a catalytic cycle with strong positive cooperativity for both ATP hydrolysis and substrate transport [11,12]. Due to its multidomain structure, fragility, intolerance of most covalent modifications and requirements of the lipid environment, MRP3 is a good test candidate for the feasibility of a proteoliposome-based biosensor.

We used the QCM-D as primary tool for the analysis of proteoliposome tethering because it simultaneously provides information

Abbreviations: ABC, ATP binding cassette; CDCF, 5(6)-carboxy-2′,7′-dichlorofluorescein; DCB, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(cap biotinyl); DDM, *n*-dodecyl β-D-maltoside; DTT, dithiothreitol; eq, stoichiometric equivalents; LUV, large unilamellar vesicle; MRP, multidrug resistance-associated protein; MTX, methotrexate; PL, proteoliposome; QCM(-D), quartz crystal microbalance (with dissipation monitoring); SA, streptavidin; SAM, self-assembled monolayer; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

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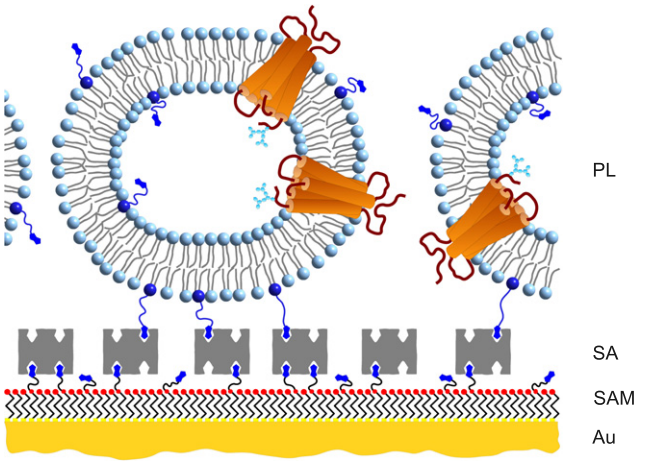

Fig. 1. Schematic illustration of a biosensoric surface with the ABC transporter MRP3 as active biological system. A gold surface (Au) as transducer is coated with streptavidin (SA) on top of a binary biotinylated thiol SAM (SAM). Proteoliposomes (PL) with the reconstituted protein MRP3 are tethered to the streptavidin coating via the biotinylated lipid DCB.

on the mass uptake and the viscoelastic properties of the immobilized mass [13,14].

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(cap biotinyl) (DCB) was purchased from Avanti Polar Lipids, asolectin from Sigma–Aldrich, streptavidin from Rockland, other chemicals from AppliChem or Sigma–Aldrich in the highest available purity. Water was purified and deionized in-house by a Sartorius arium 61316/611VF to resistivity of 18.2 MΩ.

2.2. Preparation of large unilamellar vesicles (LUVs)

Lipids were mixed in $CHCl_3$, dried under a stream of nitrogen at 40 °C and kept in high vacuum over night. The resulting lipid films were resuspended in reconstitution buffer (20 mM TES-NaOH, pH 7.4; 100 mM NaCl; 1 mM $MgCl_2$) at a concentration of 20 mM and extruded 21 times through a polycarbonate membrane with 200 nm pore size (Avestin Liposofast).

2.3. Reconstitution of MRP3

Recombinantly expressed MRP3 was reconstituted into LUVs at a lipid-to-protein ratio of 25/1 (w/w) corresponding to a molar ratio of approximately 5500/1, as described previously [12,15], and resulting PLs were stored flash-frozen at −70 °C. Prior to use, PLs were thawed on ice, incubated with 1 mM DTT and extruded five times through a polycarbonate membrane (Avestin Liposofast, 200 nm pore size).

2.4. Surface cleaning and preparation

5 MHz AT-cut QCM sensors with gold electrodes (Q-Sense QSX 301) were cleaned in an ammonia (25%)/hydrogen peroxide (30%)/water mixture (1/1/5, v/v/v) for 5 min at 80 °C, excessively rinsed with water and dried in a stream of nitrogen. Immediately before use, the sensors were exposed to an argon plasma for 5 min. Self-assembly of a biotinylated thiol monolayer was accomplished by incubation in a chloroformic solution of 16-mercaptohexadecanol and 16-mercapto-(8-biotinamido-3,6-dioxaoctyl)hexadecanamide (50/1 (n/n); 0.1 mM) as described previously [8].

2.5. QCM measurements

A Q-Sense E4 QCM-D equipped with four temperature controlled flow cells in parallel configuration and a four-channel peristaltic sucking pump (Ismatec IPC) monitored frequency and dissipation changes of odd overtones 3 to 13. Flow rates in µL/min were 100 during rinses, 50 during adsorption and 10 during thermal equilibration. Frequency and dissipation shifts are reported normalized to the overtone and relative to a sensor with a thiol SAM in pure reconstitution buffer.

2.6. LUV/PL tethering

QSX 301 QCM sensors bearing a biotinylated thiol SAM were rinsed with reconstitution buffer at 20 °C until the baseline stabilized. SA (10 µg/mL in reconstitution buffer) was allowed to adsorb to the SAM in circular flow for 15 min followed by a 5 min rinse. Subsequently, LUVs/PLs (diluted to 1 mM in reconstitution buffer) were applied for at least 30 min in circular flow followed by a rinse until readings stabilized.

2.7. ATPase assay

After warming tethered LUVs/PLs to 37 °C and equilibration in transport buffer (50 mM TES-NaOH, pH 7.4; 41 mM NaCl; 10 mM KCl; 12 mM $MgSO_4$; 10 mM Na_2ATP), the flow was stopped for 1 h. Subsequently, the liquid in the flow chamber (300 µL) was harvested, flash-frozen in liquid nitrogen, lyophilized and redissolved in 40 µL 200 mM H_2SO_4 containing 2 mM DDM. Inorganic phosphate from ATP hydrolysis was quantified according to van Veldhoven and Mannaerts [16] using a total assay volume of 56 µL.

Stimulation of ATPase activity was achieved by supplementing the transport buffer with 2 mM MTX. LUVs without MRP3 served as negative control for unspecific ATP hydrolysis.

2.8. CDCF uptake assay

After warming tethered LUVs/PLs to 37 °C and equilibration in transport buffer containing 20 µM CDCF, the flow was stopped for 1 h. The tethered (proteo)liposomes were washed with reconstitution buffer at a flow of 100 µL/min for 12 min before lysis with Triton X-100 (0.1% (w/v) in reconstitution buffer) at the same flow rate, collecting fractions of 100 µL. Fluorescence of CDCF was detected at 485 nm excitation and 535 nm emission in a Berthold Mithras LB 940 plate photometer.

3. Results and discussion

3.1. Tethering of proteoliposomes to biosensoric surfaces

We first performed QCM-D measurements on the tethering of LUVs made from asolectin, which is a suitable lipid mixture for the reconstitution of MRP3 and other ABC transporters, and doped with 0.1 mol% of the biotinylated lipid DCB. Fig. 2A shows typical time courses for frequency and dissipation shifts from a QCM-D measurement during immobilization of streptavidin and subsequent tethering of DCB-doped asolectin LUVs to a gold surface functionalized with a biotinylated SAM. In QCM-D experiments, the negative frequency shift correlates with the mass that is acoustically coupled to the surface. While this correlation is linear for rigid layers as shown by Sauerbrey [17], it becomes non-linear for