Modeling Small-Angle X-Ray Scattering Data for Low Density Lipoproteins – Insights Into The Fatty Core Phase Packing And...
Modeling Small-Angle X-ray Scattering Data for Low-Density Lipoproteins: Insights into the Fatty Core Packing and Phase Transition

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ABSTRACT: Atherosclerosis and its clinical consequences are the leading cause of death in the western hemisphere. While many studies throughout the last decades have aimed at understanding the disease, the clinical markers in use today still fail to accurately predict the risks. The role of the current main clinical indicator, low density lipoprotein (LDL), in depositing fat to the vessel wall is believed to be the onset of the process. However, many subfractions of the LDL, which differ both in structure and composition, are present in the blood and among different individuals. Understanding the relationship between LDL structure and composition is key to unravel the specific role of various LDL components in the development and/or prevention of atherosclerosis. Here, we describe a model for analyzing small-angle X-ray scattering data for rapid and robust structure determination for the LDL. The model not only gives the overall structure but also the particular internal layering of the fats inside the LDL core. Thus, the melting of the LDL can be followed in situ as a function of temperature for samples extracted from healthy human patients and purified using a double protocol based on ultracentrifugation and size-exclusion chromatography. The model provides information on: (i) the particle-specific melting temperature of the core lipids, (ii) the structural organization of the core fats inside the LDL, (iii) the overall shape of the particle, and (iv) the flexibility and overall conformation of the outer protein/hydrophilic layer at a given temperature as governed by the organization of the core. The advantage of this method over other techniques such as cryo-TEM is the possibility of in situ experiments under near-physiological conditions which can be performed relatively fast (minutes at home source, seconds at synchrotron). This approach now allows the monitoring of structural changes in the LDL upon different stresses from the environment, such as changes in temperature, oxidation, or external agents used or currently in development against atherosclerotic plaque build-up and which are targeting the LDL.

KEYWORDS: low-density lipoprotein, small-angle X-ray scattering, cholesteryl ester packing

In westernized societies, atherosclerosis and its clinical consequences (cardiovascular diseases, CVD) constitute the leading cause of death.1 In atherosclerosis, plaques of lipids and fibrous elements accumulate in the arteries, leading to heart disease and stroke.1 One of the main blood clinical indicators in use today and the main target of therapy for the prevention of CVD is low-density lipoprotein (LDL).2,3 Even though there is well-established evidence for LDL involvement in atherosclerotic plaque build-up, it is clear that many different factors influence its atherogenicity.3 For example, it is widely accepted that different forms of LDL, including small dense LDL and oxidized LDL, promote atherosclerosis,2 and thus, it is not surprising that measurements of total plasma LDL and cholesterol concentrations often fail to predict the risks for CVD.3,4 Detailed understanding of the LDL particle structure specifically related to its chemical composition is necessary to better evaluate the atherogenic capacity of various LDL subfractions and further develop robust and standardized methods for both diagnostics and therapeutic approaches against CVD.

LDL is a macromolecular complex of both proteins and fats: lipids, triglycerides, and sterols.2 Detailed understanding of the LDL particle structure specifically related to its chemical composition is necessary to better evaluate the atherogenic capacity of various LDL subfractions and further develop robust and standardized methods for both diagnostics and therapeutic approaches against CVD.
component biomolecular assemblies are some of the most challenging systems with regard to structural studies. The particles’ inherent heterogeneity has made crystallization extremely challenging, and while several attempts have led to diffracting crystals, a high-resolution structure of the LDL is yet to be reported.5–7 Instead, solution small-angle scattering (SAS) using both X-rays (SAXS) and neutrons (SANS) has been considered a useful tool for investigating the structure of the LDL.8–14 Despite their inherent orientational averaging, SAXS and SANS have been able to provide important structural information not only on the overall shape and size of the particles but also on the lipid packing inside the particle core.10,15,16 The SAS models applied in the literature assumed a spherical centrosymmetry, following the many oscillations in the LDL SAXS data, which are usually only observed in cases with such symmetry. These early models have been strengthened by data obtained through electron microscopy (EM), NMR spectroscopy, differential scanning calorimetry (DSC), IR spectroscopy, and fluorescence spectroscopy, leading to a structural model of the LDL which has been widely accepted across the scientific community: a globular core—shell particle composed of a monolayer of phospholipids and the apolipoprotein ApoB-100 surrounding a cholesteryl ester and triglyceride core.15,17

Early studies using techniques such as DSC, and supported by SAXS and SANS, showed that the cholesteryl esters inside the particle core undergo a temperature-induced transition state between 20 and 40 °C, which varies considerably for LDL particles obtained from different individuals.14,16 Below the transition temperature, the cholesteryl esters were shown to be in a liquid–crystalline arrangement and thought to be forming concentric layers, while a more disordered liquid-like state at physiological temperature was assumed.10 This ordered arrangement of the core lipids, thought to be composition dependent (triglyceride content), has been shown to have a positive effect on LDL particles’ resistance to oxidation18 and thus should have an impact on atherogenesis.

Over the past decade, the advancement of cryo-TEM revealed that LDL has rather an ellipsoidal shape with cylindrical lamellar layering of cholesteryl esters inside the particle core.19 These findings were used in the present work as a base for the development of a model for the analysis of experimental SAXS data for LDL, which in contrast to previous models does not have spherical centrosymmetry. The model allows in situ determination of the structure of LDL particles, including the internal organization, and it provides an easy approach for following the variation of the structure when varying, e.g., temperature.

Although it is possible to calculate radial electron density profiles from the TEM micrographs20,21 and also to calculate SAXS profiles from them, the results are only obtained after tedious data treatment with alignment, averaging, and filtering. In addition, the underfocusing usually applied in TEM and the selection of a threshold level can lead to some uncertainty in, e.g., size estimates. Moreover, the quenching speed of the cooling of the sample may influence the results, making high temperature state investigations challenging.19 Here, SAXS offers an important complementary alternative for characterizing the structures in situ. With the structural features of the particles known from cryo-TEM, a parametric structural model as derived here can be least-squares fitted to experimental SAXS data providing a set of structural parameter values that describe the overall size of the particle and the internal arrangement of

the particle core. The latter is of particular importance due to the current discrepancies with respect to the possible existence of an intermediate state even though a clear layering of the cholesteryl esters is present at low temperatures that disappears at high temperatures.20,21

The model presented here describes the overall structure/shape of the LDL while it gives insights into the lamellar inner packing of the cholesteryl esters and triglycerides in addition to providing the transition temperature as confirmed by DSC data. The model was successfully applied to SAXS data obtained on native LDL particles extracted and purified from human blood of three healthy males (pooled samples). The methodology established here together with detailed compositional analysis (i.e., proteonomics and lipidomics) can in the future be applied for systematic structural studies of various types of LDL subfractions, coming from larger populations, thus enabling comparisons between different risk groups.

RESULTS

LDL Composition and Heterogeneity. Human LDL are typically isolated through density gradient ultracentrifugation and include particles with densities between 1.019 and 1.050 g/mL.22 In this study, LDL was purified from the plasma of three healthy male volunteers and pooled after density gradient ultracentrifugation. The LDL fractions obtained through the same purification procedure but at two separate occasions and from different donors were further purified by size-exclusion chromatography (Supplementary Figure 1). These data showed the presence of three different populations: (1) very large particles eluting in the void volume of the column assigned to very-low density lipoprotein (vLDL) with a retention time (tR) of 20 min, (2) medium-sized particles assigned to LDL eluting at 25 min, and (3) smaller particles assigned to high-density lipoprotein (HDL) eluting after 30 min. The assignments were done in accordance with previous studies23 and confirmed through proteomic analysis (data not shown) of each of the peaks that confirmed the main presence of apoB-100 in the LDL fraction subsequently chosen for this study (Supplementary Figure 1). Mass spectrometry of the protein content also showed large amounts of human serum albumin in addition to HDL with Apo-AI as the main apolipoprotein present in the peak eluting at tR = 35 min. The concentration of the total protein for LDL was determined by the Bradford method,24 while the different fat components of LDL were analyzed enzymatically and the relative concentrations of protein, total cholesterol, triglycerides, and phospholipids are summarized in Supplementary Table 1. For reliable structural analysis using SAS, ensuring samples of high purity is necessary to minimize polydispersity. With that in mind, and after comparison with SAXS data for crude LDL after ultracentrifugation without further purification (Supplementary Figure S2), all structural data used for modeling were collected only on the SEC fractions corresponding to the size of the LDL and containing ApoB-100 as the main protein component. By a comparison to the results of Chapman et al.5 of different fractions of LDL obtained by analytical ultracentrifugation, the samples were expected to have a size polydispersity in the outer radius of less than 10%.

Structural Characterization of LDL. The recent TEM studies19 revealed a particle shape that is neither spherical nor ellipsoidal but more like a cylindrical structure with rounded edges and corners. Therefore, the model derived in this study was based on a super ellipsoid25 since it allowed more variation
of the particle form than an ellipsoid in the direction of the observed form. More specifically, an oblate super ellipsoid of revolution was used with a radius at equator, \( R \), the half-height of the ellipsoid, \( \varepsilon R \), where \( \varepsilon \) is defined as eccentricity, and the shape exponent \( t \) which is defined as the particles super-ellipticity. Figure 1a illustrates a super ellipsoid with \( t = 3 \). A usual ellipsoid of revolution is obtained for \( t = 2 \) and a super ellipsoid of revolution approaches a cylinder for \( t = \infty \). Figure 1b shows the cross-section structure of the LDL model where apolipoprotein and phospholipid head groups constitute the outer layer, the so-called hydrophilic shell, while cholesteryl esters are integrated as cylindrical disk layers within the core of the LDL particle. The protein and phospholipid head groups in the outer shell are illustrated in red.

![Figure 1](image1.png)

Figure 1. (a) Illustration of a super ellipsoid with ellipticity or shape exponent \( t = 3 \). The symmetry axis goes vertically through the center, while the size and the shape are given by the radius at equator, \( R \), the half-height of the ellipsoid, \( \varepsilon R \), and the shape exponent \( t \). (b) Illustration of a super ellipsoid with a shape component \( t = 3 \) including three ordered layers of cholesterol esters (cyan) inside the hydrophobic core (blue) of the LDL particle. The protein and phospholipid head groups in the outer shell are illustrated in red.

The experimental SAXS data for LDL at different temperatures are shown in Figure 2a. Clear minima and maxima that extend out to rather large scattering vectors were observed at all temperatures. This supports the expectation that the size polydispersity in the sample was relatively low. Upon increased temperature, a clear shift in the periodicity of the oscillations was observed between \( q \approx 0.015 \) Å\(^{-1}\) and \( q \approx 0.025 \) Å\(^{-1}\) toward smaller \( q \) values, indicating that changes in both size and shape took place (Figure 3). In addition, the Bragg peak with maxima at \( q = 0.17 \) Å\(^{-1}\), attributed to the layering of cholesteryl esters\(^{19,30}\) dramatically decreased in intensity when the temperature increased from 5 to 40 °C.

The fitting of the layered superellipsoid was performed from low to high temperatures, and the best fits are presented in Figure 2b. For the experimental data collected above the expected \( T_m \), the superellipsoid model had too many degrees of freedom. Hence, the model needed to be adjusted for the three highest temperatures to incorporate the expected physical changes within the particle core, i.e., allow a more disordered system devoid of the cholesteryl esters layers. It also became clear that the thickness constraint for the hydrophilic shell of 25 Å (protein and headgroup layer) did not hold at temperatures above the expected \( T_m \). Instead, free fitting of this parameter led to a thickness of \( \sim 46 \) Å, suggesting that the flexibility of the protein could be dependent on the disorganization of the LDL core in addition to temperature. Note that including a layer \( D_c \) of \( \sim 5 \) Å between the cholesteryl ester particle core and the protein shell below \( T_m \) improved the fits in the oscillation...
Moreover, the apolipoprotein could adopt a different configuration in contact with the lipoprotein particle core, thus giving rise to the thin extra shell layer. Additionally, restricting the model to an LDL containing only three cholesteryl ester layers was sufficient to reproduce the experimental data, obtaining a "goodness of fit" ($\chi^2$) of two or below (Table 1).

The model parameters describing the overall size and shape obtained from the best fits to the SAXS data are summarized in Figure 3: with radius (Figure 3a), eccentricity (Figure 3b), and superellipticity (Figure 3c). At 5 °C, the LDL particles displayed a radius of $\sim$125 Å, a half-height, $eR$, of $\sim$90 Å, and a superellipticity of $\sim$3 resulting in a flattened ellipsoid with the overall dimensions of $\sim$250 Å $\times$ $\sim$250 Å $\times$ $\sim$182 Å. This is in very close agreement with the LDL dimensions obtained through cryo-TEM at temperatures below LDL $T_m$.

Above the expected $T_m$, the LDL showed an increase in outer particle radius, and the eccentricity term reached unity while the superellipticity decreased, resulting in a completely spherical particle with a diameter of $\sim$262 Å. While this falls slightly out of the defined LDL size range (180–250 Å)\(^4\) the obtained diameter is reasonable, well within the limits of the technique, and presents an improvement when compared with previous SAXS-based models. These previous models showed LDL particles with diameters of >300 Å. Such overestimation of the LDL size could also be explained by the presence of larger vLDL particles after ultracentrifugation\(^5\) in the samples used for SAXS. Indeed, SAXS curves obtained from different LDL preparations before and after SEC clearly showed differences in size as well as shape of the particles that could, after further analysis, be assigned to the presence of other common blood components and simply result from insufficient lipoprotein purification (Supplementary Figure S2).

The change in the particles superellipticity and eccentricity, which shows that the LDL morphs from an ellipsoid to a sphere with increasing temperature, is reversible, although the shape is slightly different in overall dimensions (the radius becomes smaller, whereas the dimension in the perpendicular direction increases slightly). Consequently, the LDL undergoes a change from a sphere back to a slightly flattened shape upon cooling from 40 to 5 °C (Figure 3c). This specific flattening of the particles was originally thought to just be an artifact of the LDL

![Figure 3. LDL particle structure as a function of temperature upon heating (open circles) and after cooling (closed circles): (a) LDL radius, $R$ (open circles), and $eR$ (red filled circles); (b) eccentricity, $e$; (c) shape component, $t$, for the particles; (d) the cholesterol ester thickness, $T_e$; and (e) excess contrast of cholesterol ester layers, $\Delta \rho_c$.](image)

occuring at the middle $q$ range, suggesting the presence of a short phospholipid region that is not as mobile as the rest of the lipid core. This is expected as the headgroup packing imposes a restriction on the packing/mobility of the phospholipid tails.

Table 1. Fitting Parameters for SAXS Profiles of LDL Particles

<table>
<thead>
<tr>
<th>$T$ (°C)</th>
<th>shell layer thickness $D_{shell}$</th>
<th>$\Delta \rho_{core}$</th>
<th>CE thickness $T_e$</th>
<th>CE layer distance $d$</th>
<th>$\Delta \rho_l$</th>
<th>goodness of fit $\chi^2$</th>
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<td>5</td>
<td>25</td>
<td>$-1.96 \pm 0.08$</td>
<td>15 ± 2</td>
<td>36.7 ± 0.1</td>
<td>3.8 ± 0.2</td>
<td>2.24</td>
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<td>10</td>
<td>25</td>
<td>$-2.04 \pm 0.09$</td>
<td>16 ± 2</td>
<td>36.7 ± 0.1</td>
<td>3.8 ± 0.2</td>
<td>2.26</td>
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<tr>
<td>15</td>
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<td>$-2.07 \pm 0.09$</td>
<td>17 ± 2</td>
<td>36.7 ± 0.1</td>
<td>3.5 ± 0.1</td>
<td>2.21</td>
</tr>
<tr>
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<td>25</td>
<td>$-2.07 \pm 0.06$</td>
<td>17 ± 1</td>
<td>35.2 ± 0.1</td>
<td>3.45 ± 0.06</td>
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<td>25</td>
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<td>$-1.77 \pm 0.05$</td>
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<td>35</td>
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<td>–</td>
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<td>0.99</td>
</tr>
<tr>
<td>45</td>
<td>45.8 ± 0.4</td>
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<td>–</td>
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<td>0**</td>
<td>0.87</td>
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<tr>
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<td>36.0 ± 0.1</td>
<td>3.45 ± 0.03</td>
<td>1.92</td>
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</table>

*Parameter was fixed to zero as it converged to this value during the fitting and the corresponding peak disappeared. "–" indicates the parameter is not applicable as the electron density of the layers is fixed to zero. The rows indicated in boldface mark the melting of the core, where the scattering contrasts as well as the CE thickness and layer distance decrease. All dimensions are given in Angstroms. The excess scattering length density $\Delta \rho_{core}$ of the hydrocarbon core and the extra scattering of the layers $\Delta \rho_l$ (cholesterol part of cholesteryl esters) are taken relative to that of the shell.*
particles’ interaction with the EM grid. However, such flattening depends on the crystallinity of the core lipids and thus on the specific core composition.\textsuperscript{19,20,26,32,33}  

\textbf{Fatty Core Phase Transition and Cholesteryl Ester Packing.} The inner layering of the cholesteryl esters in the LDL core is described by the layer thickness $T$, the layer distance $d$, and the extra scattering contrast defined as $\Delta \rho_{c}$. The latter parameter is defined as “extra”, meaning on top of the core excess contrast of all hydrocarbons inside the particle core; thus, the higher the contrast the better defined the layering of the core. This comes from the higher electron density of the sterol part of the cholesteryl ester compared to that of the alkyl chains and triglycerides. The parameters representing the cholesteryl ester packing obtained through model fitting are summarized in Table 1 and Figure 3d,e. The interlayer distance of 36.7 Å at low temperatures (5–15 °C) is in very good agreement with the dimensions that have previously been determined for LDL cholesteryl esters at low temperature as well as for extracted cholesteryl esters in the smectic phase.\textsuperscript{10,34,35} Moreover, $\Delta \rho_{c}$ started to decrease at 15 °C as a result of the onset of the melting of the crystalline core. Free fitting of all three parameters representing the cholesteryl ester layering resulted in a successive decrease of both the $\Delta \rho_{c}$ (Figure 3e) and the cholesteryl ester thickness between 15 and 30 °C (Figure 3d). At 35 °C and temperatures above, the layered model resulted in an insignificant scattering contrast for the cholesteryl esters layering and was therefore omitted from the model (Table 1). The “disappearance” of the layered structure at 35 °C and above showed that the cholesteryl esters had transitioned from a smectic to a nematic phase or an isotropic distribution at higher temperatures. The parameter $\Delta \rho_{\text{core}}$ is the excess scattering density of the (hydrocarbon) core taken relative to that of the outer shell (which was correspondingly fixed at 1.0 as the modeling was done on a relative intensity scale). The results in Table 1 show that this parameter decreased in the temperature range 15–30 °C, where the inner layering disappeared. As the layers were defined relative to the electron density of the hydrocarbons and triglyceride ($\Delta \rho_{c}$ is positive), this was in perfect agreement with a higher electron density being distributed more evenly in the core. At 35 °C and above, the shell that should contain the hydrophilic components (lipid head groups and protein) nearly doubled in width (from 25 to ~46 Å). This means that the quite flexible apolipoprotein \textsuperscript{31,36–38} must indeed be distributed within the shell. Due to the much larger volume of the shell at high temperature, the protein concentration is decreased, and the scattering length density of the shell is greatly reduced. As the core excess scattering length was taken relative to the scattering length density of the shell (which was kept constant in the model), this led to the increase of $\Delta \rho_{\text{core}}$. Taken together, these data indicate that the LDL core had fully melted at 35 °C and that this was accompanied by an increase in flexibility of the protein in the outer shell. The observation of a thicker outer shell is in good agreement with the cryo-TEM pictures in Figure 1 of Kumar \textit{et al.}\textsuperscript{31}  

The phase transition temperature for this LDL preparation was obtained in parallel through DSC, and the particle heat capacity ($C_{p}$) as a function of temperature is shown in Figure 4. Pure LDL was heated from 5 to 45 °C and showed a clear peak starting just above 15 °C and ending at 35 °C. This is in perfect agreement with the transition temperature obtained by the modeling of the SAXS data (Figure 3). The peak was also present upon reheating of the sample after cooling to 5 °C, thus showing that the melting of the LDL was fully reversible. Such reversible thermal transition that occurs near physiological temperature in LDL was attributed to the layering of cholesteryl esters already in 1975 by Deckelbaum \textit{et al.}\textsuperscript{16,34} The physiological relevance of this transition temperature has never been fully understood,\textsuperscript{15,39} even though it has been suggested to play a role in the progression of atherosclerosis; there is, e.g., a lower susceptibility to oxidation when the lipids are packed in a crystalline state.\textsuperscript{18} Our study shows that the fatty core of this LDL preparation melted between 15 and 35 °C. For an LDL preparation with a concentration of triglycerides in the normal range as in this case (Supplementary Table 1), the obtained onset of the melting temperature of 15 °C agrees fully with previous data on the correlation between triglyceride content and the LDL $T_{m}$.\textsuperscript{16}  

In order to investigate the variation and conservation of volumes and core–shell interface upon the shape transition from a super ellipsoid toward a sphere (schematically shown in Figure 5), we calculated the volume and scattering mass (the scattering mass is the product of volume and excess scattering length) of the core and shell as well as the core–shell interface area as a function of temperature. Figure 6a shows that the core volume decreased (22%), whereas the shell volume increased by as much as 78%. This is in agreement with the protein becoming much more disordered above the transition, so that some of it leaves the surface of the particles. The ApoB-100 has a molecular mass of 550 kDa,\textsuperscript{38} and with a partial specific volume of 0.70 mL/g, the volume of a single molecule is 0.64 × $10^{4}$ Å$^{3}$. This means that at low temperature only about 20% of the total shell volume is the protein, whereas it is reduced to about 10% at high temperature. Moreover, the surface area decreased above the phase transition; see Figure 6b. Indeed, a simple calculation shows that a 25 Å thick ApoB-100 layer covers about half of the surface at low temperature. Assuming that the core consists of cholesterol, cholesteryl esters, triglycerides, and the tails of the phospholipids, and using the mass fraction of the latter, the phospholipids can be calculated to cover the rest of the surface assuming a surface area per molecule of 65 Å$^{2}$ for fluid phase at low temperature.\textsuperscript{40} Thus, these results agree well with the high flexibility of ApoB-100 and with our conclusion for a more disordered protein
configuration that partly leaves the particle surface and protrudes toward the solution at high temperatures.

The results for the scattering masses of the various components of the particles are displayed in Figure 6c, showing a clear decrease of the scattering mass of the shell at high temperature. This means that some of the protein is actually intermixed with the core and contributes with a positive scattering length density to it and that the extent of intermixing depends on the protein configuration within the shell and thus the temperature. This is in agreement with the core-scattering mass becoming less negative at high temperature, with a change that almost exactly matches the decrease in the shell-scattering mass. Note that the overall scattering mass is the sum of the two large positive (shell) and negative (core) scattering masses that nearly cancel each other, and thus, a small change in one or both contributions can lead to a large overall change. Such a change, and the corresponding change in the forward scattering $I(q = 0)$, is due to a difference in temperature dependence of the component volumes of the LDL particles. The change in the volumes and the redistribution of the components also leads to a shift of the first minimum and maximum of the form factor (Figure 2a).

As mentioned previously, although the particles are expected to have less than 10% polydispersity in outer radius, other variations in structure are possible, for example, that the particles are nonperfect super ellipsoids of resolution with some eccentricity around the symmetry axis or that the protein is nonuniformly distributed within the shell. The fluctuation term that is added to particle scattering (see the Methods for a detailed description of the model and Figure 7) is expected to describe the scattering from such variations and deviations, and this was the reason for including the term. However, in order to check the possible influence of the effects, a series of calculations were performed for the model without inclusion of the fluctuation term. The models are described in the SI, and the model curves obtained after fitting to the 5 °C SAXS data are shown in Figure 7, where the fit of the original model is also shown. For the original model without the fluctuation term (curve not shown), the first minimum in the form factor is very pronounced due to the near-spherical symmetry of the particles with a constant shell thickness. When an elliptical cross-section is introduced in the model (Figure 7) there is relatively little influence on the curve at the first minimum in the form factor. The belt model influences the minimum much more; however, there is more oscillation throughout the $q$ range and the curve also deviates from the original at the position of the peak that originates from the layering. The curve for the polydisperse model almost perfectly fits with the original model except at very high $q$ where the more gradual, power-law like decay of the original model (and the measured SAXS data) is not reproduced. The optimized model parameters for these alternative models are given in Table 2. The conclusions from the calculations are that all three effects influence the model curve and are probably present in the particle structure. However, the polydispersity alone can almost perfectly account for the smearing of the curve at the first form factor minimum. Therefore, the original model with the fluctuation term can describe all of these structural effects, which are in fact impossible to distinguish from each other. Moreover, with the already relatively large number of parameters in the original

Figure 5. Schematic representation of the LDL particle structure shown as cuts through the particles. Cuts at the center perpendicular to the cylindrical symmetry axis (top) and cuts at the center containing the cylindrical symmetry axis (bottom) are shown at different temperatures from left to right: 5, 20, 30, and 40 °C. The structures were made by generating points in agreement with the various structural constraints by Monte Carlo methods and plotted using PyMOL (PyMOL Molecular Graphics System, Version 1.8, Schrödinger, LLC.), placing atoms/spheres of different colors, depending on the component, at the positions of the points. The van der Waal radius was chosen as 4 Å. Protein and phospholipid head groups are shown in red, cholesterol esters in cyan, and hydrocarbon in blue. The cut structure for the highest temperature is in the lower panel shown in a tilted perspective.

Figure 6. (a) Volume of shell (red filled circles) and core (blue filled circles) as a function of temperature. (b) Area of core–shell interface as a function of temperature. (c) Scattering mass of shell (red filled circles), core (blue filled circles), layers (green filled circles), nonlayer hydrocarbon part of core (black filled circles), and total scattering mass (yellow filled circles).
Thus, there is very good agreement between cryo-TEM and the SAXS results at low temperature.

The high temperature values for the outer radius of the projected and the cut through the structure are, respectively, 122 and 128 Å, compared to the value of 131 Å from SAXS. Note that for the projected and center cut there was an increase in radius of 4–5 Å in the largest radius along the layers upon increasing the temperature. This increase in radius was 6 Å for the SAXS model fitting. Overall, there is very good agreement between the SAXS obtained parameters and the cryo-TEM results of Kumar et al. (Table 3).

Table 3. Comparison of Size and Shape between TEM and SAXS Data

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<tr>
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<th>low temperature</th>
<th>high temperature</th>
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<tr>
<td></td>
<td>εR (Å)</td>
<td>R (Å)</td>
</tr>
<tr>
<td>TEM projected</td>
<td>91</td>
<td>97</td>
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<td>TEM middle cut</td>
<td>97</td>
<td>128</td>
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<td>SAXS</td>
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In summary, the structural changes derived from the model fitting, illustrated in Figure 5, show that LDL overall shape, in terms of eccentricity and size, changes only upon complete melting of the LDL core, i.e., above \( T_m \). Interestingly, our data show that not only the superellipticity but also other parameters such as the thickness of the hydrophilic shell (the protein layer) are governed by the crystallinity of the inner core. This can be correlated with the higher motion of molecules in a more disordered system. The thickness of the hydrophilic shell almost doubles in size at temperatures above \( T_m \), suggesting that even the flexibility of the protein is dependent on the organization of the LDL core. The structural arrangement of the protein is of high importance as even subtle conformational changes in ApoB-100 were shown to have vast implications on the apolipoproteins’ affinity for the cellular LDL receptor and can result in lower LDL removal rates, thus promoting plaque build-up.

It is widely accepted that lipoproteins containing ApoB-100 tend to promote atherosclerosis and that their atherogenic capacity is composition/structure dependent. However, with the wide composition and size distribution associated with the LDL, several precautions are necessary when performing SAS experiments. Ensuring samples of high purity is crucial in order

Table 2. Structural Parameters for Original Model with Fluctuation Term and for the Models with Lower Symmetry without the Fluctuation Term after Adjusting the Model Parameters To Fit the 5 °C SAXS Data

<table>
<thead>
<tr>
<th>Parameter</th>
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</thead>
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<tr>
<td>R (Å)</td>
<td>124.9 ± 0.4</td>
<td>114.2 ± 1.1</td>
<td>109.7 ± 0.4</td>
</tr>
<tr>
<td>ε</td>
<td>0.731 ± 0.003</td>
<td>0.772 ± 0.008</td>
<td>0.681 ± 0.004</td>
</tr>
<tr>
<td>t</td>
<td>2.83 ± 0.08</td>
<td>2.51 ± 0.06</td>
<td>3.42 ± 2.49</td>
</tr>
<tr>
<td>Δρcore</td>
<td>−2.01 ± 0.09</td>
<td>−2.94 ± 0.08</td>
<td>−1.23 ± 0.03</td>
</tr>
<tr>
<td>Δρcyl</td>
<td>3.7 ± 0.1</td>
<td>3.77 ± 0.08</td>
<td>1.87 ± 0.02</td>
</tr>
<tr>
<td>T (Å)</td>
<td>16 ± 1</td>
<td>24.7 ± 0.3</td>
<td>20.2 ± 0.6</td>
</tr>
<tr>
<td>d (Å)</td>
<td>36.7 ± 0.1</td>
<td>36.2 ± 0.1</td>
<td>37.5 ± 0.2</td>
</tr>
<tr>
<td>εc (perp)</td>
<td>1.40 ± 0.02</td>
<td>0.79 ± 0.02</td>
<td>0.76 ± 0.03</td>
</tr>
<tr>
<td>σ(R)/R</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

“aThe protein belt was 70 Å high and 25 Å thick. The rest of the core surface was covered by a 10 Å thin shell. “bThe contrast of the shell varied as 1 + (4 × 10^−5)S, where S is the core surface area, which depends on the size of the particles. Note that the contrast was further normalized to unity for the average size particles. “cThese contrasts are lower than for the other models as the integral scattering of the shell is smaller in this model due to the smaller volume of the shell.
to obtain reliable SAS data. This should furthermore be combined with full compositional characterization of the particles through lipodemic and proteomic studies to be able to elucidate as much structural information as possible when fitting to a predefined model.

Nonetheless, the advancement in modeling presented here allows for systematic structural studies of various compositionally different LDL subfractions that are thought to promote atherogenic plaque build-up. It allows in situ measurements of the structural changes inflicted on the LDL and its core lipids by external stimuli including, e.g., different oxidation agents as well as LDL-specific drugs. This can in turn provide important information on the physiological relevance of the lipid packing (lipids packed in a crystalline state are thought to be shielded from the less mobile radicals18 and give insights into the conditions needed to protect the particles from structural modifications). The latter is essential in the quest for the development of drugs against CVD.

CONCLUSIONS

We have developed a SAXS model that describes the overall structure of LDL as well as the particular layering of lipids inside the LDL particle core. The model, when applied to experimental SAXS data, gives information on the particle-specific melting temperature of the core lipids and shows that the state of the lipid core influences the size and shape of the overall LDL particle and to which extent. The model is also able to extract information on the protein layer of the LDLs, and the results show that it undergoes large structural changes induced by the crystalline packing of the core lipids. This model will now allow systematic studies to unravel composition/structure dependency as well as LDLs susceptibility to external agents that are currently in use or are planned to be used for atherosclerosis control.

METHODS

Purification of LDL Particles from Human Plasma. Human LDL particles (densities between 1.019 and 1.050 g/mL) isolated by sequential ultracentrifugation were a kind gift from the group of Prof. Gunilla Fredrikson (Lund University). The samples conserved in 50% sucrose, 150 mM NaCl, 24 mM EDTA, pH 7.4, were buffered exchanged into 25 mM Tris, 150 mM NaCl, 0.5 mM EDTA, pH 7.4 using PD10 desalting columns (GE Healthcare) and fractionated on a Superose 6 Increase 10/300 GL column (GE Healthcare) equilibrated in the same buffer. Different fractions from SEC corresponding to LDL particle size were collected at 25 °C, stored at 4 °C, and tested individually using SAXS and used within a week of purification.

LDL Compositional Analysis. The concentration of the protein component of LDL (ApoB-100) was determined by the Bradford method and with bovine serum albumin as the standard.19,20 The concentrations of the fat components of LDL were analyzed enzymatically. Total cholesterol (TC) and triglycerides (TG) were determined on a chemistry calibration standard (Pointe Scientific, Inc.). Phosphatidylcholine was determined using the phosphatidylcholine assay kit (Sigma-Aldrich) and confirmed through determination of total phosphorus content.43

SAXS Data Collection. All SAXS data were collected at the optimized SAXS (Bruker AXS) instrument at Aarhus University that uses a powerful gallium metal jet X-ray source (Excillum)66 together with home-built scatterless slits in front of the sample with a two-pinhole geometry. The home-built flow-through sample holders were made of quartz capillaries glued into stainless steel holders, and the samples were handled by an automated injection system, based on Gilson components, which also cleaned and dried the capillary between measurements. The buffers were measured as background, and background subtraction and all necessary normalizations were made using our homemade software. The temperature was controlled by a Peltier element, and the samples were measured at nine different temperatures ranging from 5 to 40 °C.

Derivation of the SAXS Model for LDL. The SAXS intensity expressions for an LDL model were based on previously published cryo-TEM images on human LDL fractions collected and purified under similar conditions to the ones reported here.9,21 These images suggested that the shape of the LDL particles resembles that of a core-shell ellipsoid of revolution, for which the core presents layers of ordered cholesteryl esters. This layering is thought to induce a flattening of the shape, which could be well reproduced by a superellipsoid of revolution. A central cut of a super ellipsoid is shown in Figure 1a. The size and shape are given by the radius at equator, $R$, the half-height of the ellipsoid, $r$, and the shape exponent $t$. Figure 1b shows a super ellipsoid with $t = 3$. A usual ellipsoid of revolution is obtained for $t = 2$, and the super ellipsoid of revolution approaches a cylinder for $t = ∞$.

The outer shape of a super ellipsoid of revolution is in Cartesian coordinates $(x, y, z)$ given by

$$r^2 = \left(\frac{x^2 + y^2}{\epsilon^2}\right)^{1/t} + \frac{z^2}{\rho^2} = R^t$$

or in cylindrical coordinates

$$(\rho^2/\epsilon^2) + (z/\rho) = R^t$$

The form factor $P(q)$ can be calculated using the form factor of a cylinder, which is symmetric around $z = 0$, with a radius $r$ that depends on $z$

$$V^2(q,P) = \int_0^{\pi/2} \int_0^{2\pi} 2\pi(r(z)^2 \sin \theta) \cos(qz \cos \theta) \cos(\theta) \, d\theta \, dz$$

where $V$ is the volume of the super ellipsoid and

$r(z) = \left(\frac{R^2 - (z/\rho)^2}{\epsilon^2}\right)^{1/t}$

$I_1(x)$ is the Bessel function of first order and first kind. In the implementation, both integrals are calculated numerically.

A core-shell model can easily be constructed by making appropriate differences inside the integrand of the angular integration on the amplitudes before they are squared. For this purpose, we introduce the notation

$$V_{\text{core-se}}(q, R, \epsilon, \theta) = \int_0^{\pi/2} 2\pi(r(z)^2 \sin \theta) \cos(qz \cos \theta) \cos(\theta) \, dz$$

For a constant shell thickness $D_{\text{shell}}$ an excess scattering length density of $\Delta \rho_{\text{shell}}$ and a core excess scattering length density of $\Delta \rho_{\text{core}}$, leads to

$$P_{\text{core-shell}}(q) = \int_0^{\pi/2} \left(\rho_{\text{shell}} - \rho_{\text{core}}\right) V_{\text{shell}} \rho_{\text{core}} A_{\text{se}}(q, R - D_{\text{shell}}, \epsilon_{\text{core}}, \theta) \, \sin \theta \, d\theta$$

where $\epsilon_{\text{core}} = \left(e R - D_{\text{shell}}/\rho_{\text{core}} - D_{\text{shell}}\right)$, $V_{\text{shell}}$ is the volume corresponding to the outer shape of the particle and $V_{\text{core}}$ is the volume of the core. Also here the integrals in the implementation are calculated numerically. Note the outer normalization of the core-shell form factor is changed so that it contains both the excess contrasts squared and the volume squared.

The model includes the specific layering of the cholesteryl esters as has been shown in the TEM data22 and illustrated in Figure 1b. To allow that the layers are not in contact with the shell, a minimum

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distance $D_i$ between the core–shell interface and the layers is introduced. For this purpose, we consider a shell with radius $R_i = R - D_{shell} - D_i$ and eccentricity $\varepsilon_i = (e R - D_{shell} - D_i)/(R - D_{shell} - D_i)$. Note that this shell is only used as a means of calculating the radius of the layers.

The extra contrast of the layers is defined as $\Delta\rho_i$ (extra meaning on top of the core excess contrast of hydrocarbon inside the particle core). The thickness of the cholesteryl ester layers is defined as $T$, leading to the following contribution of the central layer inside the square in the integral of the core–shell form factor:

$$A_{central}(q, R, \varepsilon, L, \theta) = \Delta\rho_c R_c^2 T \int_0^{\pi/2} \frac{2J_2(q R_c \sin \theta)}{q R_c \sin \theta} \sin(q T \cos \theta/2) q R_c \sin \theta - q T \cos \theta/2$$

The two other layers are defined to be at a separation of $d$ from the central one, which then contributes the following term inside the square:

$$A_{2layer}(q, R, T, d, \theta) = \Delta\rho R^2 \int_0^{\pi/2} \frac{2J_2(q R \sin \theta)}{q R \sin \theta} \left[(2d + T) \sin (q (2d + T) \cos \theta/2) - (2d - T) \sin (q (2d - T) \cos \theta/2) \right]$$

where $R_i$ is given by

$$R_i = \left(R - d/\varepsilon_i \right)^{1/2}$$

The total form factor then becomes

$$P_{LDL}(q) = \int_0^{\pi/2} \left(\Delta\rho_{shell} V_{shell} A_{shell}(q, R, \varepsilon, \theta) - \Delta\rho_{core} V_{core} A_{core}(q, R - D_{shell}, \varepsilon_{core}, \theta) + A_{central}(q, R, \varepsilon, L, \theta) + A_{2layers}(q, R, T, d, \theta) \right)^2 \sin \theta \, d\theta + C_{bulk} P_{bulk}(q R_c)$$

where the last term, for example, describes the fluctuation scattering within the shell due to a partially disordered protein with an ensemble-average radius of gyration $R_c$. Note that calculations for particles with deviation from the ideal model in terms size polydispersity, eccentricity around the $z$ axis, or the protein being located in a belt show that these effects influence the curve in a similar way as the fluctuation term (see Figure 7 and the SI for model descriptions). The effect of deviations from the ideal model as well as of the fluctuation term is to add intensity at the minima of the form factor oscillations in a similar way. Therefore, the fluctuation term may also describe such deviations. Due to this and the fact that the model already contains a relatively large number of parameters, the model with the fluctuation term is to be preferred.

The parameter $C_{bulk}$ is a scale factor and the fluctuations are described by the form factor of Gaussian chains

$$P_{bulk}(x) = \frac{2(x^{-2} - 1 + x)}{x^2}$$

where $x = q^2 R_c^2$. The model was implemented in a home-written program which uses standard weighted least-squares methods for optimizing the model parameters when fitting to the experimental data.\(^{37}\)

The volume of a super ellipsoid of revolution is given by

$$V(R, \varepsilon, t) = \frac{4\pi}{3t} \left(\varepsilon + \frac{1}{t} \right)$$

where $\beta(x, y) = \Gamma(x) \Gamma(y) / \Gamma(x + y)$ and $\Gamma(x)$ is the gamma function.\(^{48}\)

The surface areas were calculated numerically as the volume of a thin shell with constant thickness divided by the shell thickness

$$S(R, \varepsilon, t) \approx \frac{V(R, \varepsilon, t) - V(R - \delta, e R - \delta)/R - \delta, t)}{\delta}$$

The contribution to the scattering mass of the various components of the model are given by their scattering, which is the product of the excess scattering length density $\Delta\rho$ of the component multiplied by volume $V$ of the component. As the total scattering mass is conserved, except for small variations in the changes of the volume of the component with temperature, the calculation of this quantity can reveal if there is a change in the mixing of the components, when the temperature is changed.

In the calculation of the volumes of the various components from the composition (Supporting Table S1) as given in the texts, we used a protein partial specific density of 0.7 cm\(^3\)/g, a density of 0.99 g/cm\(^3\) for cholesterol and cholesteryl esters, a density of 1.00 g/cm\(^3\) for the phospholipids, a density of 0.925 g/cm\(^3\) for triglycerides, and partial volumes of head and tail for POPC of 324 and 980 Å\(^3\) respectively.

**Schematic Representation of LDL Particle Structure.** The schematic representations of the LDL structures were made by generating points in agreement with the various structural constraints by Monte Carlo methods.\(^{42}\) The points for each component were saved in different files in PDB format. The structures were plotted using PyMOL (PyMOL Molecular Graphics System, Version 1.8, Schrödinger, LLC) placing atoms/spheres of different colors, depending on component, at the positions of the points. The van der Waal radius was chosen as 4 Å.

**Differential Scanning Calorimetry.** The heat capacity ($C_v$) for purified LDL was collected using a VP-DSC from MicroCal. The LDL sample (protein concentration 0.5 mg/mL) in 50 mM Tris buffer, 150 mM NaCl, after heating was then repeated after equilibration of the SEC column and elution of LDL as the reference. The sample was first equilibrated for 30 min at 5 °C then heated to 45 °C with a heating rate of 60 °C/h and equilibrated for 10 min. The heating process was then repeated after cooling the sample down to 5 °C with the same rate. Background was subtracted using the Origin Software.

**Size Analysis of Published Cryo-TEM Micrographs.** The micrographs of Kumar et al.\(^{21}\) were analyzed using the ImageJ software (https://imagej.nih.gov) in order to estimate the dimensions of the particles for comparison with the SAXS data. By calculating line intensities in various directions of the particles; both projected and cuts through the particle in the middle were analyzed. The separation of the cholesteryl ester layer was used for calibration of the scale of micrographs by setting it equal to the SAXS value of 37 Å. The line intensities were analyzed by fitting a series of Gaussian functions plus a constant to the data so that the peak center positions could be obtained and used for calibration. The outer dimensions were determined from where the intensity in the line profile approaches the lowest value.

**ASSOCIATED CONTENT**

**3 Supporting information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.nano.6b08089.

Size-exclusion chromatogram for LDL fractions after ultracentrifugation, SAXS profiles for LDL fractions before and after SEC, composition for LDL fraction after ultracentrifugation and SEC, description for derivation of alternative models shown in Figure 7 (PDF)

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Author Contributions

Notes
The authors declare no competing financial interest. Studies investigating human LDL and HDL involves lipid extraction from plasma obtained from healthy blood donors. Plasma is obtained from clinical immunology and transfusion medicine, Skåne University Hospital, Sweden, in accordance with the Helsinki Declaration. The plasma is anonymized and outdated and can therefore not be used for transfusion of patients.

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