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Interactions between sphingomyelin and cholesterol in low density lipoproteins and model membranes

Andrew J. Guarino [a], Sum P. Lee [b], Steven P. Wrenn [a,*]

[a] *Chemical Engineering Department, Drexel University, Philadelphia, PA 19104, USA*
[b] *School of Medicine, University of Washington, Seattle, WA 98195, USA*

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Abstract

This work examines three related, but previously unexplored, aspects of membrane biophysics and colloid science in the context of atherosclerosis. First, we show that sphingomyelinase (SMase)-induced aggregation of low density lipoproteins (LDLs), coupled with LDL exposure to cholesterol esterase (CEase), results in nucleation of cholesterol crystals, long considered the hallmark of atherosclerosis. In particular, this study reveals that the order of enzyme addition does not effect the propensity of LDL to nucleate cholesterol crystals, raising the possibility that nucleation can proceed from *either* the intra- or extracellular space. Second, we demonstrate that ceramide-rich aggregates of LDL release cholesterol to neighboring vesicles far more rapidly, and to a greater extent, than does native LDL. A likely explanation for this observation is displacement of cholesterol from SM–Chol rafts by "raft-loving" ceramide. Third, we demonstrate that a time-independent Förster resonance energy transfer (FRET) assay, based on dehydroergosterol and dansylated lecithin and used previously to study cholesterol nanodomains, can be used to measure raft sizes (on the order of 10 nm) in model membrane systems. Taken together, these observations point to the possibility of an extracellular nucleation mechanism and underscore the important role that biological colloids play in human disease.

Keywords: Sphingomyelinase; LDL; Aggregation; Sphingomyelin; Cholesterol; Rafts

1. Introduction

Low density lipoproteins (LDLs) are the main carriers of cholesterol in the blood and are the key components in cholesterol transfer and metabolism [1]. These particles contain a hydrophobic core surrounded by a phospholipid–cholesterol monolayer. The core is primarily composed of esterified cholesterol, but also contains some triglycerol and free cholesterol [1]. The monolayer is comprised of about 33 mol% cholesterol with the balance made up of phospholipids—chiefly phosphocholine (PC) and sphingomyelin (SM) in a 2:1 ratio [2]. As the monolayer of LDL is undersaturated in cholesterol, a triggering event, such as enzyme-induced aggregation, is necessary to induce cholesterol nucleation. These aggregates can also be endocytized by macrophages, which can lead to foam cell formation, as

Abbreviations: Low density lipoprotein (LDL); Sphingomyelinase (SMase); Cholesterol esterase (CEase); Egg sphingomyelin (SM); Cholesterol (Chol); Egg lecithin (Egg PC); Phosphatidylcholine (PC); Förster resonance energy transfer (FRET); Dehydroergosterol (DHE); 1-Acyl-2-[12-[(5-dimethylamino-1-naphthalenesulfonyl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (DL); 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(5-dimethylamino-1-naphthalenesulfonyl) (DLPE); 1,2-Dipalmitoleoyl-*sn*-glycero-3-phosphocholine (16:1 PC); Fluorescence ratio (R_F); Dynamic light scattering (DLS); Phospholipase C (PLC); Multilamellar vesicles (MLVs); Small unilamellar vesicles (SUVs); Atomic force microscopy (AFM); Liquid ordered (l_o).

* Corresponding author. Fax: +1 215 895 5837.
E-mail address: wrenn@coe.drexel.edu (S.P. Wrenn).

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termed by Brown and Goldstein, due to the foamy appearance of the macrophages resulting from the accumulation of cholesterol esters from LDL [3]. The foam cells may then be responsible for the generation of cholesterol crystals found in atherosclerotic plaques. Current techniques rely on crystal appearance time, which is already after the fact. Here, we use colloidal science to take a new approach to study the molecular events which are associated with atherosclerosis.

LDL aggregation and retention are essential steps in the development of atherosclerosis [4], and retention of LDL is hypothesized to be the single necessary and sufficient event to provoke atherosclerosis in an otherwise healthy artery [5]. The retention of LDL involves LDL diffusing across the walls of the arteries and becoming unable to move back across. This could be the result of the LDL aggregating and getting too large to diffuse back across the artery walls. Once retained, LDL aggregates are subject to endocytosis and deesterification of cholesterol in the LDL core. A consequence is nucleation of cholesterol (Chol) crystals [6], long considered a hallmark of advanced atherosclerotic plaques and suspected to influence atherosclerotic plaque instability. Sphingomyelinase (SMase) is an enzyme known to trigger LDL aggregation by cleavage of the SM phosphoester bond, resulting in removal of polar choline head groups, and production of ceramide moieties in the LDL monolayer. Hydrophobic ceramide moieties induce LDL aggregation so as to eliminate exposure of these regions to water. We recently developed a mass action model to describe this colloid aggregation process in terms of the SMase-to-LDL molar ratio [7].

SMase-modified LDL, once aggregated, represents a physiological example of how enhanced uptake by LDL receptor-mediated endocytosis leads to foam cell formation [8]. This is because the intracellular enzyme cholesterol esterase (CEase) converts esterified cholesterol within the LDL core to free cholesterol upon engulfment of LDL by macrophages. Aggregated LDL facilitates foam cell formation because of the larger cholesterol pool it delivers to macrophages in comparison with native LDL [8]. As cholesterol crystals have not been found to nucleate directly from native LDL, SMase and CEase are considered important in regulating how and when cholesterol nucleates from LDL or macrophages.

A related role of SMase concerns an interaction between SM and Chol, which closely associate in the LDL monolayer. A study of the interactions of cholesterol with various glycerophospholipids and SM showed the interaction between cholesterol and SM to be strongest [9]. The interaction is attributed to hydrogen bonding between the amide group of SM and the 3β-OH group of cholesterol [10]. Moreover, SM degradation in cells results in an increased cholesterol efflux from the cells to methyl-β-cyclodextrins [11], which are well known for their ability to facilitate transfer of cholesterol between cells and vesicles [12–14]. Conversion of SM to ceramide by SMase thus causes an efflux of cholesterol from LDL to neighboring vesicles or cells—the effect is supersaturation of cholesterol and nucleation of cholesterol crystals.

SM and Chol, owing to their strong interaction, form laterally phase-separated domains, known as "rafts," in biological membranes. Lateral phase separation also arises in model membranes comprising Chol and disaturated phospholipids, where discrete domains constitute a "liquid-ordered" (l_o) phase. There is a great deal of interest in determining the complete phase diagram for ternary model systems comprising SM, Chol, and unsaturated PC [15,16] as well as for analogous systems comprising a disaturated PC in place of SM [17,18]. Atomic force microscopy (AFM) studies of model membranes comprising 1:1:1 SM:Chol:dioleoylphosphatidylcholine reveal domain sizes in the range of 100–400 nm; the domains comprise primarily SM and Chol and exist in the liquid-ordered state [19,20]. AFM is not always useful for measuring liquid-ordered domain sizes, namely when chain lengths of unsaturated phospholipids (in the liquid disordered phase) and sphingomyelins and saturated phospholipids (in the l_o phase) are sufficiently similar that height differences among the two phases cannot be resolved. Moreover, AFM requires supported bilayers, which might alter domain sizes and precludes size measurements in monolayers. A complementary technique is needed for measuring domain sizes when such difficulties arise.

An ideal candidate is Förster resonance energy transfer (FRET) from dehydroergosterol (DHE) to dansylated lecithin (DL) (fluorescent analogs of cholesterol and phosphocholine, respectively), which takes advantage of the steep dependence ($1/r^6$) on the separation, r, between fluorophores [21]. DL is excluded from l_o phases so that the energy transfer efficiency provides a measure of domain size. DHE-to-DL FRET is a well established method for examining phase behavior and compositional changes in phospholipid–cholesterol systems [22–25]. Especially relevant to this work, the assay has been used to examine lateral phase separation of cholesterol nanodomains [25], nucleation of cholesterol from LDL [6], and methyl-β-cyclodextrin-mediated efflux of DHE [14]. FRET measures nucleation on the molecular level as opposed to the classical nucleation time, which is really crystal appearance time. Thus, colloidal science is necessary to study the molecular events associated with atherosclerosis.

Here we are interested in answering three fundamental questions. First, does the order of exposure (SMase followed by CEase versus CEase followed by SMase) of LDL influence the combined interaction of these two enzymes with respect to LDL aggregation and cholesterol nucleation? Second, does SMase hydrolysis cause LDL to give up its free cholesterol more readily because the SM–Chol interaction is destroyed? Third, can DHE-to-DL FRET be used to measure sizes of "model rafts" in systems comprising PC, SM, and Chol? We believe this work lays a foundation for, and underscores the importance of, applying principles of col-