Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1778 (2008) 175–184
Biochimica et Biophysica Acta
BBA
www.elsevier.com/locate/bbamem

Kinetics of cholesterol extraction from lipid membranes by methyl-β-cyclodextrin—A surface plasmon resonance approach

Mojca Podlesnik Beseničar [1], Andrej Bavdek [1], Aleš Kladnik, Peter Maček, Gregor Anderluh *

Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, 1000 Ljubljana, Slovenia

Received 8 June 2007; received in revised form 31 August 2007; accepted 20 September 2007
Available online 4 October 2007

Abstract

The kinetics of cholesterol extraction from cellular membranes is complex and not yet completely understood. In this paper we have developed an experimental approach to directly monitor the extraction of cholesterol from lipid membranes by using surface plasmon resonance and model lipid systems. Methyl-β-cyclodextrin was used to selectively remove cholesterol from large unilamellar vesicles of various compositions. The amount of extracted cholesterol is highly dependent on the composition of lipid membrane, i.e. the presence of sphingomyelin drastically reduced and slowed down cholesterol extraction by methyl-β-cyclodextrin. This was confirmed also in the erythrocyte ghosts system, where more cholesterol was extracted after erythrocytes were treated with sphingomyelinase. We further show that the kinetics of the extraction is mono-exponential for mixtures of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine and cholesterol. The kinetics is complex for ternary lipid mixtures composed of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, bovine brain sphingomyelin and cholesterol. Our results indicate that the complex kinetics observed in experiments with cells may be the consequence of lateral segregation of lipids in cell plasma membrane.

Keywords: Lipid membrane; Cholesterol extraction; Cyclodextrin; Surface plasmon resonance; Biacore

1. Introduction

Cyclodextrins (CDs) are a family of cyclic oligomers of glucose, linked by α-1,4 glycosidic bonds. They differ in the number of glucose units in the ring, i.e. they are composed of six, seven or eight glucose units in α-, β- or γ-CD, respectively [1]. They are highly hydrophilic molecules with a hydrophobic core in which they can bind and thereby solubilize hydrophobic molecules [2,3]. Derivatives of CDs, which are more soluble and less toxic [2], have been in use in pharmacological research for years as carriers of lipophilic drugs and only in last two decades has their use in membrane studies been appreciated [4]. In the latter they are mostly used in studies of cholesterol (CHO) role in the cellular membranes [4–8]. β-CD and its derivatives, methyl-β-CD (MBCD) and 2-hydoxypropyl-β-CD, are mostly chosen for these studies as they extract membrane CHO very efficiently [4,5,8,9]. MBCD is CHO-specific at concentrations below 10 mM and only negligibly binds other lipids [10,11]. At low concentrations (<1 mM) it has also been used as a catalyst for CHO transfer between lipid membranes and other CHO acceptors, such as serum lipoproteins [6,9], or for inter-vesicular CHO transfer [10].

The mechanism of CHO extraction by CD is not yet fully understood. In CHO extraction experiments most authors have observed neither significant binding to nor inserting into the membranes of the extracting agent [1,4]. It was hypothesized that CHO efflux from the membranes occurs primarily by an aqueous diffusion mechanism in which CHO molecules desorb from the cell or vesicular membrane and are incorporated in either lipoprotein or MBCD molecule after diffusion across aqueous layer [4]. The reason for much faster process mediated by CDs compared to serum lipoproteins might be much smaller

Abbreviations: CHO, cholesterol; CD, cyclodextrin; DOPC, 1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine; LUV, large unilamellar vesicles; MBCD, methyl-β-cyclodextrin; NBD-CHO, 25-(N-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-methyl]amino)-27-norcholesterol; RU, response units; SM, sphingomyelin; SPR, surface plasmon resonance

* Corresponding author. Tel.: +386 1 423 33 88; fax: +386 1 257 33 90.
E-mail address: gregor.anderluh@bf.uni-lj.si (G. Anderluh).
[1] Contributed equally to the work.

0005-2736/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2007.09.022

size of the former, which enables it to come considerably closer to the cellular membrane and/or reversible CD–membrane interactions [4,9,12]. This hypothesis has been contradicted by Steck and co-workers [8], whose results indicate the extraction proceeds via activation–collision pathway so that lipid monomers enter an activated state, e.g. partial projection from the lipid bilayer, and from this state they are either captured by collision with acceptors or return to the ground state [8,13]. The stoichiometry of the complex has been estimated to be 1:1 [14] but more recently the stoichiometry of 2:1 was suggested [11]. These are corroborated by molecular dimensions of both species, i.e. the CHO molecule is ~18 Å long, whereas the length of MBCD hydrophobic cavity is only ~8 Å [11].

Most of the literature suggests that there are »two pools« of CHO in cellular and lipid vesicle membranes [4,5,15–18]. On the basis of the kinetics of CHO extraction by CD, a fast and a slow CHO pools with half times of approximately half a minute and 10 min to half hour, respectively, were observed [4,5,17,18]. The two CHO pools have also been observed in vesicles composed of dipalmitoyl-glycero-phosphocholine, palmitoyl-oleoyl-glycero-phosphoglycerol and CHO [5]. While the fast pool is most likely the CHO present in plasma membrane, the slow pool might be attributed to intracellular CHO, CHO present in the intracellular monolayer of the plasma membrane or CHO in a separate lateral domain in the plasma membrane [5]. This has not yet been satisfactorily resolved, but studies in recent years indicated that the latter possibility is actually the most likely. The CHO efflux from cellular and model membranes is slower, if there is sphingomyelin (SM) present in the membrane [19–21]. It has been also shown that the sphingomyelinase treatment of cell membranes drastically increases CHO efflux from membranes [17,22], whereas there is no such effect when phosphatidylcholine content is changed [22]. The same phenomenon was shown to hold true for lipid monolayers, further confirming the cholesterol desorption experiments from bilayers [23] and implies that cholesterol preferentially associates with SM [24,25].

Most of the experimental procedures, which are also time and material consuming, for monitoring the extraction of cholesterol require radioactive labeling of cholesterol in cellular or vesicular membranes [4,5,8,17,18,26] and/or CD molecules [5]. One exception is very recent experimental work in which isothermal titration calorimetry was employed, which is fast and does not require radioactive labeling of the molecules [11,27]. Here, we have employed a surface plasmon resonance (SPR) approach, which allows direct monitoring of the extraction process, to address the question of kinetics of CHO extraction from membranes of different compositions. SPR on a Biacore technology is mainly used in biochemistry to study kinetics of molecular interactions, such as protein–protein, protein–small molecule, protein–membrane, etc. The most obvious advantages of SPR over other techniques are direct and rapid determination of association and dissociation rates of binding process, no need for labeling of molecules, and low amount of sample that is used in an assay [28–31]. It is possible to immobilize intact liposomes on the L1 Biacore sensor chip, which has covalently linked lipophilic anchors on a dextran matrix [32,33]. Liposomes are stably retained on this surface, when low flow-rates are applied. It is, therefore, easy to reproducibly prepare stable liposome surface under various experimental conditions, i.e. lipid composition, temperature or pH. So, in a typical experiment, a liposome surface is prepared on the surface of the sensor chip and the molecule of interest is passed over.

In this work we introduced and optimized the SPR technique to monitor real-time kinetics of CHO desorption from lipid vesicles using MBCD. We show that MBCD does not bind to dioleoyl-glycero-phosphocholine (DOPC) membranes and does not perturb lipid membranes. The extraction by MBCD is CHO-specific in the concentrations below 2 mM. It is highly dependent on the composition of lipid membrane, i.e. the presence of sphingomyelin drastically slowed down cholesterol extraction by MBCD. We finally show that the kinetics of the extraction is complex for ternary lipid mixtures composed of DOPC, SM and CHO.

2. Materials and methods

2.1. Materials

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), bovine brain sphingomyelin (SM), cholesterol (CHO), and 25-{N-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-methyl]amino}-27-norcholesterol (NBD-CHO) were obtained from Avanti Polar Lipids (USA). Methyl-β-cyclodextrin (MBCD), Triton X-100, streptolysin and *Bacillus cereus* sphingomyelinase were from Sigma. Throughout this work a following SPR buffer was used: 140 mM NaCl, 20 mM NaH_2PO_4, 1 mM EDTA, pH 7.5.

2.2. Large unilamellar vesicles preparation and characterization

The lipids were dissolved in chloroform:methanol (2:1, vol:vol) mixture and the desired composition was dried under vacuum for 3 h to form a lipid film. The buffer with or without 50 mM calcein was applied to the film and extensively vortexed with glass beads until all of the lipids were resuspended, forming multilamellar structures. The so-obtained suspension was extruded through 100 nm membranes (Avestin, Canada) to form large unilamellar vesicles (LUV) of defined size of approximately 100 nm. In the case of calcein containing vesicles, they were further applied to a small Sephadex G-50 column to separate untrapped calcein from the vesicles. The indicated lipid ratios are always molar. The lipid concentrations were determined by enzymatic tests for lipid concentration determination Phospholipids B (for choline containing DOPC and SM) and free cholesterol C (for cholesterol) (both from Wako, Germany). The size of the LUV in the absence or presence of various MBCD concentrations was determined by the dynamic light scattering using Zeta Sizer 3000 (Malvern Instruments Ltd.).

2.3. Preparation of erythrocyte ghosts

Erythrocyte ghosts were prepared essentially as described in Bavdek et al. [34]. Briefly, fresh erythrocytes from healthy donor were washed in 130 mM NaCl, 20 mM Tris–HCl, pH 7.4 (erythrocyte buffer). Erythrocytes were lysed with five volumes of cold 5 mM Na_2HPO_4, pH 8.0. Lysed erythrocytes were centrifuged in a benchtop centrifuge at 14,000×*g* and 4°C. Ghosts in the pellet were washed at the same conditions until all of the hemoglobin was removed. The final pellet was resuspended in a small volume of an SPR buffer. SM was depleted from erythrocytes by *B. cereus* sphingomyelinase. One milliliter of washed erythrocytes was incubated with 2 U/ml of *B. cereus* sphingomyelinase in erythrocyte buffer for 60 min at 37°C. Erythrocytes were washed well before use for ghost preparations. The majority of SM was removed from erythrocyte membranes according to TLC and lipid concentration measurements (as described above).

2.4. Fluorescence measurements

Permeabilisation of calcein-loaded liposomes was measured by Jasco FP-750 spectrofluorimeter. The excitation wavelength was 485 nm and the emission

was followed at 520 nm. The concentration of the lipids stirred at 25°C in the buffer was 20 μM. The permeabilisation induced by the MBCD was expressed as the percentage of the maximal permeabilisation obtained at the end of the assay by the addition of detergent Triton X-100 to a final concentration of 2 mM. For the fluorescence scattering the excitation and emission wavelengths were 400 nm. The liposomes at 20 μM lipid concentration were stirred in the cuvette and then MBCD in the buffer was added to rich the desired final concentration.

2.5. Fluorescence microscopy

NBD-CHO fluorescence on the L1 chip was imaged with the AxioImager Z1 microscope (Carl Zeiss, Germany) with a 10× objective using blue light excitation (450–490 nm bandpass excitation and 515 nm longpass emission filter). Fluorescence images were acquired with AxioCam MRm digital camera and AxioVision 4.5 software (Carl Zeiss Vision, Germany) and further processed in ImageJ 1.34 [35]. Images used for semi-quantitative comparison of gray value profiles were acquired with the same microscope and camera settings. Uneven background in the image was corrected with the Shading Corrector plug-in in ImageJ using the image of an empty field on the chip showing background fluorescence. On each of these corrected images the profile of gray values was measured in a selected area, covering approximately 50% of the image. Profiles were measured in the direction perpendicular to the flow cell on the L1 chip. The profile values represent the average fluorescence intensity in the vertical pixel lines of the profile area.

2.6. Surface plasmon resonance (SPR)

Biacore X system and L1 chip (both Biacore, Sweden) were used to monitor cholesterol depletion from the chip immobilized LUV. The 100 nm LUV were immobilized on the surface of the chip as described previously [33]. Initially, the sensor chip surface was cleaned with three consecutive injections of 50 mM NaOH: isopropanol 3:2 (vol:vol). LUV at 0.5–2 mM concentration were injected at the flow rate 1 μl/min until the signal reached approximately 10000 response units (RU). Unbound vesicles were washed off the surface by two consecutive 1-min injections of 100 mM NaOH at a flow rate of 30 μl/min flow. The cholesterol depletion was performed at 25°C by injecting the desired concentration of MBCD in the buffer for 30 min at 2 μl/min. MBCD binding to clean L1 chip and CM5 chip was performed at 40 μl/min. The carboxylic groups of CM5 chip were activated with the mixture of ethyl-N-(3-diethylaminopropyl) carbodiimide and N-hydroxysuccinimide and blocked with ethanolamine according to the producer's instructions (Biacore AB).

2.7. Data analysis

The raw sensorgrams were first normalized to 10000 RU of deposited LUV. Then they were corrected with sensorgrams that were obtained by using liposomes of the same DOPC:SM ratios, but without CHO, to account for the decrease in the signal due to competition of MBCD for the lipophilic anchors of the L1 chip (see the Results section). So corrected sensorgrams were fitted to a bi-exponential decay model by using Origin software:

$$R(RU) = R_{\text{fin}} + A_1(\exp^{-k_1 t}) + A_2(\exp^{-k_2 t})$$

where R_{fin} represents the final response, A_1 and A_2 are amplitudes of each step and k_1 and k_2 are apparent rate constants. For liposomes without SM, the second term was not used and the data were reliably fitted to a mono-exponential decay function. The linearization of the data was performed as described [36,37].

3. Results

3.1. Interaction of MBCD with DOPC membranes

In this paper we have used SPR approach to study the MBCD extraction of CHO from the lipid membranes. LUV were deposited on the surface of an L1 sensor chip, which contains lipophilic anchors used for immobilization. Such liposome-coated sensor chip is stable for hours and can be tested for the binding of various compounds, i.e. proteins or lipids [31]. In our case, MBCD solution was flowed across such a surface at low flow-rate during so-called association phase, when the ligand immobilized on the surface of the chip is in the contact with the analyte, which is flown across. We have always deposited approximately 10000 RU of LUV, which results in a homogeneously covered sensor chip [33]. In such a system, the increase or the decrease in the signal is linearly proportional to changes of the amount of the material on the surface of the sensor chip, respectively [31].

We have first checked whether MBCD itself interacts with membranes without CHO, i.e. with liposomes composed of pure DOPC. A wide concentration range of MBCD was tested, from 2 μM to 8 mM, but no interaction of MBCD with chip-immobilized liposomes was observed at any concentration (Fig. 1A). Instead, a small decrease of the signal was visible during the association phase (Fig. 1A, inset). This decrease was more pronounced at MBCD concentrations above 1 mM that are typically used in cell biology research. The decrease was significant at 4 and 8 mM MBCD, where approximately 400 and 1000 RU of material was desorbed from the chip during the association phase (Fig. 1A). These two concentrations were, therefore, not used in any of the subsequent experiments. The decrease of the signal during the association phase could be either due to extraction of DOPC by MBCD or due to competition of MBCD with chip-attached liposomes for the binding to lipophilic anchors of L1 sensor-chip. Lipophilic anchors could in principle be accommodated in the MBCD hydrophobic cavity. We have tested for these possibilities by checking the binding of MBCD to L1 chip alone (Fig. 1B) and compared it to the binding to CM5 chip, with blocked carboxylic functional groups, which do not posses lipophilic anchors (Fig. 1C). The binding was better on L1 chip (Fig. 1D), indicating for an interaction of MBCD with lipophilic anchors. This interaction is, however, not tight as the signal decreases to initial values immediately. At high MBCD concentrations the signal at the beginning of the dissociation phase is below the signal before the injection. This is most likely due to matrix effects, i.e. dextran matrix on the surface of the chip accommodates to different buffer conditions but after some time reaches the initial value (as seen in Fig. 1C).

To further test that MBCD does not interact with DOPC liposomes, we have also checked effects of MBCD on the size and permeability of liposomes composed of DOPC and DOPC containing 40 mol% of CHO. The presence of MBCD did not affect the size of DOPC liposomes, i.e. the dimensions determined by the dynamic light scattering were 122±1 nm in the absence of MBCD and 122±2 in the presence of 2 mM MBCD. MBCD reduced the diameter of DOPC:CHO 60:40 liposomes by approximately 5 nm, that is from 121±1 in the absence of MBCD to 116±1 in the presence of 2 mM MBCD. This was further confirmed by monitoring light scattering, where DOPC liposomes were not affected by the presence of MBCD, while DOPC:CHO 60:40 liposomes showed a decreased intensity of scattered light, consistent with the reduction in the size of the liposomes when MBCD was present (Fig. 2A and B). MBCD does not affect the permeability of the membranes, as there

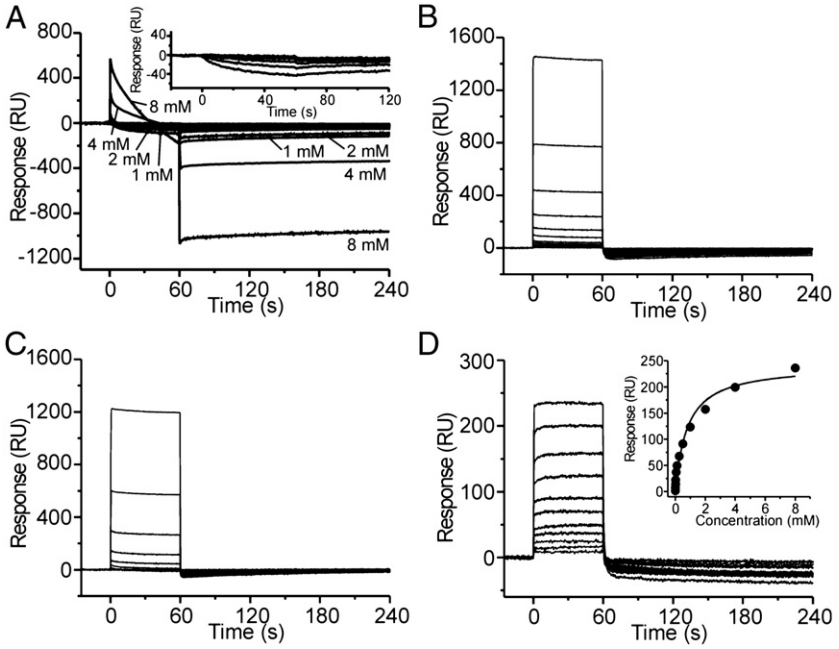

Fig. 1. Interaction of MBCD with DOPC liposomes. MBCD was dissolved in the SPR buffer (20 mM NaH_2PO_4, 140 mM NaCl, 1 mM EDTA, pH 7.5.). The flow-rate was 40 μl/min and experiments were performed at 25°C. The concentration (from bottom to top) were in all cases, 2 μM, 5 μM, 10 μM, 20 μM, 50 μM, 100 μM, 250 μM, 500 μM, 1 mM, 2 mM, 4 mM and 8 mM MBCD. (A) Binding of MBCD to DOPC liposomes. The L1 chip was covered with approximately 10000 RU of DOPC liposomes prior to measurements. (B) Binding to L1 chip. (C) Binding to CM5 chip. CM5 chip was activated and then blocked by using standard reagents and procedure recommended by the producer (Biacore). (D) The difference between the responses on L1 and CM5 blocked chips. The inset is showing the response values at equilibrium, i.e. at the end of the association phase.

was no permeabilisation of calcein observed for any of liposomes tested (Fig. 2C and D). We have, however, observed slight decrease of the fluorescence signal for calcein-loaded DOPC:CHO 60:40 liposomes, which may be explained by increased self-quenching of calcein due to the reduced volume of liposomes.

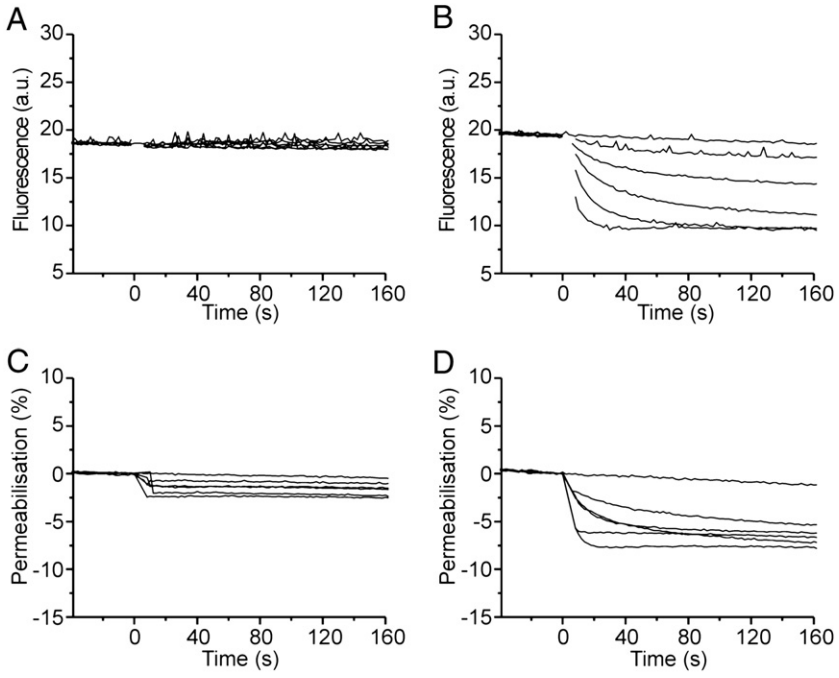

Fig. 2. Effect of MBCD on liposomes. The light scattering (A and B) and calcein fluorescence (C and D) for DOPC liposomes (A and C) and DOPC liposomes with 40 mol% of CHO (B and D). Liposomes at 20 μM concentration were stirred at 25°C. Excitation and emission wavelengths were set to 400 nm for the light scattering experiments. The excitation and emission wavelengths were 485 nm and 520 nm for calcein release experiments, respectively. Slits were in all cases set to 5 nm. At time 0 MBCD was added and fluorescence measured. The concentrations of MBCD were in all cases (from top to bottom) 0, 0.25 mM, 0.5 mM, 1 mM, 2 mM and 4 mM.

All of the data above indicated that DOPC liposomes are not affected by the concentrations of MBCD tested, i.e. MBCD does not extract DOPC molecules from the liposomes in this concentration range. The decrease of the SPR signal in the presence of high concentrations of MBCD is, therefore, due to competition of MBCD and immobilized liposomes for the binding to lipophilic anchors of L1 chip. So we had always performed controls in the absence of CHO and used them to correct the sensorgrams (see below).

3.2. Selective extraction of CHO by MBCD

We have initially characterized the selective extraction of CHO from DOPC liposomes. Conditions, used also in further experiments, were chosen to mimic the MBCD extraction protocols used by researchers in cell biology. The MBCD was injected across the liposome-covered surface of L1 chip at a very low flow rate of 2 μl/min (Fig. 3, arrow). Injection of MBCD caused a large decrease in the signal when DOPC:CHO 60:40 liposomes were used. To check for the selective extraction of CHO we have probed surface of the chip after CHO depletion for the binding of streptolysin (Fig. 3, point a), a CHO dependent pore forming toxin [38]. Streptolysin requires CHO for stable binding and does not bind to membranes in the absence of CHO. Streptolysin did not bind to such a surface (Fig. 3, trace a), however, it bound significantly to the surface that contained the same RU amount of DOPC:CHO 60:40 liposomes as depleted liposomes (point b and trace b in the inset).

We have also used a fluorescent analogue of CHO (NBD-CHO) to visualize the surface of the chip by fluorescence microscopy (Fig. 4). The extraction of NBD-CHO by MBCD (point c in Fig. 4, upper panel) efficiently removed NBD-CHO

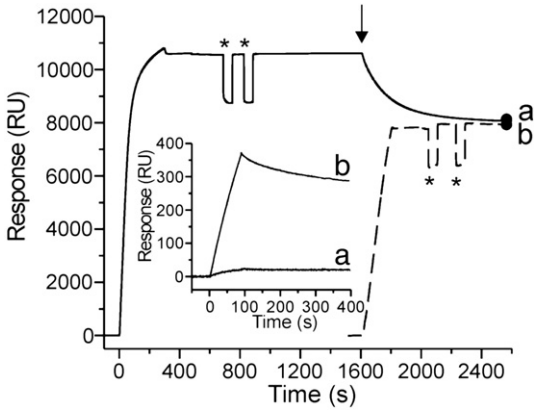

Fig. 3. Selective extraction of cholesterol from membranes as evidenced by the use of cholesterol dependent cytolysin. The liposomes composed of DOPC and CHO at 40 mol% were immobilized on a clean L1 chip to approximately 10000 RU by using a low flow-rate 1 μl/min. Liposome surface was primed two times by 100 mM NaOH injections of 1 min at 30 μl/min (denoted by asterisks). At the time, designated by an arrow, 1 mM MBCD was injected at 2 μl/min. After the depletion (point a) the surface was tested for the binding of cholesterol-dependent cytolysin streptolysin (inset) at 30 μl/min. The association was 90 s and the dissociation was followed for 5 min. The concentration of streptolysin was approximately 10 μg/ml. The binding to cholesterol depleted surface (trace a in inset) was compared to the binding to the surface that contains approximately the same RU amount of original liposomes (dashed trace and trace b in the inset).

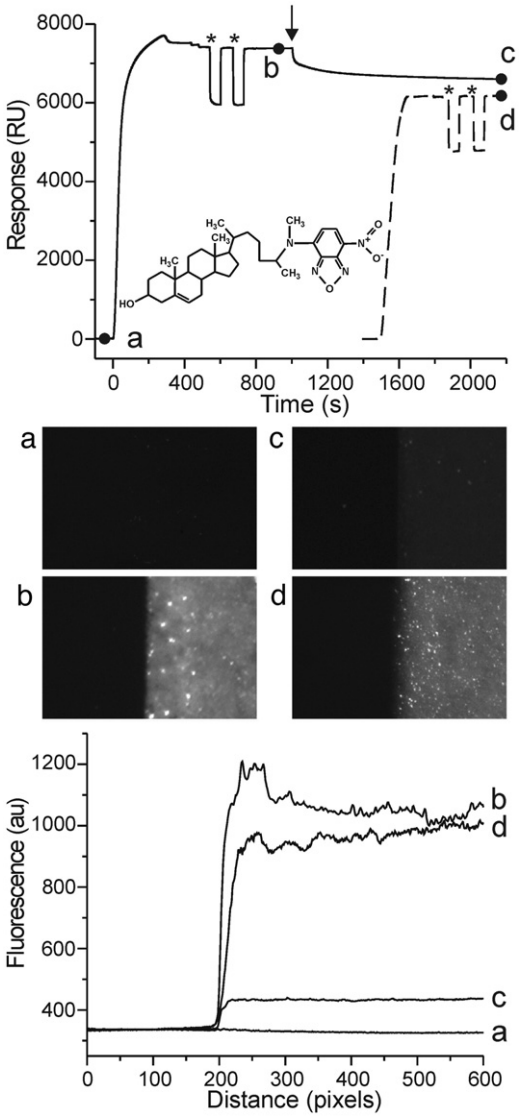

Fig. 4. Selective extraction of cholesterol from membranes as evidenced by the use of fluorescently labelled cholesterol. In the upper panel, liposomes composed of DOPC and NBD-CHO at 30 mol% were immobilized on a clean L1 chip as described in the legend to Fig. 3. The middle panel is showing the part of the flow-cell imaged by fluorescence microscopy and the lower panel is showing the profile of fluorescence intensity across the images in the middle panel. (a) The fluorescence of a sensor chip before the deposition of liposomes; (b) the fluorescence of a sensor chip when approximately 7400 RU of DOPC with NBD-CHO were deposited; (c) the fluorescence of a sensor chip after NBD-CHO depletion; (d) fluorescence intensity of a surface of a sensor chip after deposition of DOPC:NBD-CHO liposomes to the level reached after CHO depletion (i.e. point d).

from DOPC:NBD-CHO 70:30 liposomes. Only 14% of the fluorescence remained on the chip, compared to point b, which represent 100%, i.e. the total amount of NBD-CHO before the extraction. When approximately the same RU amount of DOPC:NBD-CHO 70:30 was immobilized (point d), the surface of the chip was brightly fluorescent and the amount of the fluorescence approximately corresponds to the amount of fluorescent NBD-CHO on the surface of the chip, i.e. approximately 6200 RU (point d) represents 84% of the response of point b

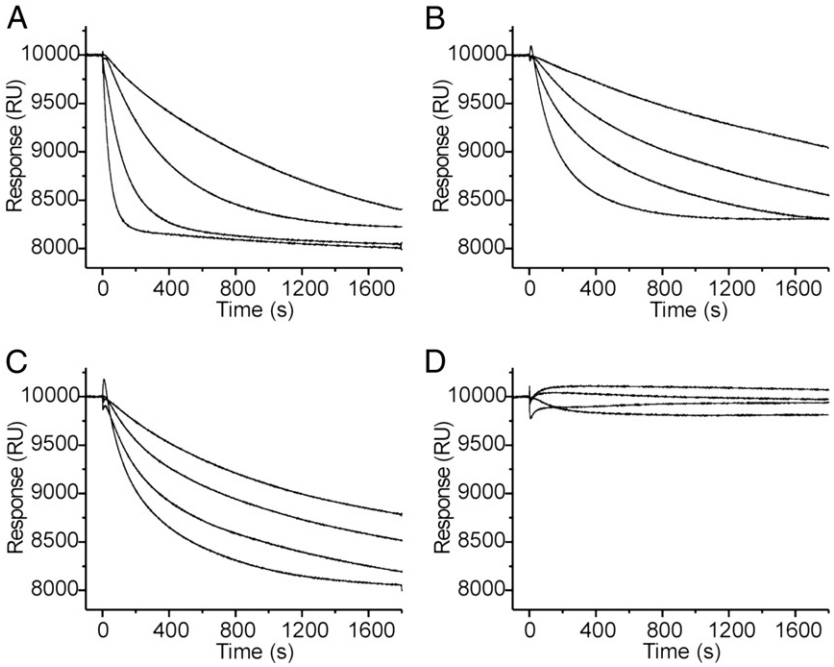

Fig. 5. Extraction of CHO from liposomes of various compositions. The liposome covered surface of L1 chip was prepared as described in the legend to Fig. 3. The cholesterol was extracted with 0.25, 0.5, 1 and 2 mM MBCD (curves from top to bottom) at 2 μl/min for 30 min. (A) DOPC:SM:CHO 70:0:30; (B) DOPC:SM:CHO 53:17:30; (C) DOPC:SM:CHO 30:30:40; (D) DOPC:SM:CHO 40:40:20. The sensorgrams for DOPC:SM:CHO 17:53:30 looked similar to those presented in panel D.

(approximately 7400 RU) and this yields 84% of the fluorescence (Fig. 4, middle panel).

These data collectively indicate that the signal decrease is due to selective removal of CHO from the liposomes.

3.3. Extraction of CHO from membranes of various composition

We have then performed the extraction of CHO with increasing concentrations of MBCD from membranes, where ratio of DOPC, SM and CHO was varied (Figs. 5 and 6). The extraction of CHO was rapid from DOPC liposomes in the absence of SM. It was almost complete in 30 min even when the lowest 0.25 mM concentration of MBCD was used (Fig. 5A). The presence of SM slowed down the extraction to quite a considerable level, and the final amount of extracted cholesterol

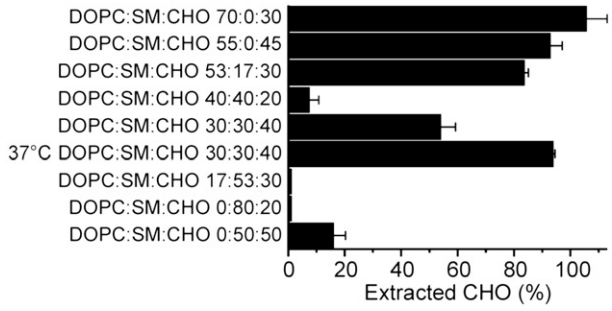

Fig. 6. Extraction of CHO from liposomes of various compositions. The amount of extracted CHO from liposomes of various compositions after 30 min depletion with 0.5 mM MBCD. Experimental conditions are the same as in Fig. 3. We have assumed that only CHO was extracted with MBCD and that the contributions of CHO and DOPC to the refractive index change were similar. $n = 3–6$, average ± S.D.

was also decreased (Fig. 6), as shown for DOPC:SM:CHO 53:17:30 (Fig. 5B) and DOPC:SM:CHO 30:30:40 (Fig. 5C). The extraction of cholesterol was almost negligible for DOPC:SM:CHO 40:40:20 (Fig. 5D), DOPC:SM:CHO 17:53:30, and DOPC:SM:CHO 0:80:20 (Fig. 6) or other SM:CHO mixtures (data not shown). We have also checked whether the temperature affected the extraction of CHO from DOPC:SM:CHO 30:30:40 liposomes. Both the final amount of desorbed material and the kinetics of the extraction were considerably changed when extraction proceeded at 37°C (Fig. 6 and see below). We have also observed that the stability of the liposomes attachment on L1 chip was dependent on the presence of SM and may be connected with the rigidity of the membrane. At high SM concentration in liposomes, controls were desorbed more than the tested liposomes that contained also CHO, i.e. DOPC:SM:CHO 40:40:20, DOPC:SM:CHO 17:53:30, or any mixture of SM and CHO. The resulting curves showed artifacts at the beginning of the association phase (see the increase at the beginning of the injection in the case of DOPC:SM:CHO 40:40:20 in Fig. 5D), but anyway allowed a clear conclusion that the amount of extracted CHO was none or extremely low for these mixtures. These curves were not used for further analysis.

3.4. The kinetics of CHO extraction

The data for 0.5 and 2 mM MBCD of some of the tested compositions were fitted to a mono- and bi-exponential model to obtain the apparent rates of cholesterol extraction (Fig. 7). It was possible to use mono-exponential curves to fit the data for DOPC:CHO 70:30, yielding reasonably minor residuals (Fig. 7A). The linear transformation of the response curves

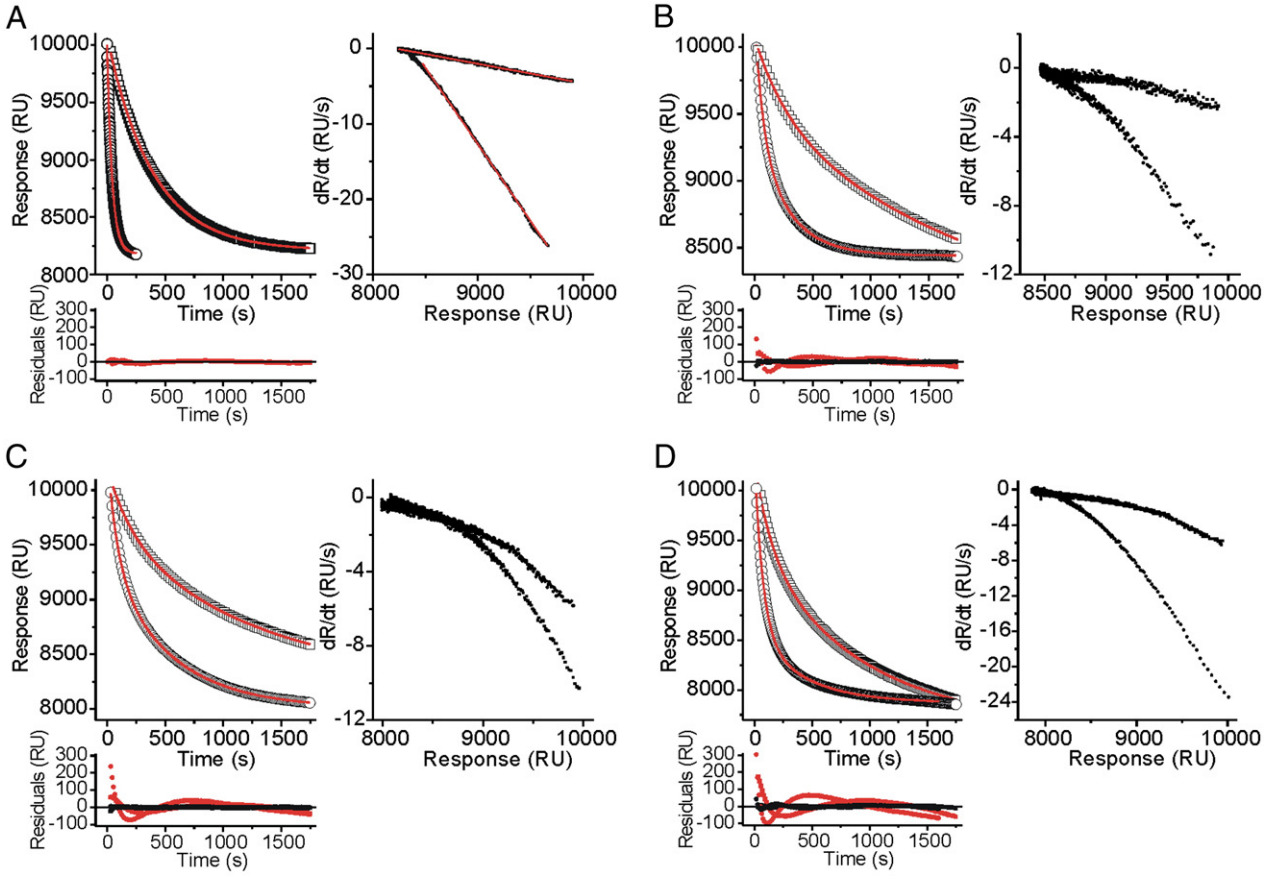

Fig. 7. Fits to the experimental data. Experimental data for the extraction of CHO with 0.5 (squares) or 2 (circles) mM MBCD were fitted to a mono-exponential decay (panel A) or bi-exponential decay (panels B–D). The plot on the right shows the linear transformation of the data on the left side. The transformed data of panel a were fitted to a linear function (red line) and the derived rates, from the slopes of the curves, are reported together with other parameters in the Table 1. The residuals are also shown for the fit to the mono-exponential decay (solid symbols) or bi-exponential decay (open symbols). (A) DOPC:SM:CHO 70:0:30; (B) DOPC:SM:CHO 53:17:30; (C) DOPC:SM:CHO 30:30:40; (D) DOPC:SM:CHO 30:30:40 when extraction was performed at 37°C.

(right hand plots in Fig. 7) confirmed the fitting model selection, as the data for DOPC:CHO 70:30 yielded the linear relationship. The derived parameters of the fits are reported in Table 1. The apparent rates of extraction in the case of DOPC:CHO 70:30, determined from the slopes of curves after linear transformation, were similar to rates obtained by fitting the experimental data to a mono-exponential curve. At 2 mM MBCD half of the cholesterol was extracted in approximately 30 s. The kinetics of extraction was more complex for compositions that contain SM and fitting to mono-exponential curve produced poor fits and

Table 1
The kinetic parameters for the extraction of CHO with 0.5 or 2 mM MBCD derived from the data presented in Fig. 7

Lipid composition	Fast			Slow			n [a]	Linearisation	n [a]
	k	t_{50}	A	k	t_{50}	A		k	
	$s^{-1} \times 10^{-3}$	s	RU	$s^{-1} \times 10^{-3}$	s	RU		$s^{-1} \times 10^{-3}$	
0.5 mM MBCD									
DOPC:SM:CHO 70:0:30	2.8±0.9	271±83	1814±84				4	2.8±1.0	4
DOPC:SM:CHO 53:17:30	3.4±0.6	220±24	575±125	0.4±0.2	2276±797	1894±377	3	n.d.	
DOPC:SM:CHO 30:30:40	5.8±1.9	131±46	389±134	0.9±0.3	832±310	1485±102	4	n.d.	
DOPC:SM:CHO 30:30:40 37°C	6.7±0.4	105±7	871±36	1.1±0.02	648±17	1789±36	3	n.d.	
2 mM MBCD									
DOPC:SM:CHO 70:0:30	20.9±2.1	33±4	1828±43				3	22.4±2.5	3
DOPC:SM:CHO 53:17:30	15.8±0.8	46±2	814±6	3.7±0.3	192±6	982±10	4	n.d.	
DOPC:SM:CHO 30:30:40	10.8±1.0	65±6	850±83	1.7±0.2	411±56	1456±77	6	n.d.	
DOPC:SM:CHO 30:30:40 37°C	13.3±0	52±0	1797±16	2.0±0.2	344±28	745±104	2	n.d.	

n.d., not determined.
[a] Number of experiments.

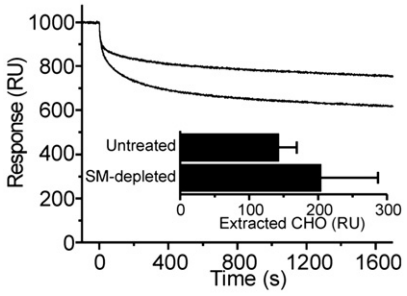

Fig. 8. Extraction of CHO from erythrocyte ghosts. Erythrocyte ghosts were prepared from human erythrocytes and immobilized on the surface of an L1 sensor chip to approximately 1000 RU. The depletion of cholesterol was performed with 0.5 mM MBCD for 30 min at 25°C. The ghosts were prepared from untreated erythrocytes (upper curve) or from SM-depleted erythrocytes (lower curve). The curves are corrected for the contribution of MBCD to the refractive index. The representative curves of five experiments from two independent ghost preparations are shown. Inset: the amount of extracted cholesterol after 30 min of depletion. $n=5$, average±S.D.

high residuals; so bi-exponential decay model was used instead (Fig. 7B–D and Table 1). The two phases had t_{50} of 46–65 s and 3–7 min, respectively, when 2 mM MBCD was used. We performed extraction also at 37°C for the mixture DOPC:SM:CHO 30:30:40. In comparison to 25°C the extraction was more rapid and more CHO could be extracted when 0.5 mM MBCD was used (Fig. 7C, D and Table 1).

3.5. The extraction of CHO from erythrocyte ghosts

Finally, we have checked the extraction of cholesterol from human erythrocyte ghosts. Approximately 150 RU were extracted from untreated erythrocytes, when approximately 1000 RU of ghosts were immobilized on the surface of L1 chip (Fig. 8). The experimental curves could be fitted to a bi-exponential model with apparent rate constants of 0.035 s^{-1} and 1.2×10^{-3} s^{-1} for the fast and the slow step, respectively (data for the curve in Fig. 8). The removal of SM from erythrocyte membranes by bacterial sphingomyelinase resulted in the increase of extracted cholesterol (Fig. 8), which is in the agreement with the data obtained on liposomes.

4. Discussion

In this paper we have developed an SPR approach to monitor the extraction of CHO by MBCD. The obvious advantage of SPR approach in comparison to isothermal titration calorimetry [11,27] and other approaches [4,5,8,17,18,26] is that it is possible to obtain data in real time and without radioactive or fluorescent labeling. The developed approach allowed us to test the hypothesis that the slow pool of extracted cholesterol in cell culture studies may be due to lateral segregation of lipids.

Our data indicate that MBCD does not partition in the DOPC membrane (Fig. 1), does not change the size of the DOPC LUV (Fig. 2), and neither perturbs membrane bilayer and induces calcein release from liposomes (Fig. 2). When CHO was present the vesicles shrank upon removal of CHO, but again without any calcein release (Fig. 2). Eventual escape of calcein was tested by on-chip permeabilisation assay, when calcein loaded vesicles immobilized on the surface of L1 chip were treated by MBCD, buffer coming from the cell collected and checked for the fluorescence [33]. In that case calcein release was always less than 10% from calcein-loaded liposomes composed of DOPC or DOPC:CHO 60:40, when up to 2 mM concentrations of MBCD were used. Our data are thus in accordance with other studies that showed no binding of MBCD to lipid membranes [1,4] and no appreciable extraction of other lipids [10,11,27].

The removal of CHO from mixed DOPC:CHO membranes was fast, and proceeded in seconds when mM concentrations of MBCD were used. According to experiment with cholesterol-dependent cytolysin streptolysin and fluorescent CHO analogue, NBD-CHO, it was also complete. The extraction of NBD-CHO was extremely rapid, as the majority of NBD-CHO present in liposomes was extracted in approximately 2 min. It is interesting to note that a bulky fluorophore group attached to the isoalkyl chain of CHO did not affect much the interaction with MBCD. It seems that the main interaction of CHO with MBCD is mediated by the steroid ring system. As we could observe hardly any fluorescence after the extraction, i.e. only 14% of NBD-fluorescence remained (Fig. 4), clearly both monolayers were depleted of NBD-CHO, indicating that the flip-flop diffusion for this particular lipid occurs within 1 min. When CHO was used in DOPC liposomes we could extract almost complete CHO in less than 2 min with 2 mM MBCD, i.e. the t_{50} was 33 s (Fig. 5 and Table 1). The observed decrease in response signal approximately corresponds to the amount of CHO present in lipid membranes (we have assumed that the contributions of CHO and DOPC to the refractive index change were similar). Hence, from the described results we could estimate that the CHO flip-flop diffusion in DOPC membranes is completed in less than 1 min. This estimate is similar to those of other authors who used MBCD; they have also estimated the half times for interbilayer cholesterol flip-flop movement to less than 1–2 min [10], while Steck and coworkers have it estimated to be less than 1 s when using MBCD [8]. In either case, the cholesterol flip-flop diffusion seems to be a relatively fast and not rate-limiting process, and thus does not influence significantly the rate of cholesterol extraction from membranes [10].

The presence of SM drastically slowed down the extraction of CHO from liposomes (Fig. 5) and erythrocyte ghosts (Fig. 8), in accordance with the notion that the CHO efflux from cellular and model membranes is slower in the presence of SM [17,19–22]. The bi-exponential kinetics was used to evaluate the data for mixtures DOPC:SM:CHO 53:17:30 and DOPC:SM:CHO 30:30:40. The two phases could conceptually correspond to the existence of two CHO populations, one that is associated with SM and another that is not. The CHO population not associated with SM should be extracted rapidly, and indeed, the t_{50} roughly corresponds to that for CHO extracted from DOPC liposomes. The t_{50} for the other CHO fraction was from approximately 3 to 7 min. The relative amount of each population depended on the amount of SM in the liposomes. In DOPC:SM:CHO 53:17:30 mixture both populations were equally represented, as the RU amplitudes were similar when CHO was

extracted with 2 mM MBCD. In contrast, in DOPC:SM:CHO 30:30:40, more CHO was associated with SM and this was reflected in the amplitude of the slow phase, which was almost twice as big as the amplitude of the fast phase. When the extraction of CHO from this mixture was performed at 37°C, the ratio actually reversed and more CHO was extracted during the fast phase, since the membrane was more fluid and CHO was less tightly associated with SM. Our study also suggests that liquid-ordered and liquid-disordered lipid domains, resulting on de-mixing of CHO and SM combined with phosphatidylcholine, account for differential CHO extraction by MBCD. One reason for the slower depletion of CHO from liquid ordered phases, typical for SM:CHO mixtures, may be a higher affinity of CHO for SM than for phosphatidylcholine [39]. However, another possibility could not be excluded at present. Namely, as compared to DOPC headgroup that of SM may be oriented differently, thus hindering CHO in an umbrella-like manner from the MBCD accession [40].

We have performed experiments at 25°C and by using lower concentrations of MBCD (up to 2 mM). In cell biology, a CHO-depletion experiment is usually performed at 37°C, with higher MBCD concentrations (not uncommon up to 10 mM) and even by using other CD. Despite of this, our data clearly indicate that the kinetics of CHO extraction by MBCD from lipid membranes of composition that favors formation of lipid domains is complex and indicate that the complex kinetics observed in experiments with cells may be the consequence of lateral segregation of lipids and CHO interaction with SM [4,5,17,18]. As seen in Table 1, less CHO was associated with the fast pool if a higher amount of SM was used. In some cases, even no extraction of CHO was observed (Fig. 6), indicating that CHO associated with SM is harder to extract then CHO associated with DOPC. This suggests that one should take extra care when performing the MBCD extraction on cells. It is quite possible that only CHO in lipid disordered domains, i.e. associated with unsaturated lipids in cell membrane, would be preferentially extracted, if low MBCD concentration or short extraction times are used.

Acknowledgements

This work was supported by grants from the Slovenian Research Agency.

References

[1] Y. Ohtani, T. Irie, K. Uekama, K. Fukunaga, J. Pitha, Differential effects of alpha-, beta- and gamma-cyclodextrins on human erythrocytes, Eur. J. Biochem. 186 (1989) 17–22.
[2] J. Pitha, T. Irie, P.B. Sklar, J.S. Nye, Drug solubilizers to aid pharmacologists: amorphous cyclodextrin derivatives, Life Sci. 43 (1988) 493–502.
[3] T. Irie, K. Fukunaga, J. Pitha, Hydroxypropylcyclodextrins in parenteral use. I: lipid dissolution and effects on lipid transfers in vitro, J. Pharm. Sci. 81 (1992) 521–523.
[4] E.P. Kilsdonk, P.G. Yancey, G.W. Stoudt, F.W. Bangerter, W.J. Johnson, M.C. Phillips, G.H. Rothblat, Cellular cholesterol efflux mediated by cyclodextrins, J. Biol. Chem. 270 (1995) 17250–17256.
[5] P.G. Yancey, W.V. Rodrigueza, E.P. Kilsdonk, G.W. Stoudt, W.J. Johnson, M.C. Philips, G.H. Rothblat, Cellular cholesterol efflux mediated by cyclodextrins: demonstration of kinetic pools and mechanism of efflux, J. Biol. Chem. 271 (1996) 16026–16034.
[6] V.M. Atger, M.M. de la Llera, G.W. Stoudt, W.V. Rodrigueza, M.C. Phillips, G.H. Rothblat, Cyclodextrins as catalysts for the removal of cholesterol from macrophage foam cells, J. Clin. Invest. 99 (1997) 773–780.
[7] S.K. Rodal, G. Skretting, O. Garred, F. Vilhardt, B. van Deurs, K. Sandvig, Extraction of cholesterol with methyl-β-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles, Mol. Biol. Cell 10 (1999) 961–974.
[8] T.L. Steck, J. Ye, Y. Lange, Probing red cell membrane cholesterol movement with cyclodextrin, Biophys. J. 83 (2002) 2118–2125.
[9] G.H. Rothblat, M. de la Llera-Moya, V.M. Atger, G. Kellner-Weibel, D.L. Williams, M.C. Philips, Cell cholesterol efflux: integration of old and new observations provides new insights, J. Lipid Res. 40 (1999) 781–796.
[10] R. Leventis, J.R. Silvius, Use of cyclodextrins to monitor transbilayer movement and differential lipid affinities of cholesterol, Biophys. J. 81 (2001) 2257–2267.
[11] A. Tsamaloukas, H. Szadkowska, P.J. Slotte, H. Heerklotz, Interactions of cholesterol with lipid membranes and cyclodextrin characterized by calorimetry, Biophys. J. 89 (2005) 1109–1119.
[12] W.J. Johnson, F.H. Mahlberg, G.H. Rothblat, M.C. Philips, Cholesterol transport between cells and high-density lipoproteins, Biochim. Biophys. Acta 1085 (1991) 273–298.
[13] T.L. Steck, F.J. Kedzy, Y. Lange, An activation–collision mechanism for cholesterol transfer between membranes, J. Biol. Chem. 263 (1988) 13023–13031.
[14] S.L. Niu, B.J. Litman, Determination of membrane cholesterol partition coefficient using a lipid vesicle–cyclodextrin binary system: effect of phospholipid acyl chain unsaturation and headgroup composition, Biophys. J. 83 (2002) 3408–3415.
[15] F.H. Mahlberg, G.H. Rothblat, Cellular cholesterol efflux. Role of cell membrane kinetic pools and interaction with apolipoproteins AI, AII, and Cs, J. Biol. Chem. 267 (1992) 4541–4550.
[16] G.H. Rothblat, F.H. Mahlberg, W.J. Johnson, M.C. Philips, Apolipoproteins, membrane cholesterol domains, and the regulation of cholesterol flux, J. Lipid Res. 33 (1992) 1091–1097.
[17] M.P. Haynes, M.C. Philips, G.H. Rothblat, Efflux of cholesterol from different cellular pools, Biochemistry 39 (2000) 4508–4517.
[18] M. Hao, S.X. Lin, O.J. Karylowski, D. Wustner, T.E. McGraw, F.R. Maxfield, Vesicular and non-vesicular sterol transport in living cells. The endocytic recycling compartment is a major sterol storage organelle, J. Biol. Chem. 277 (2002) 609–617.
[19] L. Fugler, S. Clejan, R. Bittman, Movement of cholesterol between vesicles prepared with different phospholipids or sizes, J. Biol. Chem. 260 (1985) 4098–4102.
[20] S. Lund-Katz, H.M. Laboda, L.R. McLean, M.C. Philips, Influence of molecular packing and phospholipid type on rates of cholesterol exchange, Biochemistry 27 (1988) 3416–3423.
[21] L.K. Bar, Y. Barenholz, T.E. Thompson, Dependence on phospholipid composition of the fraction of cholesterol undergoing spontaneous exchange between small unilamellar vesicles, Biochemistry 26 (1987) 5460–5465.
[22] H. Ohvo, C. Olsio, P.J. Slotte, Effects of sphingomyelin and phosphatidylcholine degradation on cyclodextrin-mediated cholesterol efflux in cultured fibroblasts, Biochim. Biophys. Acta 1349 (1997) 131–141.
[23] H. Ohvo, P.J. Slotte, Cyclodextrin-mediated removal of sterols from monolayers: effects of sterol structure and phospholipids on desorption rate, Biochemistry 35 (1996) 8018–8024.
[24] P.J. Slotte, Sphingomyelin–cholesterol interactions in biological and model membranes, Chem. Phys. Lipids 102 (1999) 13–27.
[25] R.E. Brown, Sphingolipid organization in biomembranes: what physical studies of model membranes reveal, J. Cell Sci. 111 (1998) 1–9.
[26] Y. Lange, J. Ye, T.L. Steck, How cholesterol homeostasis is regulated by plasma membrane cholesterol in excess of phospholipids, Proc. Natl. Acad. Sci. U. S. A 101 (2004) 11664–11667.
[27] T.G. Anderson, A. Tan, P. Ganz, J. Seelig, Calorimetric measurement of phospholipid interaction with methyl-beta-cyclodextrin, Biochemistry 43 (2004) 2251–2261.

[28] D.G. Myszka, Kinetic analysis of macromolecular interactions using surface plasmon resonance biosensors, Curr. Opin. Biotechnol. 8 (1997) 50–57.
[29] J.H. Lakey, E.M. Raggett, Measuring protein–protein interactions, Curr. Opin. Struct. Biol. 8 (1998) 119–123.
[30] R.L. Rich, D.G. Myszka, Advances in surface plasmon resonance biosensor analysis, Curr. Opin. Biotechnol. 11 (2000) 54–61.
[31] M. Beseničar, P. Maček, J.H. Lakey, G. Anderluh, Surface plasmon resonance in protein–membrane interactions, Chem. Phys. Lipids 141 (2006) 169–178.
[32] M.A. Cooper, A. Hansson, S. Lofas, D.H. Williams, A vesicle capture sensor chip for kinetic analysis of interactions with membrane-bound receptors, Anal. Biochem. 277 (2000) 196–205.
[33] G. Anderluh, M. Beseničar, A. Kladnik, J.H. Lakey, P. Maček, Properties of nonfused liposomes immobilized on an L1 Biacore chip and their permeabilization by a eukaryotic pore-forming toxin, Anal. Biochem. 344 (2005) 43–52.
[34] A. Bavdek, N.O. Gekara, D. Priselac, I. Gutiérrez-Aguirre, A. Darji, T. Chakraborty, P. Maček, J.H. Lakey, S. Weiss, G. Anderluh, Sterol and pH interdependence in the binding, oligomerization, and pore formation of Listeriolysin O, Biochemistry 46 (2007) 4425–4437.
[35] W.S. Rasband, National Institutes of Health, Bethesda, MD, 1997–2007.
[36] R. Karlsson, A. Michaelsson, L. Mattsson, Kinetic analysis of monoclonal antibody–antigen interactions with a new biosensor based analytical system, J. Immunol. Methods 145 (1991) 229–240.
[37] T.A. Morton, D.G. Myszka, I.M. Chaiken, Interpreting complex binding kinetics from optical biosensors: a comparison of analysis by linearization, the integrated rate equation, and numerical integration, Anal. Biochem. 227 (1995) 176–185.
[38] R.K. Tweten, Cholesterol-dependent cytolysins, a family of versatile pore-forming toxins, Infect. Immun. 73 (2005) 6199–6209.
[39] H.M. McConnell, M. Vrljic, Liquid–liquid immiscibility in membranes, Annu. Rev. Biophys. Biomol. Struct. 32 (2003) 469–492.
[40] J. Huang, G.W. Feigenson, A microscopic interaction model of maximum solubility of cholesterol in lipid bilayers, Biophys. J. 76 (1999) 2142–2157.