

Lipoprotein ability to exchange and remove lipids from model membranes as a function of fatty acid saturation and presence of cholesterol

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ABSTRACT

Lipoproteins play a central role in the development of atherosclerosis. High and low-density lipoproteins (HDL and LDL), known as ‘good’ and ‘bad’ cholesterol, respectively, remove and/or deposit lipids into the artery wall. Hence, insight into lipid exchange processes between lipoproteins and cell membranes is of particular importance in understanding the onset and development of cardiovascular disease. In order to elucidate the impact of phospholipid tail saturation and the presence of cholesterol in cell membranes on these processes, neutron reflection was employed in the present investigation to follow lipid exchange with both HDL and LDL against model membranes. Mirroring clinical risk factors for the development of atherosclerosis, lower exchange was observed in the presence of cholesterol, as well as for an unsaturated phospholipid, compared to faster exchange when using a fully saturated phospholipid. These results highlight the importance of membrane composition on the interaction with lipoproteins, chiefly the saturation level of the lipids and presence of cholesterol, and provide novel insight into factors of importance for build-up and reversibility of atherosclerotic plaque. In addition, the correlation between the results and well-established clinical risk factors suggests that the approach taken can be employed also for understanding a broader set of risk factors including, e.g., effects of triglycerides and oxidative stress, as well as local effects of drugs on atherosclerotic plaque formation.

1. Introduction

Atherosclerosis is the largest killer in the west and accounted for an estimated 17.9 million deaths in 2016 [1]. It is a leading cause of cardiovascular disease (CVD), which in turn originates from the build-up of plaque on artery walls. This plaque build-up arises from the deposition of lipoproteins, which are subsequently oxidised, in turn

initiating an active inflammatory process and taken up by macrophages. The macrophages develop into foam cells filled with lipids and cholesterol, over time forming a hard plaque. If this plaque ruptures, blood will be exposed to thrombogenic material, which can result in a heart attack or stroke [2]. Although plaque formation is a complex process, involving not only endothelial cells but also connective tissue, as well as calcification and onset of oxidative and other processes [3,4],

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lipoprotein deposition nevertheless constitutes a key initial step. Despite this, the initial interaction of lipoprotein particles with the membrane wall remains poorly understood, notably in relation to lipid exchange from lipoprotein to the cell membrane and vice versa.

Lipoproteins are particle-like aggregates which consist of a core of cholesterol esters and triglycerides, encased by a monolayer of lipids and apolipoproteins [5]. Among the diverse family of lipoproteins [5], high- and low-density lipoproteins (HDL and LDL) are particularly important in atherosclerosis. HDL and LDL differ in structure, composition and their role in atherogenic processes [6]. It has been shown clinically that LDL deposits into artery walls, aiding the development of atherosclerosis, whilst HDL plays a preventive role by removing cholesterol from the lipid-filled foam cells in a process known as reverse cholesterol transport (RCT) [7,8]. For these reasons, HDL and LDL are frequently referred to as 'good' and 'bad' cholesterol, respectively.

In RCT, HDL removes free cholesterol from blood vessel walls via various pathways [9,10] and eventually transports it to the liver where it is removed from the body. An inverse relationship has been demonstrated between RCT efficiency and the risk of CVD [10], illustrating the importance of this step and the role of HDL in the prevention of atherosclerosis. However, the situation is complex as the quantity of HDL alone does not determine suppression of the risk of CVD, and increased levels of HDL have also been found to result in no effect [11] or even in an increased risk for CVD [12]. Having said that, the ratio of HDL to LDL is of importance [13]. This suggests that there is a delicate equilibrium in the transport of cholesterol and other fats in both directions between the lipoproteins and the endothelial membranes, which determines the onset of atherosclerosis. There are also several other fat components important in the development of atherosclerosis, also transported by lipoproteins. These include triglycerides and lipids with either saturated or unsaturated fatty acid tails.

Whilst the dietary guidelines for the prevention of CVD include the reduction of saturated fats with preference for unsaturated fats, there are further layers of complexity in the types of fats present in foods and their origin in the diet. Reflecting this, there is a striking lack of consensus of whether or not dietary saturated fats can be directly correlated to the development of various lipoprotein types and in turn CVD [14]. There is also little evidence that the presence of cholesterol in the diet impacts the risk of CVD [15].

In our previous work, we developed methodologies to quantify lipid exchange from model cell membranes by lipoproteins (Fig. 1) using both neutron reflection [16] and small angle neutron scattering [17]. Such methodologies allow mechanistic studies of the impact of membrane composition on the capacity of lipoproteins to exchange and remove lipids. For example, neutron reflection results have demonstrated that increasing the content of negatively charged lipids in the membrane (as in cancerous cells [18]) resulted in an increased removal of lipids from the cell membrane bilayer in both HDL and LDL systems [19]. This mirrors the strong interaction of LDL with negatively charged surfaces, mediated by electrostatic interactions with the positive residues in the ApoB protein in LDL [20], and has clinical implications related to calcium signalling in atherosclerotic development [21]. While thus showing promise as a tool for mechanistic studies of lipoprotein-membrane exchange, these previous investigations did not address effects of cholesterol and lipid saturation, widely important for atherosclerotic plaque formation, but with poorly understood underlying mechanisms. Considering this, we hereby employ neutron reflection to distinguish the effects of acyl chain saturation and the presence of cholesterol on the ability of lipoproteins to exchange lipids to/from model membranes.

2. Materials and methods

D₂O (99.9% deuterated, Sigma-Aldrich) was provided by the Institut Laue-Langevin, Grenoble, France and by the ISIS Neutron Source, Didcot, UK. MilliQ water (18.2 Ω cm⁻¹) was used for all cleaning

procedures and solvent preparations. Calcium chloride (CaCl₂) and Tris buffer saline tablets were from Sigma-Aldrich. Tris buffer (50 mM Tris, 150 mM NaCl, pH 7.4) was prepared by dissolving a tablet in H₂O or D₂O as specified by the producer. 1,2-Dimyristoyl-d54-sn-glycero-3-phosphocholine (dDMPC; > 99%) was from Avanti Polar Lipids (Alabaster, AL). 1-palmitoyl, 2-oleoyl-sn-glycero-3-phosphocholine (POPC) in perdeuterated form (dPOdPC, d77; overall purity 95%) and tail-deuterated form (dPOhPC, d67; overall purity 95%) were both provided by the deuteration facility at ANSTO, produced and purified as previously described [22]. Bradford was from Sigma while *infinite* triglyceride and cholesterol enzymatic assays were from ThermoFisher.

2.1. D₂O-matched PC production

Selectively deuterated phosphatidylcholine (dPC) was produced as reported previously [23]. The *Escherichia coli* strain AL95 (pssA::kan^R lacY::Tn9 cam^R), containing the plasmid pAC-PCS_{ip}-Sp-Gm (P_{araB}-pcs Sp^R Gm^R), was grown in 100% deuterated minimal medium [24] with the incorporation of deuterated d8-glycerol, 0.2% arabinose (unlabelled; Sigma-Aldrich) and 2 mM deuterated choline chloride (tri-methyl-d9, 98%; Eurisotop) [23]. After incubation at 37 °C for 24 h, cells were harvested by centrifugation (10,000g, 20 min, 4 °C). Total lipids were extracted by the Bligh and Dyer method [25] and separated according to phospholipid head group via chromatography on a silica-gel column using varying ratios of chloroform and methanol [23].

Thin-layer chromatography was used to further characterise the phospholipids by comparison to known standards [26] and quantitatively assessed through phosphate analysis [27]. The fatty acid composition of the purified dPC was determined previously to be PC 16:0/17:0cyc (where 'cyc' refers to the cyclic unsaturation, also known as propyl unsaturation), PC 16:0/15:0cyc and PC 16:0/18:1 in order of abundance [28].

2.2. Deuterated cholesterol production

Perdeuterated cholesterol was produced and purified as reported previously [29]. The *Pichia pastoris* strain CBS7435 Δ his4 Δ ku70 Δ erg5::pPpGAP-ZeocinTM-[DHCR7] Δ erg6::pGAP-G418[DHCR24] was grown in 100% deuterated basal salts medium in the presence of d8-glycerol (Eurisotop). After 7 days in a fermenter at 28 °C, the batch phase was complete and the fed-batch phase was initiated by constant feeding of d8-glycerol for a further 12 days. The cells were harvested and then isolated using an organic solvent extraction method followed by HPLC to obtain pure cholesterol, verified by GCMS.

The production of dPC and deuterated cholesterol made full use of the Deuteration Laboratory (D-lab) within ILL's Life Sciences Group [30]; this facility is widely used for in vitro production of biomolecules in the interests of biological neutron scattering [31–37].

2.3. Model membrane preparation

Lipid films were prepared from chloroform stocks of the different PC lipids and cholesterol to obtain the desired compositions. Chloroform was evaporated under a stream of nitrogen and the resulting films placed under vacuum overnight.

Prior to use, the lipid films were hydrated to a concentration of 0.2 mg mL⁻¹ in MilliQ water and bath sonicated for 1 h. The lipids were then tip sonicated for 5 min (20% power, 5 s on, 5 s off) immediately before use. To optimise vesicle fusion, lipids were deposited at a concentration of 0.1 mg mL⁻¹ in the presence of 2 mM CaCl₂ [38,39]; freshly tip-sonicated lipids were mixed with an equal volume of 4 mM CaCl₂ solution [16,28], and immediately introduced into the solid-liquid flow cell (pre-equilibrated at 37 °C) by syringe injection. The lipids were incubated for 20 min before rinsing with water, followed by 50 mM Tris saline buffer, pH 7.4. This process leads to a supported lipid bilayer or 'model membrane'.

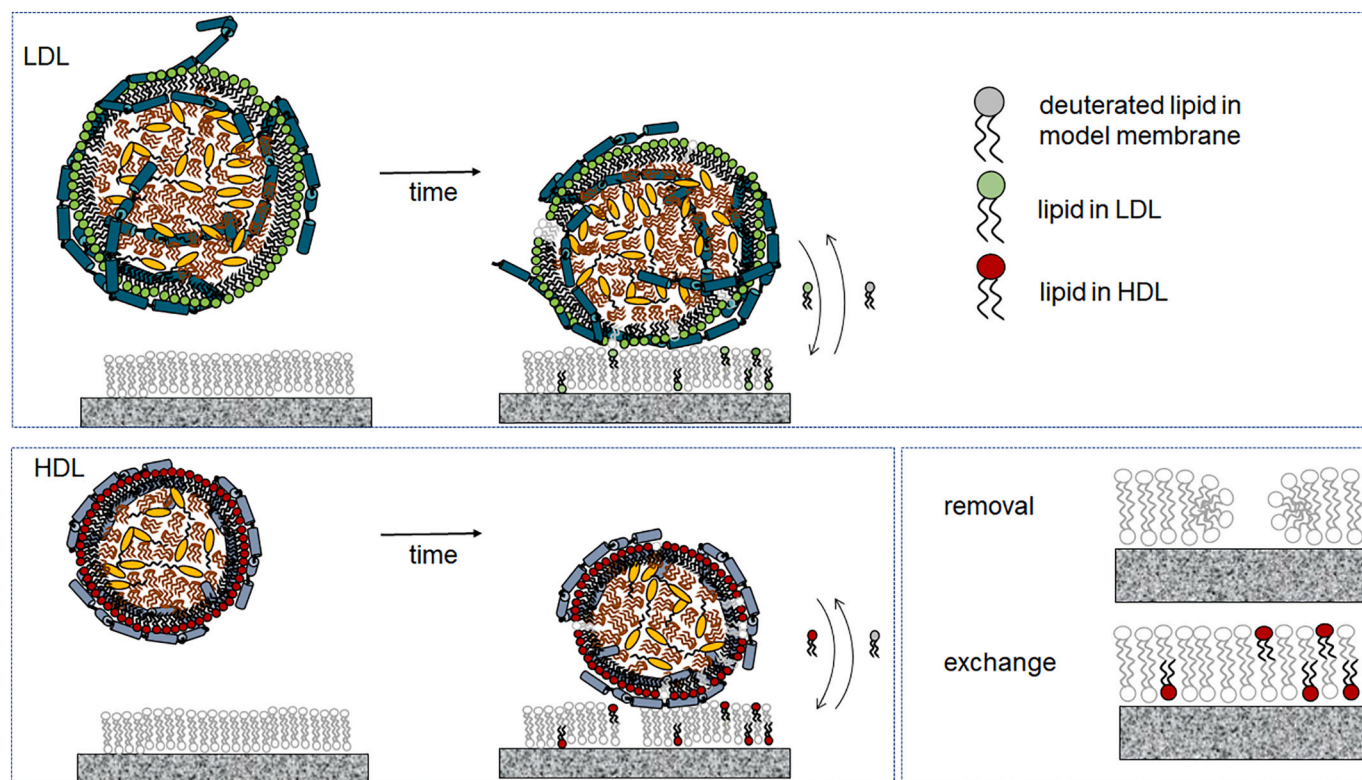


Fig. 1. Schematic illustration of the incubation of lipoprotein particles with the model membranes (not-to-scale: LDL is two to three times larger in diameter than HDL). Deuterated lipids in the model membrane can be exchanged by hydrogenated lipids from the lipoproteins and also removed via uptake by lipoproteins. Neutron reflection can distinguish between deuterated and hydrogenated lipids in the bilayer, and allows quantification of these processes (Cholesterol has not been included in the schematic illustration for clarity, since some systems contain cholesterol and others do not).

2.4. Lipoprotein preparation

Lipoprotein particle preparation was carried out as described before [40]. Human plasma from three healthy males was purified by sequential ultracentrifugation resulting in isolated LDL and HDL at densities of 1.019 and 1.065 g mL⁻¹, respectively. These purified samples were then pooled and stored in 50% sucrose, 150 mM NaCl, 24 mM EDTA, pH 7.4 at -80 °C, and further fractionated via size exclusion chromatography at 25 °C (Superose 6 Increase 10/300 GL column, GE Healthcare) before use in 25 mM Tris, 150 mM NaCl at pH 7.4. Samples were stored at 4 °C, in an inert atmosphere away from light, ready to be used within one week. The Bradford assay was used to determine protein concentration, which in turn was used to calculate lipoprotein concentration. Triglyceride and total cholesterol assays were used as described by the manufacturer. Three different pooled preparations were used for various neutron experiments and the protein, triglyceride, and cholesterol concentrations were used to calculate the total composition in mass ratio (Supporting Information, Table S1). Prior to injection, the lipoprotein solution was diluted to a protein concentration of 0.1 mg mL⁻¹ for LDL and 0.132 mg mL⁻¹ for HDL in 50 mM Tris saline buffer, pH 7.4. These values were chosen to ensure constant lipoprotein particle concentration giving the same number of HDL and LDL particles incubating with each bilayer. The same values were also used in previous studies, enabling direct comparison [16,19]. Samples were injected into the solid liquid flow cells at 1 mL min⁻¹ by a syringe pump via an injection port. The experiments were carried out at 37 °C to resemble the physiological environment and to ensure the fluidity of lipids in the model biomembrane as well as in the core in low density lipoprotein, the latter being solid below 20 °C [40].

2.5. Neutron reflectometry

Neutron reflection data were collected on FIGARO [41,42], a time-of-flight reflectometer, at the Institut Laue-Langevin (Grenoble, France), and at INTER [43] and OFFSPEC [44], both at the ISIS Neutron Facility (Didcot, UK). In specular conditions, an incident neutron beam with wavelength, λ , is directed towards the solid/liquid interface at an incident angle, θ . The reflectivity as a function of the momentum transfer, $q = 4\pi \sin(\theta)/\lambda$, is measured and corresponds to the ratio of incident and reflected intensities. The resulting $R(q)$ profile is linked to a plane-averaged scattering length density (SLD) profile perpendicular to the surface. SLD profiles describe the structure and composition in the direction perpendicular to the surface plane and can be modelled by a series of layers normal to the substrate characterised by a thickness, roughness and SLD.

Momentum transfer ranges of $0.01 > q > 0.3 \text{ \AA}^{-1}$ were measured using wavelengths $2 < \lambda < 20 \text{ \AA}$ and two incident angles, 0.8° and 2.3° on FIGARO, $1.5 < \lambda < 16 \text{ \AA}$ and 0.7° and 2.3° on INTER, and $1.0 < \lambda < 14 \text{ \AA}$ and 0.55° and 1.8° on OFFSPEC, with a spatial resolution ($\Delta q/q$) of 7% at the ILL and 3% at ISIS. The area exposed to the neutron beam was $30 \times 60 \text{ mm}^2$. The experiments were carried out in reflection-up mode to ensure that any aggregated particles settled at the bottom of the cell, away from the surface being measured.

The experiments were carried out using bespoke solid-liquid flow cells at 37 °C. The alternative experimental design of performing the experiments at a fixed temperature difference above the melting transition (e.g., $T_m + 10 \text{ °C}$) of the respective lipid system was not deemed suitable for the present investigation due to the vastly different transition temperatures in the systems investigated. Thus, DMPC has $T_m = 24 \text{ °C}$ and hence the experimental temperature used here is not too far off $T_m + 10 \text{ °C}$. However, $T_m = -2 \text{ °C}$ for POPC and thus $T_m + 10 \text{ °C} = 8 \text{ °C}$. At 8 °C, lipoproteins would behave entirely

differently with, for example, the fluidity of the LDL core depending on temperature which for healthy individuals has a $T_m \approx 30^\circ\text{C}$ [40]. Thus, at 8°C the core in LDL is effectively a gel, dramatically skewing exchange kinetics.

The silicon (111) blocks were cleaned in Piranha solution ($\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$, 7:3 in volume) for 10 min at 80°C before being thoroughly rinsed with MilliQ water. The polyether ether ketone (PEEK) and O-ring components of the cells were intensively cleaned in 2% (v/v) Hellmanex solution twice and MilliQ water via bath sonication, with MilliQ rinsing between each sonication. Buffer contrasts were changed via HPLC pump. Contrasts used were 50 mM Tris saline buffers prepared in D_2O , H_2O , and a mixture of 38:62 $\text{D}_2\text{O}:\text{H}_2\text{O}$ in volume to contrast match the SLD of the silicon block (cmSi). The use of the deuterium isotope increases the SLD of components and can be used to highlight specific parts of the molecule, while the buffer contrasts allow differences to be seen.

Experimental data for the bilayers in the three isotropic contrasts were fitted simultaneously using the MOTOFIT software [45], while the genetic Monte Carlo minimisation algorithm within the software was used to determine error estimation of the fits. Error bars are calculated from the error of the fit and correspond to a single measurement. Initially the silicon surfaces were characterised to ensure they were clean and to determine the thickness and roughness of the oxide layer; these were then fixed alongside the three solvent contrasts for fitting the model membranes. This resulted in a five-layer model consisting of a silicon oxide layer in contact with the silicon block, a small solvent layer, followed by the bilayer. The bilayer itself is split into 3 layers: an inner headgroup (closest to the silicon block), a tail region, and an outer headgroup exposed to the solvent. The three contrasts were fitted simultaneously in order to constrain the fit. The bilayer was fitted to be symmetrical, i.e., the thickness, SLD, and coverage of the inner and outer headgroups were constrained to be equal to each other during the fitting process, whilst the roughness was constrained to be the same across the whole bilayer. These initial parameters were then used as the starting point to fit the characterisations of the membranes after the incubation with the lipoprotein particles. Upon lipoprotein interaction, the silicon oxide layer was kept constant, while the parameters of the other layers were allowed to vary to accommodate the changes (details in Supporting Information; Tables S3 and S4). In order to obtain an acceptable fit and in agreement with our previous work [16], membranes incubated with HDL required one extra layer while the membranes incubated with LDL required two extra layers, likely due to the larger size of LDL over HDL. The size of the extra HDL layer and the first LDL layer were found to be similar (ca. 60 \AA), however, the second layer required for the LDL fit was slightly thicker (ca. 120 \AA). All neutron reflectivity results summarized in the figures in the main manuscript are also given in Supporting Information Tables S3 and S4 to enable the reader to enjoy the specific details so that own calculations can be made. All figures on neutron reflectometry results in the paper are based on the data in these tables.

3. Results

In this work, neutron reflection was used to monitor HDL and LDL lipid exchange with model membranes consisting of either saturated or unsaturated phospholipids, both in the absence and presence of cholesterol. Neutron reflection can differentiate between deuterium-labelled and hydrogen-rich lipids as shown schematically in Fig. 1, allowing quantification of both lipid exchange and lipid removal from the model membranes by lipoproteins. Neutrons can thus differentiate between lipid exchanged by another lipid, and lipid replaced by solvent, by following the SLD change and the solvent content of the lipid tail region, respectively. The use of tail-deuterated lipids in a non-deuterated solvent (i.e., H_2O buffer) provides the best contrast to follow these phenomena by neutron reflection. The neutron reflection profiles were analysed via a layer model as described in the Materials and methods

section. During the fitting process, the model was constrained to describe the three isotropic contrasts measured simultaneously (the bulk solution was exchanged from H_2O buffer, to a mixture of H_2O and D_2O buffer (38% D_2O), to pure D_2O buffer). This increases sensitivity of the method and allows decoupling of lipids exchanged (deuterated membrane lipids being replaced by hydrogenated lipoprotein lipids) from those removed (deuterated lipids removed and replaced by solvent). All pristine bilayers prior to incubation were found to have a surface coverage of 95% or more. Any minor defects below this were found not to influence resulting trends in lipoprotein interactions. The neutron reflection data, best fits, and layer models used to analyse the model membrane neutron reflectometry profiles can be found as Supporting Information (Tables S3 and S4, Figs. S1 and S2).

3.1. Effect of phospholipid unsaturation

Using the approach and model described above, saturated and unsaturated model membranes were found to behave very differently in terms of lipid exchange. First, we compared the interaction of HDL with saturated and unsaturated phospholipid model membranes. The results show a vast difference in the relative quantity of lipids exchanged and removed depending on phospholipid fatty acid saturation (Fig. 2). As different pooled lipoprotein samples varied slightly in composition (Supporting Information Fig. S1), Fig. 2 shows data for each model membrane relative to the incubation with the dDMPC membrane for the same lipoprotein sample. Corresponding absolute values are found in Supporting Information Table S4.

Overall, the quantities of lipids exchanged and removed were lower for the unsaturated phospholipids compared to the saturated phospholipids, regardless of lipoprotein type. For HDL, the amount of lipids exchanged with the unsaturated dPC membrane was ca. 80% lower than for saturated dDMPC. Analogously, the quantity of lipids removed (and replaced by solvent), for the unsaturated dPC membrane was significantly reduced compared to dDMPC (ca. 90%). For LDL, incubation with unsaturated dPC membranes led to a similar reduction in lipids exchanged as for HDL (ca. 80%). However, a smaller difference (ca. 60%) was observed between the saturated and unsaturated phospholipids removed. The absolute quantities of lipids exchanged and removed for dDMPC were also lower for LDL than for HDL (Supporting Information, Table S4). Both types of lipoproteins display preferential uptake of saturated lipids as compared to unsaturated ones, although HDL has a greater affinity for these than LDL. This is in agreement with previous results, showing HDL to have a higher affinity than LDL for exchanging and removing saturated lipids [16].

3.2. Effect of cholesterol in model membranes

Endothelial cellular membranes contain between 10 and 20 mol% of cholesterol [46]. Consequently, we investigated the effect of 10 or 20 mol% cholesterol on the capacity of lipoproteins to exchange lipids from model membranes (Fig. 2). The presence of cholesterol reduces the quantity of lipids exchanged and removed by HDL. For cholesterol-containing saturated membranes, there was an inverse relationship between the quantity of lipids exchanged and the amount of cholesterol in the model membrane. The presence of cholesterol also reduced the amount of lipids removed compared to dDMPC alone. In contrast, the presence of cholesterol in unsaturated dPC membranes did not affect the extent of lipids exchanged, while the amount removed was slightly increased.

A slightly different trend was observed in the presence of LDL. For saturated dDMPC membranes, exchange in the presence of 20 mol% cholesterol was comparable to that for dDMPC alone, while the quantity of lipids removed decreased with increased levels of cholesterol in the membranes. For unsaturated dPC containing 20 mol% cholesterol, there was little effect on the level of lipids exchanged as compared to the unsaturated dPC membrane alone. However, the amount of lipids

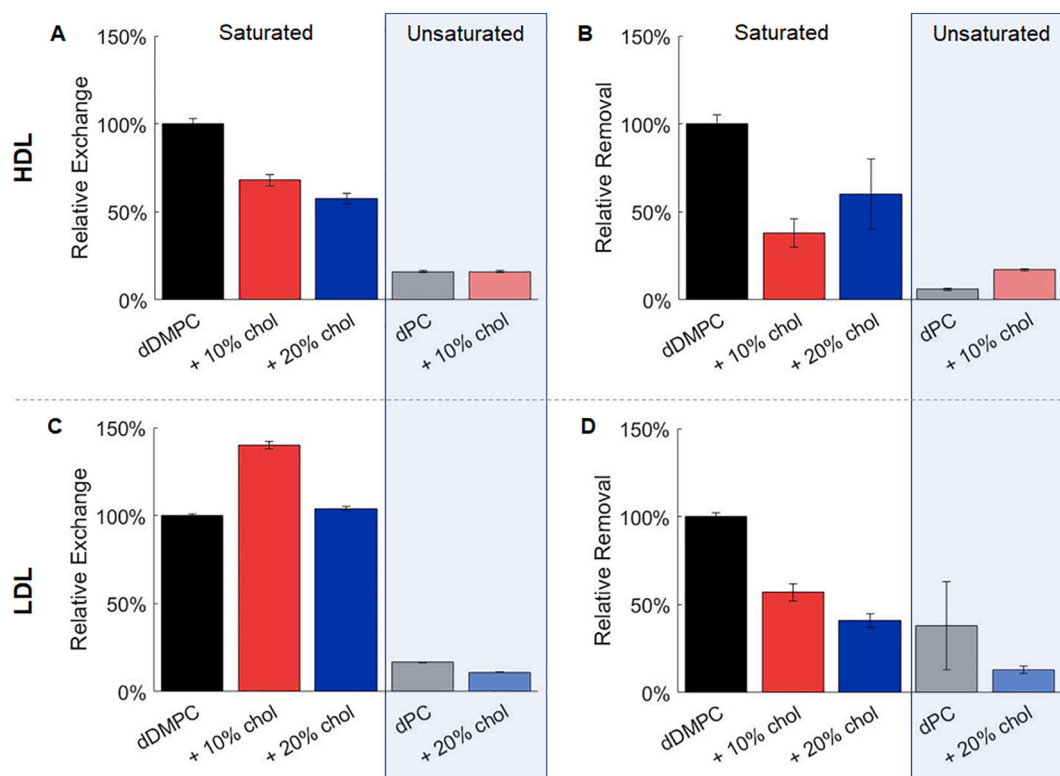


Fig. 2. Relative lipid exchange and removal for HDL or LDL particles interacting with membranes formed with saturated (dDMPC) and unsaturated (dPC) phospholipids with and without cholesterol. Results are shown for HDL (A, B) and LDL (C, D) regarding exchange (A, C) and removal (B, D).

removed by LDL decreased when including 20 mol% cholesterol in unsaturated phospholipid membranes.

3.3. Effect of lipid exchange by LDL as a function of LDL composition

Fig. 3 compares absolute values of lipid exchange according to lipoprotein particle content (the fat and protein content in the HDL and LDL fractions hereby used are given in Supporting Information Table S1). As seen, an increase in lipid exchange was found with increasing protein content in LDL, paralleled by a decreasing total fat content (cholesterol and triglyceride). In contrast, no pronounced dependency on lipoprotein composition was seen for phospholipid removal from the membrane (Supporting Information Table S4). For HDL, in turn, lipoprotein composition had only minor effects on lipids both exchanged and removed (Supporting Information Table S4).

In these experiments, the protein concentration was kept constant. This implies that the increase in protein content across the particles

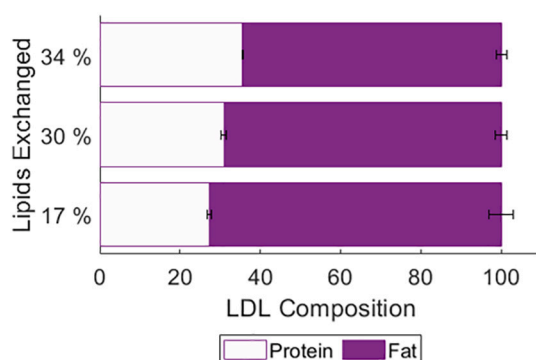


Fig. 3. HDL-induced lipid exchange and removal, for unsaturated dPOdPC, dPC and dPOhPC.

corresponds to a decrease in particle size, assuming a constant number of proteins per particle [47]. Thus, smaller LDL particles seem to have an improved ability to exchange lipids. This is well in line with clinical observations, in which smaller sized LDL sub-fractions were reported to display higher atherosclerotic effects, which can more easily penetrate artery walls and become a source of fat and cholesterol for the development of plaque [48,49].

3.4. Effect of headgroup deuteration on lipid exchange and removal by HDL

Since deuteration is a pre-requisite for performing these studies, we investigated the effect that the degree of headgroup deuteration have on lipid exchange by HDL. Fig. 4 gives the percentage of lipids exchanged or removed by HDL in model membranes consisting of either dPC (composed of a mixture of unsaturated PC and cyclopropane-containing PC), perdeuterated POPC (dPOdPC) or tail-deuterated POPC

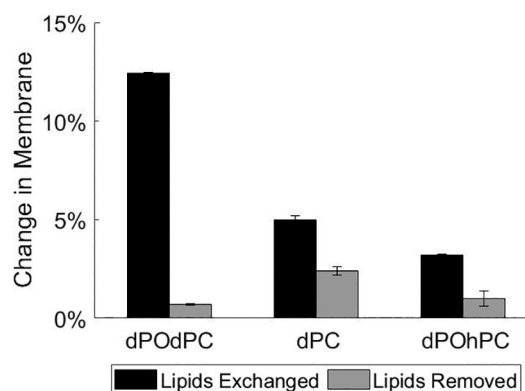


Fig. 4. Exchange of dDMPC by LDL as a function of the protein and total fat content of the latter.

(dPOhPC). These deuterated phospholipids have different levels of deuteration in their phosphatidylcholine groups: 100%, 78%, and 0% in dPOdPC, dPC, and dPOhPC, respectively. Since the experimental temperature ensured full fluidity in all model membranes, the interaction data highlight the difference in the exchange and removal induced by deuteration of the headgroup of the model membranes. We found that the higher level of deuteration in the headgroup correlated to a stronger capacity of HDL to exchange lipids. However, phospholipid removal from the membrane (i.e., lipids being replaced by solvent) is not significantly influenced by headgroup deuteration, all samples showing 0.5–2.5% phospholipid removal from the model membranes. Hence, headgroup deuteration does have an effect on this type of exchange study in terms of absolute values of lipids exchanged. This can be explained by deuteration affecting bond vibrations, molecular motions and hydrogen bonding. Thus, we hypothesise that specific protein-lipid interactions are directly involved in the recognition of lipids within the model membrane by lipoproteins [50,51]. In particular, the minor effects observed for dPC as compared to dPOhPC could be correlated with this lipid having deuteration only on the amine methyl groups of the phosphatidylcholine headgroup [23], this amine group is not involved on hydrogen bonding. Even though deuteration in the headgroups could impact lipid-protein interactions, deuteration in the lipid tails can be used with no influence on the exchange data since these are not involved on specific interactions with apolipoproteins in LDL or HDL.

4. Discussion

Summarising the lipid exchange data for dDMPC with HDL and LDL (Table S4), and comparing these with previously reported data [19], we note that the absolute quantities are similar for most preparations and thus there is good reproducibility despite small variability in the lipoprotein composition. For HDL, the absolute quantities of lipids removed and exchanged are within error to those reported by Browning et al. [19]. For LDL, on the other hand, we observe a dependency of lipid exchange with the lipoprotein composition for denser LDL (Fig. 3). Therefore, our results suggest that lipoproteins have similar capacities to exchange and remove lipids, except for LDL with total protein content lower than 30%. This implies that the pooled LDL samples (presenting higher lipid exchange capacity) could be enriched in the more atherogenic [52], smaller and denser type of LDL, even though the individuals were considered to be healthy. Thus, our data may reflect the properties of different LDL sub-types to exchange fats [14]. This is the first time that differences in lipid exchange capacity have been reported for LDL fractions presenting distinctive compositions.

Our data clearly show that the saturation level of the phospholipids present in the bilayers strongly affects the ability of lipoproteins to exchange and further remove lipids from model membranes (Fig. 2). This is seen most prominently with HDL. The difference in the ease of lipid removal may be attributed to protein-lipid binding affinity of HDL and LDL particles with the model membranes. Indeed, the conformation of the protein present in reconstituted HDL particles greatly impacts lipoprotein binding affinity for unsaturated phospholipids [53]. The lipoprotein particle composition may impose conformational restrictions on apolipoproteins, which could translate as modified binding to lipid molecules present in the model membranes. It may also be noted here that HDL therapy, based on the re-modelling of the plaque by fat-poor HDL, results in a decrease of both saturated fats and cholesterol in atherosclerotic plaque [54,55], although studies suggest that the total amount of HDL alone does not suppress the risk for atherosclerosis [11]. This highlights the need to understand which molecular properties of HDL contribute to its lipid exchange capacity.

The different affinity for saturated and unsaturated phospholipids may be related to their different mobility. A recent inelastic neutron scattering study showed that saturated phospholipids have greater mobility both in the fluid and gel phases compared to unsaturated phospholipids [56]. Our data show that saturated lipids are more easily

taken up by lipoproteins compared to unsaturated fats. Thus, lipids with greater mobility seem to be more accessible to lipoprotein binding, in turn facilitating lipid exchange. Earlier studies for lipid exchange between lipid aggregates (bicelles versus vesicles) showed that lipid exchange strongly depended on the length of the acyl chain as well as the type and size of the lipid aggregate [57]. However, these experiments were performed at 10 °C at which strong van der Waals interactions are expected. Moreover, in these experiments the molecular identity of the lipid aggregates did not change over time (bicelles exchange lipids with bicelles of the same composition or vesicles exchange lipids with vesicles of the same composition). This is clearly not the case for our experiments where lipids from the lipoproteins (presenting a very complex lipid type and acyl chain composition as measured by lipidomics [17]), replace the model membrane lipids, and vice versa. Thus, the findings from the present study on increased exchange and removal of saturated lipids add important new insight into lipid exchange between lipoprotein and lipid membranes.

The presence of cholesterol in the model membranes impacts the quantity of lipids exchanged and further removed. As with the level of saturation, this is most noticeable in the presence of HDL (Fig. 2). The normal cholesterol content in plasma membranes is 10–20 mol% [46]. Even though cholesterol is known to increase the order and hydrophobicity of membranes [46,58], differential scanning calorimetry (DSC) data of the model membranes hereby used (Supporting Information, Fig. S3) indicate that these membranes are fluid at 37 °C, in agreement with literature [59]. It is possible, however, that the cholesterol suppresses the mobility of saturated phospholipids to form liquid ordered bilayers, as previously demonstrated from in-plane diffusion studies [60]. At the same time, however, cholesterol may induce complex local ordering and other effects as well [61], which may influence lipoprotein-membrane lipid exchange. Experimentally, we did not observe any systematic trends for either lipoproteins due to cholesterol (Fig. 2), thus, it is unlikely that the effects observed are related alone to decreased mobility in the model membrane by increased cholesterol content. Furthermore, cholesterol molecules were reported to interact preferentially with saturated phospholipids [62,63]. Additionally, LDL was shown to bind preferentially to raft domains, rich in saturated fats and cholesterol [64], and to modify the cell membrane area covered by rafts [65]. Therefore, it seems likely that specific interactions between the apolipoproteins in the lipoproteins and the lipids in the model membrane determine the exchange of fats observed. We observed different lipid exchange by denser LDL (Fig. 3), suggesting that the properties of ApoB-100 (the main apolipoprotein in LDL) may be influenced by LDL density. Indeed, it was reported that small and medium-sized LDLs have altered epitopes between ApoB-100 residues 4342 and 4536, as compared with VLDLs, IDLs, and large LDL [47]. The altered expression of these epitopes in small and medium dense LDL coincides with altered accessibility of protease-sensitive sites elsewhere in the ApoB structure [66]. These findings suggest a major change in the configuration of the ApoB on the LDL surface due to increased density. Moreover, the sequence region that includes these epitopes is believed to form alpha helices which are the motives found in the exchangeable apolipoproteins (mainly A, C, E) that are known to bind lipids [67]. Indeed, we showed recently that lipoprotein binding to the model membrane is an important step for lipid exchange, since the exchange via solution or via simple collision is limited [17]. Such direct contact might favour protein-lipid interactions. However, further studies with reconstituted lipoproteins using specific apolipoproteins are needed to clarify this point.

Although debated, it has been shown that whilst dietary cholesterol intake does not in itself increase the risk of CVD [68], the intake of dietary saturated fats increases the risk of CVD [69]. Conversely, the replacement of saturated fats with mono- and polyunsaturated ones has been found to decrease the risk of CVD [70]. Such replacement has further been shown to lower total cholesterol, LDL cholesterol, and triglyceride levels in blood [70,71]: known markers for CVD risk

[49,72,73]. Furthermore, a diet with increased fat content has been shown to induce HDL production and also result in an increase in the total liver cholesterol content [74]. Mirroring this, our data show efficient lipid removal of unsaturated phospholipids also in the presence of cholesterol. Therefore, cholesterol intake together with a fatty diet based on unsaturated fats may give rise to high HDL values despite high cholesterol values in the liver. In contrast, as suggested by a pronounced effect of cholesterol content in saturated membranes in the present investigation as well as an overall large lipid exchange in the case of saturated phospholipids, a diet rich in saturated fats and cholesterol may result not only in high cholesterol values, but also affect HDL exchange in atherosclerotic plaque remodelling.

While neutron reflectivity provides novel insight on lipid exchange and removal on lipoprotein interactions with bilayers of different saturation and cholesterol content, it should be recognized that these findings have been observed for highly simplified models, which obviously do not fully represent the full biological system. For example, DMPC is present in endothelial membranes only in very small quantities, and DPPC would have represented a physiologically more relevant lipid. However, the melting temperature of the latter lipid is 41 °C, and DPPC is therefore not fluid at 37 °C. To use another temperature than 37 °C is unsuitable not only from a physiological relevance perspective, but also since other temperatures might induce phase transition of the core in low density lipoproteins to become gel-like [40]. Sphingomyelin, in turn, has a high melting temperature but currently we lack known biosynthetic/organic approaches for its deuterated form. The use of DPPC or ideally sphingomyelin in combination with POPC and cholesterol can give rise to “raft like” domains which are of interest to study in the future due to their biological relevance. Furthermore, deuteration in the backbone of the phosphatidylcholine headgroup has some effect on lipid exchange, which also demonstrates a limit on to how far one should draw the comparison with the full biological system endorsing the use of mainly tail deuterated lipids instead. With such considerations in mind, however, it seems clear that the experimental approach taken captures key effects of lipid saturation and presence of cholesterol for lipoprotein-associated lipid exchange and removal, well suited for comparison with biological results of larger biological relevance, but for which mechanistic studies are precluded.

5. Conclusions

In this work, we present data of lipid exchange between pooled lipoprotein fractions extracted from human blood (of healthy donors) and model membranes composed of either saturated or unsaturated phospholipids, in the presence or absence of cholesterol. Despite the simplicity of the model system, the exchange data mirror key clinical findings since: 1) HDL was found to remove lipids to a larger extent than LDL, 2) saturated fats were cleared to a larger extent by HDL than by LDL, while the presence of cholesterol in the membrane significantly reduced the ability of lipoproteins to exchange lipids, 3) lipoproteins present low affinity for unsaturated phospholipids which explains why HDL therapy is able to re-model plaque composition, and 4) denser LDL (with higher protein content) deposit more lipids on model membranes in agreement with the atherogenic characteristics of smaller and denser LDL sub-fractions. This suggests that the approach captures key physiological aspects of atherosclerotic plaque formation, while still allowing the mechanisms of lipid exchange to be investigated in detail. As such, it can likely be applied to unravel mechanistic information and to investigate other contexts relating to atherosclerosis such as LDL fractions arising from patients with a defined clinical condition such as high cholesterol, high triglyceride, or presence of lipoprotein(A); and to determine the capacity of these fractions to deposit and remove fats, as well as systematic studies with reconstituted HDL fractions of known composition both in terms of lipids and apolipoproteins.

CRedit authorship contribution statement

Sarah Waldie: Investigation, Project administration, Formal analysis, Writing - original draft. **Federica Sebastiani:** Formal analysis, Writing - original draft. **Kathryn Browning:** Investigation, Writing - original draft. **Selma Maric:** Investigation, Writing - original draft. **Tania K. Lind:** Investigation, Writing - original draft. **Nageshwar Yepuri:** Investigation, Writing - original draft. **Tamim A. Darwish:** Investigation, Writing - original draft. **Martine Moulin:** Investigation, Project administration, Supervision, Formal analysis, Writing - original draft. **Gernot Strohmeier:** Investigation, Writing - original draft. **Harald Pichler:** Writing - original draft. **Maximilian W.A. Skoda:** Investigation, Writing - original draft. **Armando Maestro:** Investigation, Writing - original draft. **Michael Haertlein:** Supervision, Writing - original draft. **V. Trevor Forsyth:** Supervision, Writing - original draft. **Eva Bengtsson:** Writing - original draft. **Martin Malmsten:** Writing - original draft. **Marité Cárdenas:** Project administration, Investigation, Formal analysis, Writing - original draft, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbalip.2020.158769>.

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