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Changes in biophysical membrane properties induced by the Budesonide/Hydroxy- β -Cyclodextrin complex.

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Abstract

Budesonide (BUD), a poorly soluble anti-inflammatory drug, is used to treat patients suffering from asthma and COPD (Chronic Obstructive Pulmonary Disease). Hydroxypropyl- β -cyclodextrin (HP β CD), a biocompatible cyclodextrin known to interact with cholesterol, is used as a drug-solubilizing agent in pharmaceutical formulations. Budesonide administered as an inclusion complex within HP β CD (BUD:HP β CD) required a quarter of the nominal dose of the suspension formulation and significantly reduced neutrophil induced inflammation in a COPD mouse model exceeding the effect of each molecule administered individually. This suggests the role of lipid domains enriched in cholesterol for inflammatory signaling activation.

In this context, we investigated the effect of BUD:HP β CD on the biophysical properties of membrane lipids. On cellular models (A549, lung epithelial cells), BUD:HP β CD extracted cholesterol similarly to HP β CD. On large unilamellar vesicles (LUVs), by using the fluorescent probes diphenylhexatriene (DPH) and calcein, we demonstrated an increase in membrane fluidity and permeability induced by BUD:HP β CD in vesicles containing cholesterol. On giant unilamellar vesicles (GUVs) and lipid monolayers, BUD:HP β CD induced the disruption of cholesterol-enriched raft-like liquid ordered domains as well as changes in lipid packing and lipid desorption from the cholesterol monolayers, respectively. Except for membrane fluidity, all these effects were enhanced when HP β CD was complexed with budesonide as compared with HP β CD. Since cholesterol-enriched domains have been linked to membrane signaling including pathways involved in inflammation processes, we hypothesized the effects of BUD:HP β CD could be partly mediated by changes in the biophysical properties of cholesterol-enriched domains.

Introduction

The concept of biological membranes has evolved from simple physical barriers providing individualization of the cell and subcellular compartments. This concept evolved to encompass cellular membrane complexity [1–5] regarding its (i) composition, including hundreds of lipid species, glycolipids and proteins; (ii) organization, including asymmetry and lateral domains; and (iii) function, *e.g.* signaling cascades, modulation of protein function and folding, cellular communication, pathogen and drug interaction, among many others.

The presence of non-random domains within the lipid bilayer, *e.g.* the so-called cholesterol and sphingolipid-enriched lipid rafts [6], further supports the functional character of the membrane over a simple structural role. Cholesterol and sphingomyelin (SM)-enriched domains show particular biophysical properties [7] creating an ordered (liquid ordered, l_o) lipid phase within the bulk membrane. Removal of cholesterol by a randomly methylated- β -cyclodextrin (Me β CD) from the lipid bilayer was shown to induce alterations in membrane biophysical properties [8]. Lipid rafts have been linked to several membrane functions including signaling activation by immune receptors such as TLR4 and CD44 [9] involved in inflammation and cancer [10]. Furthermore, changes in lipid membrane composition and/or biophysical properties leading to significant membrane reorganization have been linked to consequent disruption of cell signaling [11].

Cyclodextrins (CD) are cyclic oligosaccharides consisting of six (α CD), seven (β CD) or eight (γ CD) glucopyranose units linked with α -1,4 glycosidic linkages [12]. The toroidal shape and hydrophobic cavity of cyclodextrins allows the formation of inclusion complexes with hydrophobic molecules of adequate size and shape

through non covalent interactions [13]. Hydroxypropyl- β -cyclodextrine (HP β CD) is an FDA/EMA approved β -cyclodextrin derivative with increased water solubility and low toxicity [14]. HP β CD is able to form complexes with surfactants [15] and polymers [16] as well as with drug molecules, such as curcumin [17] and budesonide [18].

Budesonide (BUD), a well-known anti-inflammatory drug is a glucocorticoid commercially available as Pulmicort®. Budesonide is recommended for the treatment of asthma [19,20], acute onset of Chronic Obstructive Pulmonary Disease (COPD) [21], allergic rhinitis [22] and Crohn's disease [23] among others, acting through the direct inhibition of expression of pro-inflammatory mediators [24]. Unfortunately, with a $\log P$ of 3.2, budesonide is practically insoluble in water at physiological pH, leading to low pulmonary deposition [25–27] and reduced bioavailability, requiring the use of relatively high doses in clinical use.

In patients with mild to moderate persistent asthma, budesonide administered as an inclusion complex within HP β CD (BUD:HP β CD) required a quarter of the nominal dose of the suspension formulation due to a marked reduction in nebulization time [25,28]. Moreover, co-administration of budesonide solubilized within HP β CD has been shown to significantly reduce neutrophil induced inflammation in a COPD mouse model exceeding the effect of each molecule administered individually (Rocks et. al, unpublished data).

In this context, a clear understanding of the molecular mechanism of action of the BUD:HP β CD complex is essential to design tailored and optimized therapeutic formulations.

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This work focused on studying the effect of the BUD:HP β CD complex on biophysical properties of lipid membrane. Since the BUD:HP β CD complex is envisaged for aerial administration by nebulization, lung epithelial cells (A549) are used for the study of its cellular toxicity and cholesterol extraction potential.

The effects of BUD:HP β CD on membrane biophysical properties were evaluated using membrane model systems. Large unilamellar vesicles (LUVs) were used to study the interaction

with membrane cholesterol, via a fluorescent analogue of cholesterol (DHE), and changes in membrane fluidity and permeability, by using the fluorescent probes diphenylhexatriene (DPH) and calcein, respectively. Giant unilamellar vesicles (GUVs) were used to visualize the effect on lateral phase separation and lipid organization using fluorescence microscopy. Finally, Langmuir studies characterized the effect on lipid packing and desorption from the lipid monolayer.

Experimental procedures

Chemicals

The L- α -phosphatidylcholine (PC - Egg, Chicken), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), egg sphingomyelin (SM - Egg, Chicken), *N*-palmitoyl-D-erythro-sphingosyl phosphoryl choline (pSM - 16:0 SM d18:1/16:0), L- α -phosphatidylinositol (PI - Liver, Bovine), cholesterol (Chol - ovine wool), ergosta-5,7,9(11),22-tetraen-3 β -ol (DHE - dehydroergosterol), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt) (18:1 Liss Rho-PE) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(biotinyl) (sodium salt) (16:0 Biotinyl PE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). *N*-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)-1,2-Dihexadecanoyl-*sn*-Glycero-3-Phosphoethanolamine, Triethylammonium Salt (NBD-PE) was purchased from Life Technologies (Leusden, Netherlands). 1,6-diphenyl-1,3,5-hexatriene (DPH), avidin from egg white, 16,17-Butyldienebis(oxy)-11,21-dihydroxypregna-1,4-diene-3,20-dione (Budesonide, BUD), Fluorescein-bis(methyliminodiacetic acid) (Calcein), and Sephadex® G-50 were purchased from Sigma-Aldrich (St. Louis, MO-USA). Methyl β -cyclodextrin (Me β CD, Crysmeb®) and Hydroxypropyl- β -cyclodextrin (HP β CD, Kleptose® Oral Grade) were purchased from Roquette (Lestrem, France).

Lipids and lipid probes were dissolved in chloroform, except DPH, which was dissolved in tetrahydrofuran (THF), and were kept at -20°C. The cyclodextrins were solubilized in PBS (NaCl 137 mM, KCl 2.7 mM, Na₂HPO₄ 9.6 mM and KH₂PO₄ 1.15 mM, pH 7.4) at their maximal concentration of 30 mM for Me β CD and 250 mM for HP β CD. Budesonide was firstly dissolved in

DMSO at 100 mM and then diluted to 0.1 mM in a PBS solution (DMSO 0.1% v/v). All organic solvents used were Spectronorm grade from VWR (Radnor, PA, USA) or Emsure grade from Merck (Darmstadt, Germany).

Preparation of the Budesonide-Cyclodextrin complex

The Budesonide-Cyclodextrin complex (BUD:HP β CD) was prepared by adding budesonide to a HP β CD solution in PBS during 48 h under magnetic agitation or 2 h using a T-25 Ultra-Turrax® laboratory mixer from IKA (Staufen, Germany). The amount of budesonide effectively encapsulated was determined using HPLC-MS quantification as described by Dufour *et. al* (2015) [26].

A549 cell culturing, cytotoxicity assay and cholesterol dosage

A549 cells were cultured in DMEM medium – from Thermo-Fisher Scientific (Waltham, MA-USA) – supplemented with 10% of Fetal Bovine Serum (FBS) at 37°C and under 5% CO₂. A549 cells, grown to 80% confluence in 96-well plates, were exposed to Me β CD, HP β CD, BUD:HP β CD complex and budesonide in low-serum conditions (1% FBS). Cell death was inferred from cell membrane permeabilization to cytoplasmic Lactate Dehydrogenase (LDH). LDH activity was measured in triplicate using the Cytotoxicity Detection Kit^{plus} (LDH) version 06 from Sigma-Aldrich (St. Louis, MO-USA).

The amount of protein was determined using the DCTM Protein Assay Kit from Bio-Rad (Hercules, CA-USA).

After extraction, total cholesterol [29] was quantified using the Amplex® Red Cholesterol Assay Kit from Thermo-Fisher Scientific (Waltham, MA-USA).

Preparation of Large Unilamellar Vesicles (LUVs)

Large unilamellar vesicles (LUVs) were prepared by extrusion from multilamellar vesicles (MLVs). Lipids were mixed at the molar ratios of PC:SM:PI (4:4:3) and PC:SM:PI:Chol (4:4:3:5.5) with a probe-to-lipid ratio of 1:100 for DHE and 1:300 for DPH and a final lipid concentration of 10 mM. A lipid film was obtained after solvent evaporation over 2 h, using a R-210 rotavapor from Buchi (Flawil, Switzerland) coupled to a vacuum pump HZ 2C from Vacuubrand (Wertheim, Germany), followed by minimum 2 h in an exsiccator under vacuum. The lipid film was hydrated in Tris-HCl buffer (Tris-HCl 10 mM, NaCl 135 mM, pH 7.4). MLVs were obtained by repeated cycles (x7) of vortex/freezing/thawing. LUVs were obtained by MLV extrusion (x21) using a mini-extruder system from Avanti Polar Lipids (Alabaster, AL, USA) with a 100 nm pore size polycarbonate Nuclepore Track Etch membrane filter from Whatman® (GE Healthcare, Little Chalfont, UK). Total lipids were quantified using the method from Rouser [30] and diluted to the desired final concentration in PBS.

Vesicle Size and ζ -Potential Determinations

LUV mean size and ζ -potential were determined using a Zetasizer Nano SZ equipment from Malvern Instruments (Grovewood Road, UK) with patented NIBS (non-invasive back scatter) technology and the recommended software. Particle size distribution and the polydispersion index (PDI) measurements were performed by Dynamic Light Scattering (DLS) technology using 12 mm square polystyrene cuvettes in a thermostated chamber at 25°C. Particle charge (ζ -potential) was measured using Dynamic Electrophoretic Mobility (DEM) using a disposable folded capillary cell in a thermostated chamber at 25°C.

Fluorescence spectroscopy measurements

All fluorescence measurements were carried out with a LS55 spectrofluorimeter from Perkin Elmer (Waltham, MA-USA) in right angle geometry. Temperature was stabilized at 25°C using a C25P Phoenix II thermostating water bath from Thermo Scientific (Waltham, MA-USA).

Dehydroergosterol (DHE) Spectroscopy

The ability of BUD:HP β CD and HP β CD to bind to cholesterol was investigated using DHE fluorescence spectroscopy.

DHE (10 μ M) was prepared in PBS pH 7.4 containing 0.1% DMSO [31]. Fluorescence emission spectra of DHE in buffer solution were recorded at increasing concentrations of BUD:HP β CD or HP β CD, and the intensity of the monomeric versus microcrystalline peak ratio was plotted against log10 concentration. The excitation monochromator was set at 328 nm, and the emission spectra were recorded from 340 to 545 nm [32,33]. The influence of DMSO and DHE concentration was controlled.

To probe the interaction between BUD:HP β CD or HP β CD with cholesterol in a lipid environment, 1 mol% of DHE was incorporated in LUVs composed of PC:SM:PI:Chol (4:4:3:5.5). Maximal emission of DHE was observed around 372, 404 and 424 nm, as described previously in membrane systems [34]. LUVs (5 μ M) were incubated with increasing BUD:HP β CD or HP β CD concentrations for 3h at 25°C.

Diphenylhexatriene Fluorescence Polarization

Molecule polarization was quantified using steady state fluorescence anisotropy, $\langle r \rangle$, measurements calculated using equation 1:

$$\langle r \rangle = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \text{ (Eq. 1)}$$

where the different intensities I_{ij} are the steady state polarized vertical and horizontal components

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of fluorescence emission with excitation vertical (I_{VV} and I_{VH}) and horizontal (I_{HV} and I_{HH}) to the emission axis. The latter pair of components was used to calculate the G factor ($G = I_{HV}/I_{HH}$).

DPH concentration was determined by UV spectroscopy and adjusted to 100 μM in tetrahydrofuran. Final lipid concentration was adjusted to 50 μM in PBS pH 7.4. LUVs were incubated with BUD:HP β CD or HP β CD for 60 min at 25°C shielded from light.

Calcein Release

Changes in the membrane permeability were followed by determining the leakage of entrapped calcein at self-quenching concentrations, from liposomes [35]. Briefly, the dried lipid films were hydrated with a solution of purified calcein (73 mM) in Tris-HCl buffer at pH 7.4 and osmolarity of 404 mOsm/kg. The un-encapsulated dye was removed by the mini-column centrifugation technique using Sephadex® G-50 [36]. The liposomes were diluted to a final lipid concentration of 5 μM in an isosmotic Tris-HCl (Tris 10 mM and NaCl 188 mM) pH 7.4 buffer and stabilized for 10 min at 25°C. Values were recorded for 30 s before addition of BUD:HP β CD or HP β CD at increasing final concentrations of 10 and 20 mM. After the addition of the compounds, the fluorescence intensities were continuously recorded as a function of time for up to 500 s. The percentage of calcein released was determined according to equation 2:

$$[(F_t - F_{\text{contr}})/(F_{\text{tot}} - F_{\text{contr}})] \times 100 \text{ (Eq. 2)}$$

where F_t is the fluorescence signal measured at a time t in the presence of compounds, F_{contr} is the fluorescence signal measured at the same time t for control liposomes, and F_{tot} is the total fluorescence signal obtained after complete disruption of the liposomes by 0.02% Triton X-100.

Preparation of Giant Unilamellar Vesicles (GUVs)

Giant unilamellar vesicles (GUVs) were prepared using the electroformation method [37–39]. In brief, mixtures of DOPC:pSM (1:1) and DOPC:pSM:Chol (1:1:3) with biotinylated lipid-to-lipid ratio of 1:10⁶ Biotinyl-PE and probe-to-lipid ratio of 1:750 for Rho-DOPE and 1:250 for NBD-PE were prepared. A small volume (4 μL) of lipid mixture (4 mM) was evenly spread on the surface of an ITO coated glass lamella and the solvent was allowed to evaporate over 5 min. A 1 mm thick silicon gasket was used to form a sealed reaction chamber. Sucrose-Tris (475 μL) was added and a second ITO covered glass lamella was overlaid. The GUVs were formed at 60°C over a 2 h exposure to a sinusoidal signal with a peak-to-peak intensity of 1 V and frequency of 500 Hz. The GUVs were used within the day.

Fluorescence Microscopy Measurements

GUVs were used to visualize the lipid lateral segregation and phase separation. GUVs were placed in a μ -Slide 8-well chamber from Ibidi (Martinsried, Germany) previously coated with avidin 0.1% for a minimum of 2 h. GUVs were observed using an Axio Observer Z1 inverted microscope (Carl Zeiss, Jena, Germany) equipped with a model CSU-X1 spinning disk (Yokogawa Electric Corporation, Tokyo, Japan) and a Plan-Apochromat 100x/1.40 Oil DIC M27 objective (Carl Zeiss, Jena, Germany). Images were recorded and analyzed with an AxioCamMR3 camera using Carl Zeiss AxioVision® 4.8.2 software. The red channel was used for Rho-DOPE (excitation/emission at 561/617 nm) and in the green channel for NBD-PE (excitation/emission at 488/530 nm).

Surface Pressure–Area (π -A) Compression Isotherms

To examine the effect of BUD:HP β CD and HP β CD, on lipid packing, surface pressure–area

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(π -A), compression isotherms were recorded with an automated Langmuir trough (KSV Mini-trough KSV Instruments Ltd., Helsinki, Finland-width = 7.5 cm, length = 37 cm), two hydrophilic Delrin mobile barriers (symmetric compression), a platinum Wilhelmy plate, and a temperature probe. The system was enclosed in a Plexiglas® box, and the temperature was maintained at $22.0 \pm 1.0^\circ\text{C}$.

The cleanliness of the surface was ensured by aspiration of the sub-phase surface before each experiment. Once the temperature was stabilized, the barriers were fully closed and reopened and, if a variation in surface pressure of less than 0.5 mN/m was observed, the lipid was deposited on the air-liquid interface surface with a micro-syringe (Hamilton, USA). The platinum plate was cleaned by rinsing with isopropanol and heating to red glow in-between experiments. PBS pH 7.4 was used as the subphase. Lipids (Chol, SM, POPC, or the negatively-charged lipid, PI) were dissolved at a concentration of 2 mM in CHCl_3 :MeOH (2:1 v/v) and were spread at the liquid/air interface with a micro-syringe (Hamilton, USA). The volume was chosen in order to obtain an optimal isotherm compression curve (starting at zero mN/m and showing a collapse at the end of the compression).

After an equilibration time of 15 min, the film was compressed at a rate of 10 mm/min. BUD:HP β CD (0.04:1 mM:mM) or HP β CD (1 mM) were solubilized in the subphase before spreading the lipid using the same amount of lipid as for the control assays. The same procedure as the one used for experiments without cyclodextrin was applied. Each compression isotherm was repeated at least two times; the relative standard deviation in surface pressure and area was $\leq 5\%$.

Surface Pressure–Time (π -t) Adsorption Isotherms

BUD:HP β CD or HP β CD effect on lipid molecular area were assessed by measuring lipid molecular area over time upon incubation with the compounds (π -t isotherms). To this end, the same set-up as described for the surface pressure–area (π -A) compression isotherms was used with a different automated Langmuir trough (KSV Mini-trough KSV Instruments Ltd., Helsinki, Finland-width = 7.5 cm, length = 20 cm).

The lipids (Chol, SM, POPC, or PI) were added until a surface pressure of 30 mN/m was achieved and, after a stabilization period of 15 min, surface pressure over time was recorded. After the acquisition of a 200 s baseline to verify the stability of the monolayer, the compounds were injected into the subphase using specialized injection supports to a final concentration of 0.04:1 mM:mM of BUD:HP β CD or 1 mM of HP β CD. Surface pressure was recorded until a plateau was observed.

The obtained curves were analyzed by a fitting on a 2-phase exponential regression from where the estimated plateau values were extracted.

Statistical Analysis

All data manipulation, graphical presentation and statistical analysis was performed using Microsoft® Excel® (2016, Microsoft®, Redmond – Washington USA) and GraphPad Prism® (version 4.03 for Windows, GraphPad Software Inc., La Jolla - California USA, www.graphpad.com).

Results

Cell toxicity and cell cholesterol depletion

We first evaluated the cytotoxicity of the complex BUD:HP β CD in comparison with the highly hydrophobic anti-inflammatory drug, budesonide (BUD) and HP β CD.

The cytotoxic effect was determined by following LDH release on lung epithelial cells (A549). For concentrations in cyclodextrin varying from 0 to 10 mM and after 4 h of incubation (Fig. 1.a), the cytotoxicity induced by BUD:HP β CD or HP β CD was lower (*ca.* 10%) as compared with budesonide. At 25 mM in cyclodextrin and over, significant toxicity (*ca.* 40%) was observed. Regardless the concentrations in cyclodextrin (from 0 to 25 mM), the cytotoxicity induced by the complex (BUD:HP β CD) was lower as compared to that observed with budesonide alone. At 50 mM in cyclodextrin, cytotoxicity was comparable for HP β CD, BUD:HP β CD and budesonide. Regarding the time dependency cytotoxic effect induced by BUD:HP β CD (0.41:10 mM:mM), budesonide (0.41 mM), and HP β CD (10 mM) (Fig. 1.b), we didn't have any effect for 2 hours of incubation. After 4 hours, LDH release started and after 24 hours of incubation, BUD:HP β CD and budesonide, showed more than 35% of LDH release. In comparison, HP β CD induced less than 10% LDH release.

Selecting non-toxic conditions, we quantified the extraction of cholesterol from cells (Fig. 1.c).

Interaction with sterols in aqueous solution

The interaction of BUD:HP β CD and HP β CD with cholesterol was studied using a cholesterol analogue presenting similar behavior in aqueous solution and biological membranes [33,34,40], the dehydroergosterol (DHE). Especially, it shows similar properties regarding lateral phase separation compared to cholesterol [41]. Lateral

Me β CD (5 mM) was used as a positive control. For BUD:HP β CD (0.41:10 mM:mM) and HP β CD (10 mM), after 45 min of incubation, the amount of total cholesterol (normalized to total protein) was reduced by *ca.* 45%. HP β CD was able to significantly extract cholesterol from the membrane regardless of previous complexation with budesonide.

Interaction with membrane model systems

The effect of the extraction of cholesterol induced by BUD:HP β CD and HP β CD on membrane biophysical properties was further characterized in membrane model systems.

To determine the concentration range of HP β CD without vesicle destabilization, vesicle mean size and ζ -potential were measured upon incubation with increasing concentration of HP β CD (up to 100 mM, Fig. S1). Briefly, at ratios of HP β CD:lipid exceeding 5000:1 (HP β CD 25 mM to lipids 5 μ M), the fraction of vesicles within the diameter range of the control samples became significantly reduced. Therefore, a 25 mM threshold of HP β CD was set to avoid experimental artefacts and/or skewed results.

interactions between sterols are responsible for an increase in DHE fluorescence quantum yield at higher wavelengths of emission [32,42,43]. DHE is a self-quenching molecule and fluorescence emission of the monomeric peak (I_{372}) occurs upon the dissolution of cholesterol-enriched domains [44] or desorption of DHE from the lipid bilayer

[45] by β CDs. DHE aggregates in solution can be quantified using the fluorescence emission ratio I_{372}/I_{424} [34], corresponding to the monomeric species over the aggregate forms, such as DHE microcrystals in solution [34,42]. The spectral properties of DHE, namely fluorescence intensity and peak ratios (I_{372}/I_{424}), are used to infer upon the microenvironment of cholesterol, in agreement with results showing comparable, although slightly faster, extraction of DHE in mixed monolayers (sterols:POPC 30:70) by HP β CD, to that of cholesterol [45].

The interaction of increasing concentrations of BUD:HP β CD and HP β CD with DHE in solution was characterized (Fig. 2) and comparative control studies were performed using Me β CD (Fig. S2, a,b). An increase of the intensity of the monomeric peak at 372 nm (Fig. 2.a), concomitant with an

increase in the ratio of the monomeric over the aggregate form of DHE (Fig. 2.b) was observed. The effect started at 1 mM HP β CD and a plateau value was reached at 10 mM. Regarding the ratio of the monomeric over the aggregate form of DHE (I_{372}/I_{424}), the effect was observed at slightly lower concentrations of HP β CD when complexed with the budesonide. No effect was observed for budesonide alone (not shown). Overall, the increase in fluorescence of the DHE monomeric peak indicates the solubilisation of DHE by the HP β CDs in a concentration dependent manner, which is likely due to the formation of HP β CD-sterol complexes as previously described [45].

Interaction with sterols in a lipid membrane

In order to study the effect of increasing concentrations of cyclodextrin (HP β CD and BUD:HP β CD) on the microenvironment of cholesterol within lipid bilayers, a small fraction of DHE was incorporated into LUVs mimicking the lipid composition of plasma membrane (PC:SM:PI:Chol 4:4:3:5.5) (Fig.3) and comparative control studies were performed using Me β CD (Fig. S2, c,d).

Fig. 3 presents the fluorescence intensity at the 372 nm emission peak of DHE monomeric form (Fig. 3.a) and the ratio of emission at 372 nm over 424 nm (Fig. 3.b) upon interaction with the BUD-HP β CD complex and HP β CD. An increase in fluorescence intensity of DHE (Fig. 3.a) was observed, which was less pronounced as compared with DHE microcrystals in solution. This can be due to the lower amount of DHE within the membranes (200x less concentrated). Moreover, DHE microenvironment can affect β CD potential for complexing with cholesterol [45], the

dissolution of an aggregate form in solution being likely facilitated over the extraction of DHE stabilized within a lipid membrane. The results showed that, regardless of complexation with budesonide, HP β CD was able to increase the fluorescence of DHE monomeric peak. Regarding the ratio of the monomeric over the aggregate form of DHE within the bilayer (Fig. 3.b), it was comparable to that of DHE microcrystals solubilized by the HP β CD (Fig. 2.b). No major difference was observed when the effect of BUD-HP β CD was compared to that of HP β CD.

DHE de-quenching can be due to either the disruption of cholesterol-enriched domains [34] or to extraction from membrane by the HP β CD [45]. Measurements of emission intensity over time (data not shown) showing an instantaneous endpoint de-quenching of DHE, suggest DHE extraction from the membrane instead of the rearrangement of lipid lateral organization, which would occur over several minutes. Overall,

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increase in DHE monomeric peak emission by BUD:HP β CD or HP β CD demonstrated changes of the sterol environment in agreement with DHE extraction from the membrane [44]. Because

cholesterol is a main modulator of membrane fluidity, its extraction from membrane by BUD:HP β CD and HP β CD could modify membrane fluidity.

Effect on membrane fluidity

Cholesterol increases the fluidity of very ordered domains and increases the rigidity of very disordered domains. Moreover, cholesterol and sphingolipid-enriched domains, *i.e.* raft-like domains, present decreased fluidity and hydration when compared to the bulk membrane.

The lipid dynamics of acyl lipid chains can be monitored using diphenylhexatriene (DPH), a dye that probes the hydrophobic core of the membrane [46]. The degree of polarization of DPH, measured by its fluorescence anisotropy ($\langle r \rangle$), increases as membrane fluidity decreases. Fig. 4 shows the variation of DPH anisotropy in LUVs lacking cholesterol (PC:SM:PI 4:4:3) and LUVs containing cholesterol (PC:SM:PI:Chol 4:4:3:5.5) upon interaction with the BUD:HP β CD complex or HP β CD.

For vesicles lacking cholesterol, the results (Fig. 4.a) show a fluid membrane (l_d) in the absence of cholesterol ($\langle r \rangle$ of *ca.* 0.15), as is expected for a lipid mixture containing phospholipid:sphingolipid at a molar ratio of 7:4 at 25°C [47]. Adding cholesterol (Fig. 4.b) reduced membrane fluidity ($\langle r \rangle$ of *ca.* 0.25), indicating the presence of a liquid ordered (l_o) phase typical of a mixture containing *ca.* 33 mol% of cholesterol and 25 mol% of SM at 25°C [47].

Upon incubation of cholesterol-free vesicles with HP β CD (Fig. 4.a), an increase in DPH anisotropy to values suggesting a gel phase ($\langle r \rangle$ of *ca.* 0.30) was observed at the lower concentration of HP β CD (10 mM). Increasing HP β CD concentration caused a concentration dependent increase in membrane fluidity back to control

values. These results are in agreement with literature [48; 49; 50]. Since we excluded increase in average size of liposomes (Fig. S1) as suggested in literature [48; 49], the formation of supra-molecular cyclodextrine structures on the surface of the lipid bilayer is likely the mechanism involved in decrease in membrane fluidity [50].

The incubation of BUD:HP β CD complex or HP β CD with cholesterol-enriched membranes (Fig. 4.b) resulted in a dose-dependent decrease of anisotropy indicating an increase in membrane fluidity. This effect reached a plateau at a value of anisotropy indicative of a very fluid cholesterol-free membrane ($\langle r \rangle$ of *ca.* 0.10) at 30 mM of CD. Moreover, the cholesterol depleted membrane by HP β CD became more fluid ($\langle r \rangle$ of *ca.* 0.10) than the control mixture without cholesterol ($\langle r \rangle$ of *ca.* 0.15). As for the cholesterol-free LUVs, the extraction of other membrane rigidifying lipids, such as the high T_m sphingolipid, sphingomyelin also located within the lipid raft, is likely. Despite a larger affinity of β CD towards cholesterol, other lipids may also be extracted from the membrane with varying affinities [45].

As was observed for DHE extraction from the membrane, HP β CD complexation with budesonide did not show any effect on the induced changes to membrane fluidity as compared with HP β CD. The presence of free budesonide did not change the membrane fluidity.

In conclusion, BUD:HP β CD or HP β CD incubation with cholesterol-enriched membranes lead to increased membrane fluidity. This is in agreement with the extraction of cholesterol and

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destabilization of the liquid ordered cholesterol- and sphingolipid-enriched raft-like domains. Altogether, this suggests a lateral reorganization of

the lipids, which could be associated with increased membrane permeability.

Effect on membrane permeability

Permeation of the plasma membrane is often the first barrier for drug entry into the cell, as is the case for budesonide. Therefore, increased membrane permeability may be one of the possible modulators of drug bioavailability and efficacy. The permeabilization of the membrane can be determined by quantifying the increase in fluorescence emission of the self-quenching calcein upon release from permeabilized membranes. Calcein fluorescence intensity was measured upon interaction of BUD-HP β CD (0.41:10 mM:mM and 0.82:20 mM:mM) or HP β CD (10 and 20 mM) with vesicles lacking cholesterol (PC:SM:PI 4:4:3) and vesicles containing cholesterol (PC:SM:PI:Chol 4:4:3:5.5) (Fig.5 and Fig.S3).

In the absence of cholesterol, BUD:HP β CD (20 mM in cyclodextrins) did not induce calcein leakage. In the presence of cholesterol, the extent of permeabilization increased to reach a plateau value (around 30% of calcein release) after 200s (Fig. 5.top). The effect was dependent upon the concentration of cyclodextrins (10mM < 20 mM in cyclodextrins) (Fig. 5.middle). These results were consistent with the increase in lipid extraction and consequent leakage of encapsulated calcein.

As compared to HP β CD alone, incubation with the BUD:HP β CD increased the rate of calcein release (Fig. 5.bottom) without affecting the percentage of calcein released at equilibrium. The increase in rate of membrane permeabilization may be explained by either (i) permeation of budesonide through the membrane or (ii) increase in HP β CD affinity towards the membrane induced by budesonide.

Effect on membrane phase separation

The interaction of BUD:HP β CD and HP β CD with cholesterol may lead to the disruption of the lipid raft domains, typically composed of cholesterol and sphingolipids.

Confocal fluorescence microscopy was used to observe the changes in lipid phase separation over time upon interaction with BUD:HP β CD or HP β CD. Control GUVs and lipid-raft model GUVs, composed of DOPC:pSM (1:1) and DOPC:pSM:Chol (1:1:3)[50], were labeled with

Rho-DOPE (red channel) and NBD-PE (green channel) for the liquid disordered (l_d) and liquid ordered (l_o) domains, respectively.

Typically, the GUV population was relatively heterogeneous regarding vesicle size with diameters centered on *ca.* 15 μ m ($\pm$ 5 μ m). Overall, no effect was observed by incubation with budesonide.

While the majority of the GUVs immediately presented changes in lipid phase separation upon interaction with the HP β CD, a subpopulation,

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mostly comprised of the very small GUVS (under 10 μm in diameter) began to exhibit observable changes only after more than 30 min of incubation or remained apparently unaffected.

In the absence of cholesterol (Fig. 6-left), the DOPC:pSM 1:1 membranes show a l_d/s_o (red/dark) phase separation as expected [47]. Interaction with either BUD:HP β CD or HP β CD removed any microscopic phase separation and a single l_d phase (red) was observed. As was observed for the kinetics of membrane permeabilization (Fig. 5.c), the effect occurred at earlier incubation times for the BUD:HP β CD (under 5 min) when compared with the HP β CD alone (*ca.* 15 min).

In the presence of cholesterol (Fig. 6-right), no microscopic l_d/l_o phase separation was visible as

expected for a mixture containing 60 mol% of cholesterol. Interaction with the HP β CD, and BUD:HP β CD alike, caused the appearance of observable l_o domains after 5 min of incubation, congruent with a decrease in the molar fraction of cholesterol. Longer incubation times showed further decrease of the l_o phase giving yield to l_d domains (eventually a single l_d phase was visible). Within 45 min, a l_d/s_o phase separation was observed, indicating a negligible amount or the absence of cholesterol within the membrane.

The decrease and, ultimately, the disappearance, of the liquid ordered phase (l_o), a phase enriched in cholesterol and sphingomyelin, is consistent with removal of cholesterol from the membrane by interaction with HP β CD [51].

Effect on lipid mean molecular area

To further characterize the effect of BUD:HP β CD and HP β CD on the biophysical membrane properties, we compared the isotherms of lipid monolayers spread with BUD:HP β CD or HP β CD aqueous solutions to those deposited on PBS buffer (Fig. 7).

In presence of BUD:HP β CD or HP β CD, a long plateau at a non-zero surface pressure was observed at large molecular areas whatever the composition of the monolayer. It suggests that BUD:HP β CD or HP β CD are able to adsorb to the lipid monolayer in a gaseous state [52,53] despite the fact that they do not change the surface pressure by themselves in the absence of lipids.

For all the lipids, further compression of monolayers in presence of BUD:HP β CD and HP β CD induced a progressive increase of the surface pressure indicating the formation of a

liquid-expanded monolayer. Finally, at low molecular areas, the isotherms showed either a final plateau at constant surface pressure or a sharp decrease in surface pressure corresponding to the collapse of the monolayer. In the case of cholesterol (Fig. 7.a) and SM (Fig.7.b) monolayers and in a lesser extent for PI (Fig.7.d) monolayers, the profile of the lipid isotherm in the presence of BUD:HP β CD or HP β CD is different from that expected if the lipid molecules were simply removed from the interface. The lipid/BUD:HP β CD or HP β CD interactions leads to the formation of new system at the interface.

The molecular area and surface pressure at which the membrane collapsed (referred to as A_C and SP_C , respectively) were used to quantitatively compare the interfacial behavior of lipids in presence or in absence of BUD:HP β CD or HP β CD (Fig. 7.e and 7.f).

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In the case of cholesterol monolayers (Fig. 7.a and 7.e), a decrease in A_C (Fig. 7.a and 7.e) was observed with BUD:HP β CD to a higher extent as compared with HP β CD. A reduction of the lipid molecular area in presence of exogenous drug can arise from desorption of the interfacial material into the subphase and/or from a reorganization of the interfacial monolayer (domain packing, nucleation) [53]. The significant decrease in SP_C (Fig. 7.f) in presence of HP β CD and even much more in presence of BUD:HP β CD means that the interaction of HP β CD destabilizes the liquid-condensed phase of the cholesterol monolayer, which is in accordance with the fluidification effect shown by DPH fluorescence polarization.

The injection of BUD:HP β CD or HP β CD beneath the cholesterol monolayer initially spread at the air-water interface until a surface pressure of 30 mN/m and maintained at a constant spreading surface gave rise to a rapid and important decrease of the surface pressure (Fig. 7.a-inset). These results are in favor of cholesterol depletion from the interface, as also shown for β CD in previous studies [45,52,53].

HP β CD and BUD:HP β CD also reduces the A_C of SM monolayers (Fig. 7.b and 7.e) without significantly affecting SP_C (Fig. 7.f) suggesting some lipid desorption from the interface without changing lipid packing. The extraction of SM from the interface by HP β CD was in agreement with the decrease of surface pressure in the time-dependence surface pressure experiments. In

contrast, BUD:HP β CD increased the surface pressure when it is injected under the SM monolayer. This suggests that two parallel phenomena occurs when BUD:HP β CD interacts with the SM monolayer, (i) a limited depletion of SM from the monolayer and (ii) an adsorption of BUD:HP β CD molecules to the interface.

In the case of POPC monolayers (Fig. 7.c,d,e), the presence of HP β CD decreased A_C (Fig. 7 e) but in a lesser extent than in the case of cholesterol and SM (reduction of ~17% for POPC vs ~ 30% for cholesterol and SM). No effect on A_C was observed for the BUD:HP β CD complex with POPC or for either compound with PI monolayer. As for SM monolayer, HP β CD or BUD:HP β CD did not greatly affect the interfacial stability of POPC or PI monolayers (Fig. 7. f). However, while the HP β CD caused lipid desorption from the monolayer (Fig. 7.b-d-insets), the BUD:HP β CD complex increased slightly the surface pressure when injected under the POPC or PI monolayers. Insertion of budesonide and/or BUD:HP β CD into the monolayer can also occurs in these cases.

Overall, the BUD:HPBCD complex shows i) increased extent of destabilization of cholesterol monolayers and ii) adsorption into the phospholipid monolayers when compared to the free HPBCD. This might be due to i) a possible increase in affinity/efficacy of the HP β CD regarding cholesterol-containing membranes due to the presence of budesonide and/or ii) a possible insertion of budesonide into the monolayer.

Discussion

Membrane cholesterol has several important properties including the lateral segregation into cholesterol and sphingolipid-enriched domains known as lipid rafts [6]. These ordered domains have been shown to be essential for creating an appropriate microenvironment for signal reception and transduction. Lipid rafts are able to stabilize and cluster the receptor structures. This provides a sorting mechanism, and co-localizing receptors and cofactors thus being responsible for the fine-tuning of signal transduction [54]. Cyclodextrins and β -cyclodextrin are able to form inclusion complexes with cholesterol. They are commonly used to extract or insert cholesterol from membranes [55–58]. In addition, β CDs are also known to form inclusion complexes with several hydrophobic drugs [17,18,59,60] including budesonide (BUD).

Interestingly, clinical studies [14] showed a lower cellular toxicity of budesonide when budesonide was complexed with HP β CD. The aim of the present study was therefore to understand the potential effect of the extraction of cholesterol in lipid-raft domains in relation with regulation of the inflammatory response induced by the BUD:HP β CD complex. Thus, we characterized the interaction of BUD:HP β CD and HP β CD with lipid model membranes containing and lacking cholesterol and determined changes in biophysical membrane properties.

In model membranes containing cholesterol, we demonstrated an effect of BUD:HP β CD with cholesterol-enriched membranes leading to changes in membrane biophysical properties in agreement with cholesterol extraction, such as increased membrane fluidity and permeability, changes in lipid packing and lipid desorption from the lipid interface as well as the disruption of cholesterol-enriched raft-like liquid ordered domains. In comparison with HP β CD and except

for membrane fluidity, all these effects were enhanced and/or observed earlier with the complex BUD:HP β CD.

The molecular mechanisms involved in cholesterol extraction by HP β CD and BUD:HP β CD was unknown but as demonstrated by Lopez et al, 2011 [50], the distribution of the cyclodextrins on the surface of the monolayer could play a critical role. Spontaneous cholesterol extraction on a nanosecond time scale might be related with a suitably oriented dimer. Moreover, free energy calculations reveal that the cyclodextrins have a strong affinity to bind to the membrane surface, and, by doing so, destabilize the local packing of cholesterol molecules making their extraction favorable [50].

For the model systems lacking cholesterol, the BUD:HP β CD and HP β CD caused an increase in DPH anisotropy for the lowest concentrations and the disappearance of liquid disordered/solid ordered phase separation in GUVs. The increase of membrane rigidity is surprising but can reasonably be ascribed to the formation of a relatively thick polymer layer around the phospholipid bilayers [48]. In the same line, Lopez et al, (2011) [50], reported from simulations studies, the formation of supra-molecular cyclodextrin structures on the surface of cholesterol monolayers. The mechanism of interaction of cyclodextrins with the lipid membrane is suggested to depend on the molecular ratio of cyclodextrins to lipid. Since cyclodextrins possess a greater affinity towards cholesterol when compared to phospholipids, it is possible that the observed increase in membrane rigidity observed for the lowest HP β CD:lipid ratio might reflect non-specific stabilizing cyclodextrin interaction with the surface of the cholesterol-free membrane. For higher HP β CD concentrations the increase in membrane fluidity coming back to values similar to those obtained for controls might

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be explained by extraction of lipids other than cholesterol, namely sphingomyelin. Moreover, at higher HP β CD concentrations, HP β CD:HP β CD interactions could be promoted in comparison with HP β CD:membrane interactions explaining why no global effect was observed on membrane fluidity. Regarding the effect on membrane phase separation, in absence of cholesterol, BUD:HP β CD affected membrane phase separation without increasing membrane permeability and affecting (or only slightly) the stability of SM, POPC or PI monolayers.

A critical question is the potential competition between budesonide and lipid for the HP β CD cavity which may occur at the interface. Focusing on the mechanism involved, the BUD:HP β CD complex induced a greater destabilization of the cholesterol monolayer without affecting HP β CD cholesterol extraction potential (Fig. 7a+inset). It resulted in an increase in surface pressure over time in monolayers composed of phospholipids. These results suggest an insertion of budesonide into the air:liquid interface, an exchange between the budesonide and cholesterol in favor of cholesterol. This could be related to the increased kinetics of membrane permeabilization to calcein induced by the BUD:HP β CD complex when compared with the HP β CD.

Altogether, the results showed that BUD:HP β CD and HP β CD can effectively induce significant changes in the composition and biophysical properties of cholesterol-enriched raft-like domains in model systems. Destabilization of these domains might explain the anti-inflammatory effect observed for the HP β CD (preliminary data), probably by modifying the lipid environment of receptors involved in inflammatory processes. The co-administration of budesonide and HP β CD might provide a higher therapeutic effect acting through complementary anti-inflammatory mechanisms. Thus HP β CD could play a critical role for administration of poorly soluble drug like budesonide by increasing

cellular delivery of budesonide in vivo as well as for regulation of critical biophysical membrane properties of cholesterol-enriched domains where immune receptors are located. HP β CD can be both a targeted delivery vehicle and an anti-inflammatory agent by own.

Further studies about the modulation of the inflammatory response, focusing particularly on the relevance of lipid rafts in signal activation, are required to evaluate the mechanism involved in BUD:HP β CD and HP β CD anti-inflammatory properties.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

AGS and MPML wrote the manuscript and designed experiments.

JB contributed for the membrane fluidity and performed the membrane permeability assays.

GD, DC and BE prepared and characterized the BUD:HP β CD complex used in this study.

MD provided the expertise, equipment and resources to perform the surface pressure–area compression isotherms- and surface pressure–time (π -t) adsorption isotherms- measurements.

All authors discussed the results.

Figure legends

Figure 1: Cell toxicity and effect on cholesterol of BUD:HP β CD, HP β CD, and BUD on lung epithelial cells. Percentage of LDH released from A549 lung epithelial cells **(a)** at 4 h for BUD:HP β CD (squares) 0.04:1 to 2.03:50 mM:mM, HP β CD (circles) 1 to 50 mM in cyclodextrin, or BUD (triangles) 0.04 to 2.03 mM and **(b)** for BUD:HP β CD 0.41:10 mM:mM (squares), HP β CD 10 mM (circles), or BUD 0.41 mM (triangles) at 1, 2, 4 and 24 h. **(c)** Total cellular cholesterol (normalized to total amount of proteins) for A549 lung epithelial cells treated with BUD:HP β CD 0.41:10 mM:mM, HP β CD 10 mM, or Me β CD 5 mM, for 45 min normalized to untreated A549 cells.

Figure 2: Fluorescence of DHE in aqueous solution upon interaction with BUD-HP β CD, and HP β CD. **(a)** Fluorescence emission intensity at 372 nm and **(b)** ratio of peak intensity between 372 and 424 nm upon addition of BUD:HP β CD (squares; dotted line), or HP β CD (circles; solid line). The lines correspond to a non-linear fitting of a non-logarithmic sigmoidal Hill growth function to the data.

Figure 3: Fluorescence of DHE within LUVs upon interaction with BUD:HP β CD, and HP β CD. **(a)** Emission at the monomeric 372 nm peak and **(b)** ratio of emission at 372 nm over 424 nm upon interaction with BUD-HP β CD (squares; dotted line), or HP β CD (circles; solid line), with LUVs composed of PC:SM:PI:Chol 4:4:3:5.5. DHE was present at 1 mol% of total lipid concentration. Measurements were performed at 25°C in triplicate. The lines correspond to a non-linear fitting of a non-logarithmic sigmoidal Hill growth function to the data.

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Figure 6: Confocal fluorescence microscopy imaging of membrane phase separation in GUVs upon incubation with BUD:HP β CD, and HP β CD. Imaging of membrane domains in GUVs composed of (left) DOPC:pSM (1:1) and (right) DOPC:pSM:Chol (1:1:3) before (top, control) and after (descending) 5, 15 and 45 min with the BUD:HP β CD complex or HP β CD. DOPC:pSM vesicles were labeled with Rho-DOPE (red channel) to visualize the liquid disordered (l_d) / solid ordered (s_o) phase separation in red/dark. DOPC:pSM:Chol were labeled with Rho-DOPE (red channel) and NBD-PE (green channel) to visualize the liquid disordered (l_d) / liquid ordered (l_o) phase separation in red/green channels, respectively. The absence of fluorescent labelling indicates a solid ordered phase (s_o) and the co-localization of both probes (yellow) indicates a lack of observable phase separation.

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Figures

Figure 1:

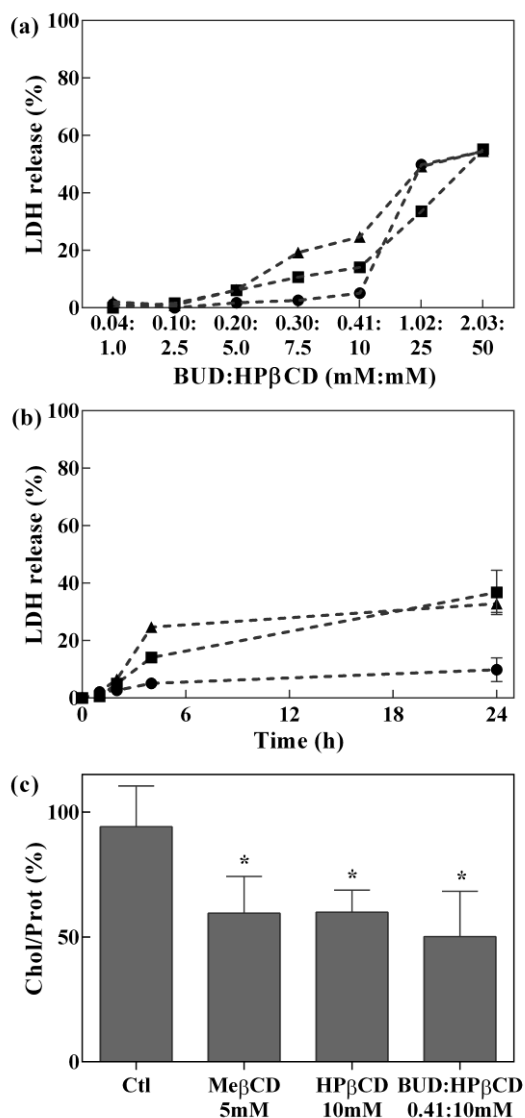


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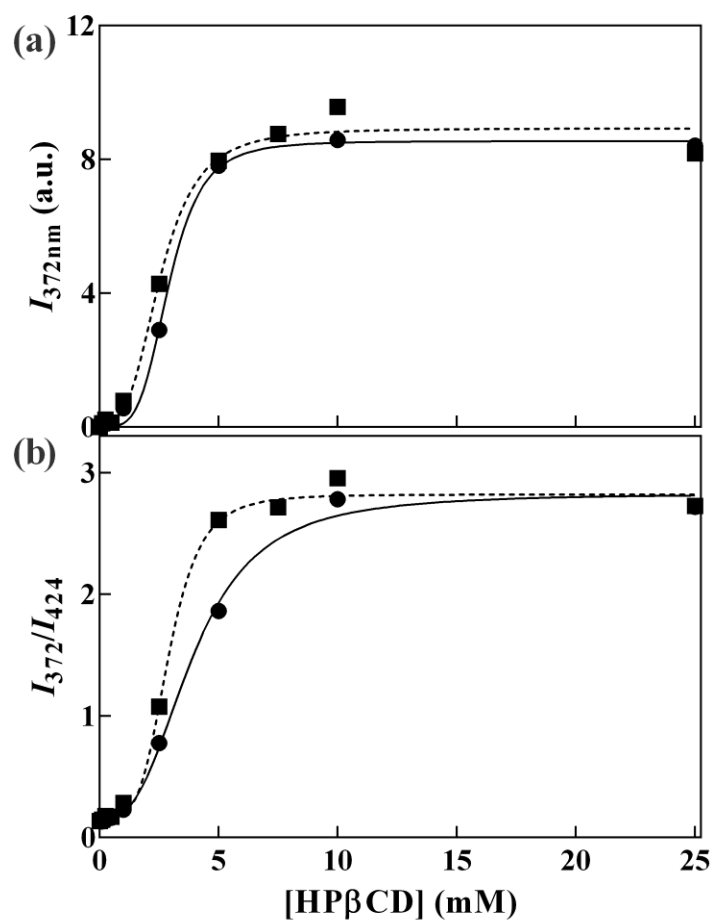


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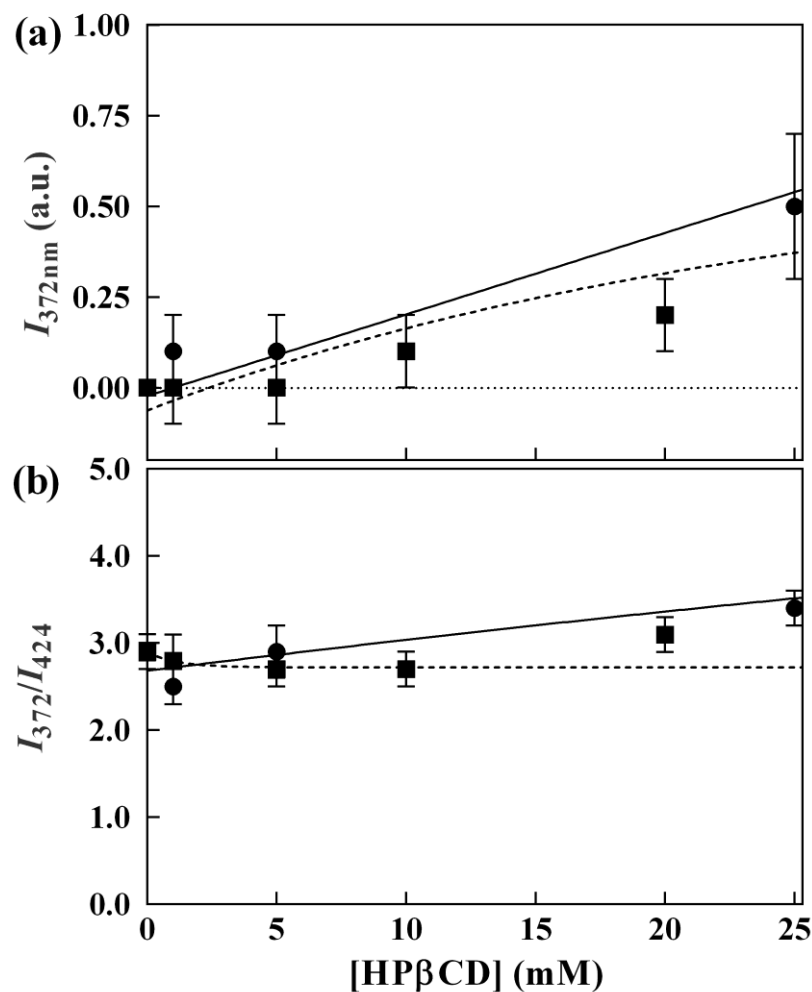


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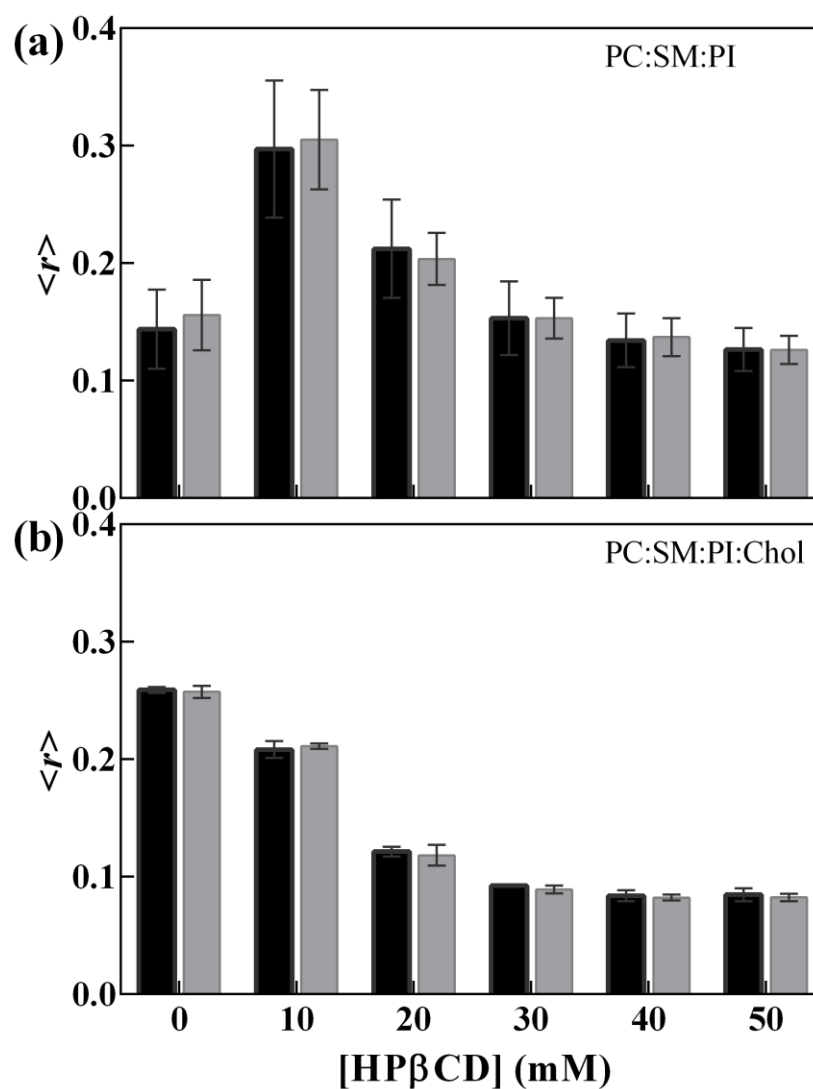


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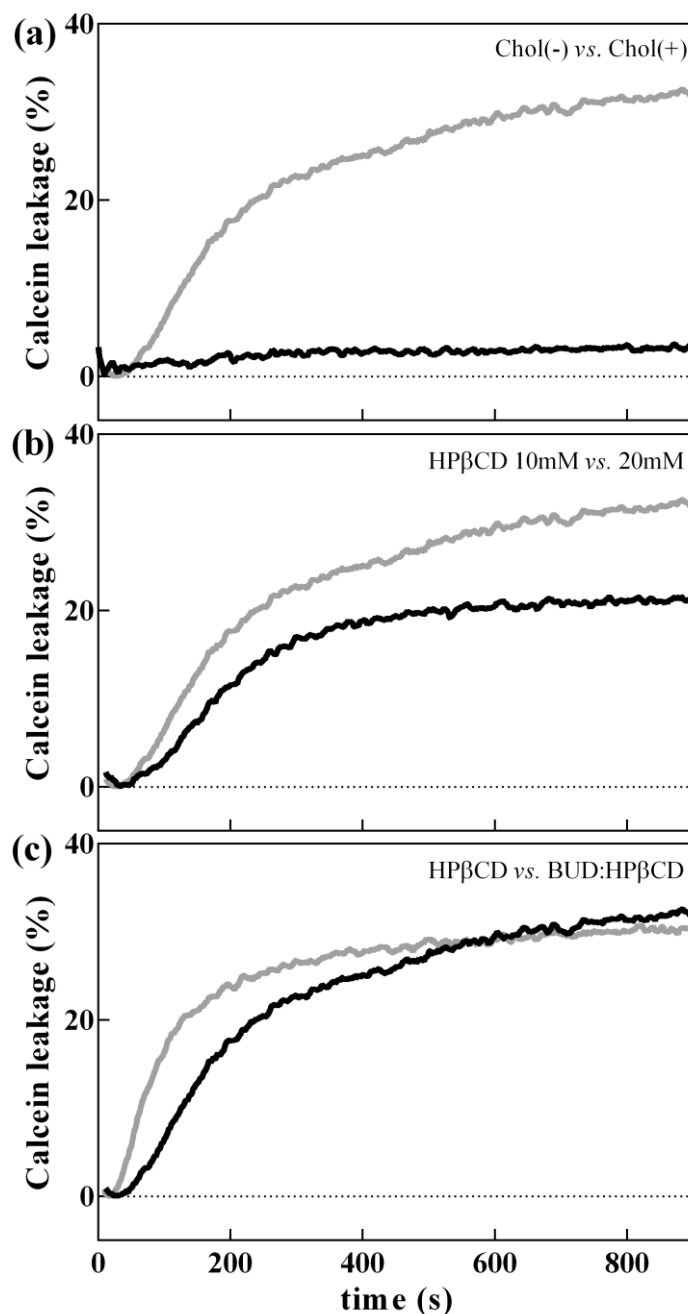


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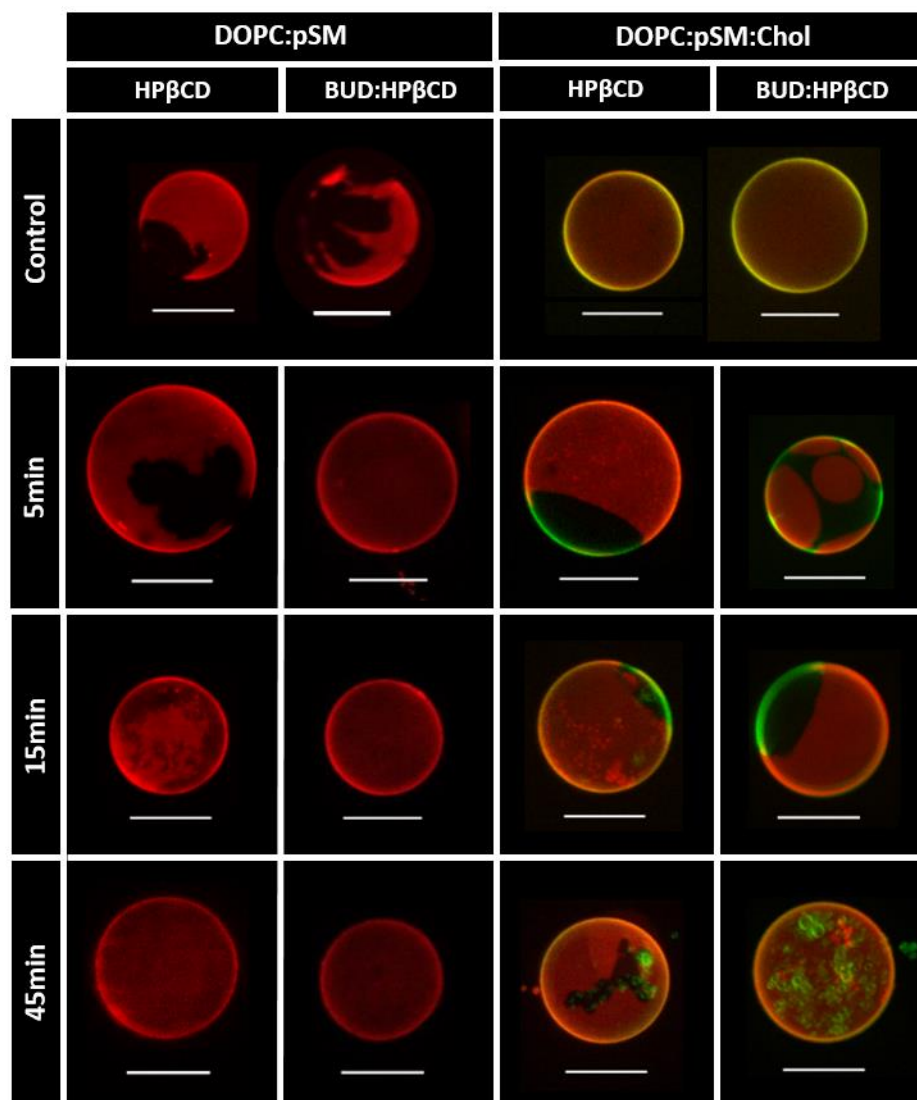


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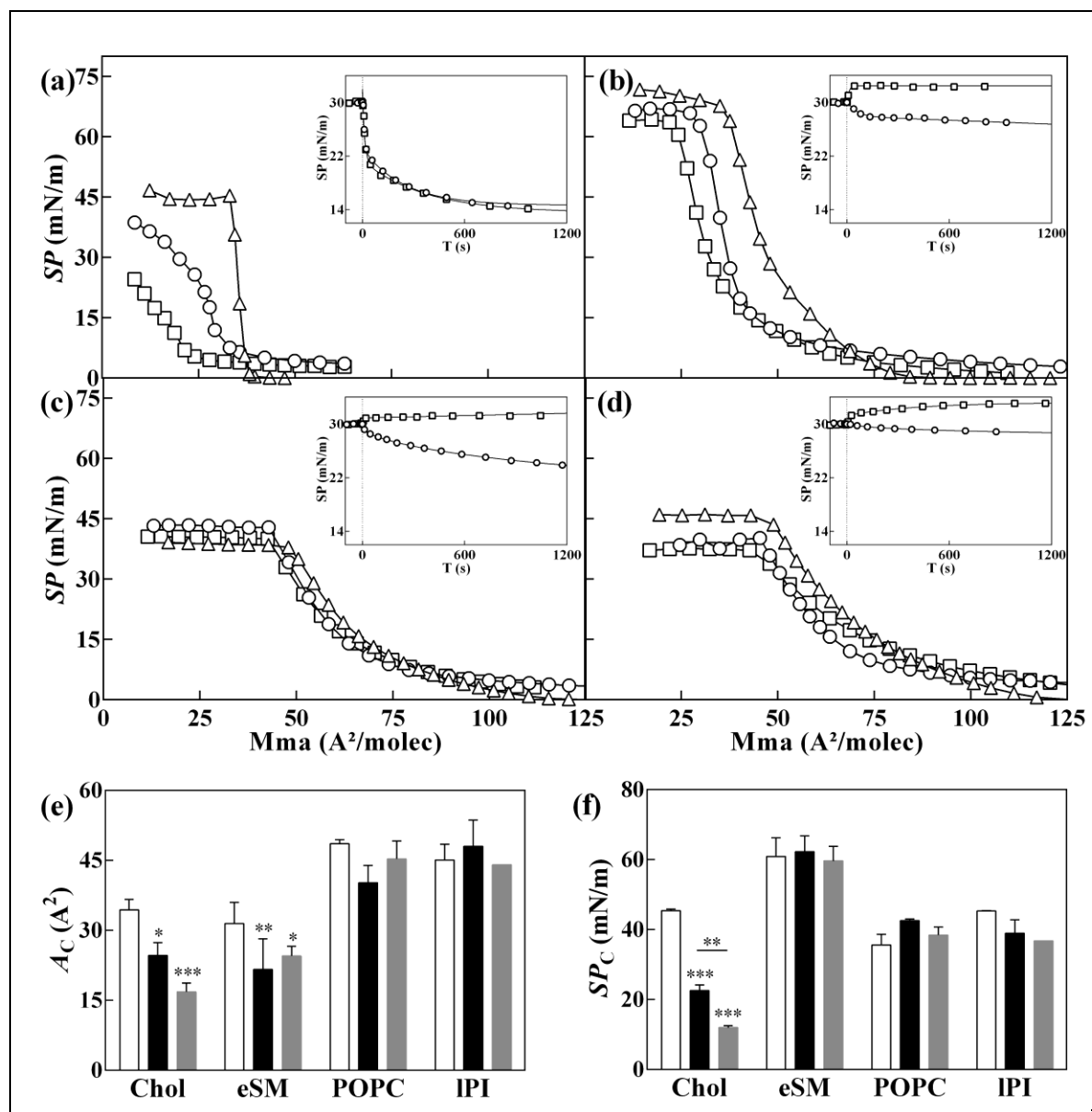


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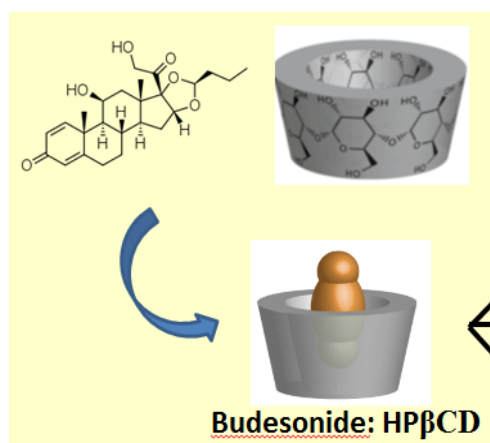
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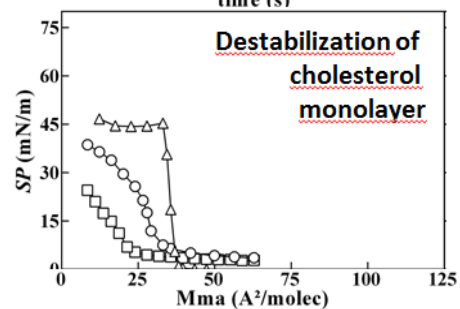
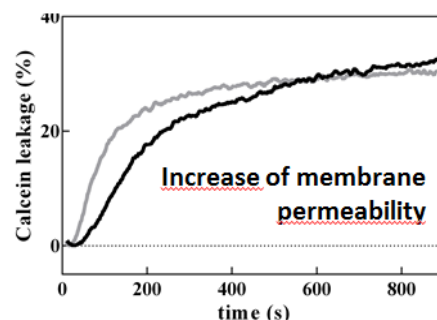
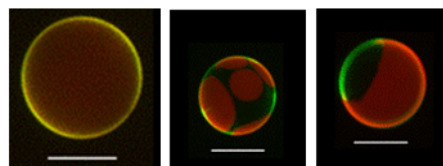
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Decrease of lipid ordered phase



Graphical abstract

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Highlights

The Budesonide-HP β CD complex interacts with cholesterol in a higher extent as HP β CD.

The interaction induces

- increase in fluidity and permeability (LUVs)
- disappearance of the liquid-ordered phase (GUVs)
- cholesterol monolayer destabilization