Effects of Orientational Order and Particle Size on the NMR Line Positions of Lipoproteins

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We demonstrate that information on internal orientational order and size of lipoprotein particles can be extracted from the positions of their NMR spectral lines. The magnetic field obtained by solving the field equations for a model lipoprotein particle is shown to account for the hitherto unexplained size dependence of the experimental NMR frequencies. The predicted sign, magnitude, and functional form of the frequency shifts are verified by novel experimental ^{1}H NMR data from size-specific lipoprotein samples.

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The lipoprotein particles transport water-insoluble lipid molecules in the circulation of the human body. They have a spherical structure with a core of hydrophobic lipids (cholesteryl esters, triglycerides, and some cholesterol) surrounded by an amphipathic surface of apolipoproteins, phospholipids, and cholesterol. An understanding of their structure and function requires detailed knowledge of their organization, e.g., internal orientational order. NMR spectroscopy is uniquely suited to address such problems, and has been extensively applied to the study of the location and motion of the lipoprotein lipid molecules [1]. However, obtaining information on the orientational order within the lipoproteins is complicated by the fact that the distribution of their local alignment axes (normal to the particle surface) is isotropic. This means that the anisotropies of the conventional NMR parameters such as nuclear shielding and quadrupolar coupling do not affect the positions of the NMR spectral lines, but must be roughly estimated from the relaxation rates (e.g., from the viscosity or magnetic field dependence of the linewidths, temperature dependence of the spin-lattice relaxation times T_1, or by combining the linewidth and T_1 data) or from the line shapes in samples where particle reorientation has been reduced sufficiently to give powderlike line shapes [1].

On the other hand, there has been a search for an explanation [2,3] for the curious observation [2–6] that the ^{1}H NMR signals from the methyl $-C\underline{H}_3$ and methylene $(C\underline{H}_2)_n$ groups in the hydrocarbon chains of the lipids in the lipoproteins shift systematically to lower frequencies with the decrease of the lipoprotein particle size. The consistency of this behavior points to the inference that there is some general physical mechanism behind it. In fact, it has been suggested [3] that the lipids in the particle core and surface shell have different magnetic susceptibilities and the size-related frequency shifts arise because the ratio of the core to surface lipids varies continuously with the particle diameter.

We show in this Letter that *isotropic* magnetic susceptibilities of the lipids are not capable of explaining the observed behavior. We introduce a simple model for the lipoprotein particles involving the *anisotropy* of the magnetic susceptibility, which leads in a natural way to a size-dependent frequency shift of correct sign, magnitude, and functional form for all the NMR resonances of the lipoprotein lipids. As the susceptibility anisotropy is directly proportional to the internal orientational order parameter of the particle, the frequency shift can be used to probe the ordering. We verify the predictions of the model by novel experimental ^{1}H NMR data from size-specific lipoprotein samples.

We model a lipoprotein particle by a spherically symmetric micelle of radius R_2 consisting of a core of radius R_1 and a spherical surface shell of thickness $\Delta = R_2 - R_1$ (see Fig. 1). The hydrophobic core (region 1) and the

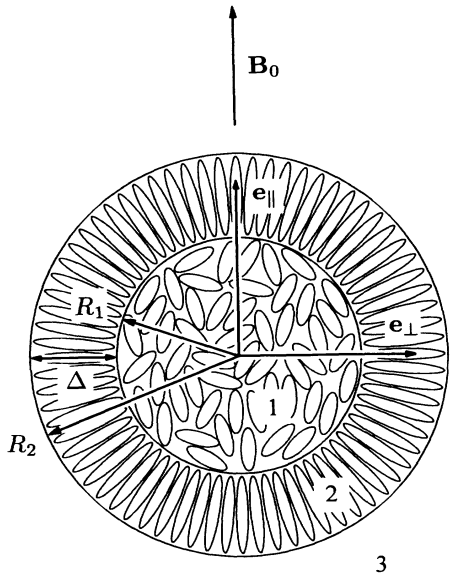

FIG. 1. Schematic representation of the model for the lipoprotein particles.

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medium surrounding the micelle (region 3) are taken to be in an isotropic liquid state, while the molecules in the shell (region 2) are taken to be radially oriented (water molecules hydrating the lipids are included in the shell). This means that the magnetic susceptibility of the shell material is anisotropic: $\Delta\chi \equiv \chi_{\parallel} - \chi_{\perp} \neq 0$, where $\chi_{\parallel}$ and $\chi_{\perp}$ are the volume susceptibilities parallel and perpendicular to the radius vector $\mathbf{r}$ of the micelle, respectively. Hence the shell magnetization $\mathbf{M}$ (the magnetic dipole moment per unit volume) induced by the externally applied magnetic field $\mathbf{B}_0 = \mu_0 \mathbf{H}_0$ is not parallel with $\mathbf{B}_0$: To first order in $\chi_{\parallel}$ and $\chi_{\perp}$ (in SI units),

$$\mathbf{M}(\theta) = H_0[\chi + \tfrac{2}{3}\Delta\chi P_2(\cos\theta)]\mathbf{e}_{\parallel} + \tfrac{1}{2} H_0 \Delta\chi \sin 2\theta \mathbf{e}_{\perp}, \quad (1)$$

where $\chi = \frac{1}{3}(2\chi_{\perp} + \chi_{\parallel})$ is the isotropic part of the susceptibility tensor, $P_2(\cos\theta) = \frac{1}{2}(3\cos^2\theta - 1)$ is the second-order Legendre polynomial, θ is the angle between $\mathbf{H}_0$ and the radius vector $\mathbf{r}$, and $\mathbf{e}_{\parallel}$ and $\mathbf{e}_{\perp}$ are the unit vectors parallel and perpendicular to $\mathbf{H}_0$ in the plane of $\mathbf{H}_0$ and $\mathbf{r}$.

The problem is to determine the magnetic field strengths $\mathbf{H}_i$ in the regions $i = 1$, 2, and 3 which satisfy the following conditions: (a) $\nabla \times \mathbf{H}_i = 0$, (b) $\nabla \cdot (\mathbf{H}_i + \mathbf{M}_i) = 0$, (c) the tangential components of $\mathbf{H}_i$ are continuous at each interface, (d) the normal components of $\mathbf{H}_i + \mathbf{M}_i$ are continuous at each interface, and (e) at large distances from the particle $\mathbf{H}_i = \mathbf{H}_3 = (1 - a\chi_3)\mathbf{H}_0$ where a is the demagnetization factor which depends on the shape of the body of the material surrounding the micelle ($a = \frac{1}{3}$ for a sphere and 0 for a long cylinder parallel with $\mathbf{H}_0$). The conditions (a) and (b) imply the existence of a scalar potential ϕ_i satisfying the equations $\mathbf{H}_i = -\nabla\phi_i$ and $\nabla^2\phi_i = \nabla \cdot \mathbf{M}_i = 2H_0\Delta\chi_i \cos\theta / r$. The solution is

$$\mathbf{H}_i(r,\theta) = H_0\left[1 - a\chi_3 + A_i + \frac{2}{3}\Delta\chi_i \ln\frac{R_2}{r} + \frac{4}{9}\left(\frac{C_i}{r^3} - \Delta\chi_i\right)P_2(\cos\theta)\right]\mathbf{e}_{\parallel} + \frac{1}{3}H_0\left(\frac{C_i}{r^3} - \Delta\chi_i\right)\sin 2\theta \mathbf{e}_{\perp}, \quad (2)$$

where $A_3 = C_1 = 0$ and A_1, A_2, C_2, and C_3 are given by the expressions

$$A_i = \frac{1}{3}\left(\chi_3 - \chi_i - \frac{2}{3}\Delta\chi_i + 2\Delta\chi_2 \ln\frac{R_2}{R_i}\right), \quad (3)$$

$$C_i = \frac{3}{2}\sum_{j=1}^{i-1}(\chi_j - \chi_{j+1})R_j^3. \quad (4)$$

The *microscopic* magnetic field strength at the vicinity of a particular molecule is $\mathbf{H}_m = \mathbf{H} + \frac{1}{3}\mathbf{M}$, provided that the contribution of the molecules inside an infinitesimal sphere (Lorentz sphere) surrounding the molecule in question can be neglected. Thus the microscopic flux density in the core region of the micelle is

$$\mathbf{B}_1^m = \mu_0 \mathbf{H}_1^m = \left[1 + \left(\frac{1}{3} - a\right)\chi_3 + \frac{2}{3}\Delta\chi_2 \ln\frac{R_2}{R_1}\right]\mathbf{B}_0. \quad (5)$$

Since this field is uniform, it does not cause line broadening in the NMR spectrum. Note that $\mathbf{B}_1^m$ does not depend on χ_1 or χ_2, i.e., the *isotropic* parts of the magnetic susceptibilities of the core and surface lipids have no effect on the resonance frequencies of the nuclei in the core. This is the case also for the nuclei in the surface shell, because χ_1 and χ_2 enter into the expression of $\mathbf{B}_2^m$ within the functions $(\chi_1 - \chi_2)P_2(\cos\theta)$ and $(\chi_1 - \chi_2)\sin 2\theta$ whose values cancel out when integrated over the shell. The frequency of the ith NMR line originating from the core region of the micelle can be written as

$$\nu_i(R_2) = \nu_i^0 + \frac{2}{3}\nu_0\Delta\chi_2 \ln\frac{R_2}{R_2 - \Delta}, \quad (6)$$

where ν_i^0 is the frequency of the line in the absence of the contribution due to $\Delta\chi_2$, and ν_0 is the operating frequency of the spectrometer. As ν_i is an explicit function of the radius R_2, the model leads in a natural way to a systematic dependence of the frequency on the size of the micelle. Note that this dependence is absent if $\Delta\chi_2 = 0$, i.e., if there is no orientational order in the micelle. However, there may also be an *implicit* dependence of ν_i on R_2, due to size-associated changes in ν_i^0, $\Delta\chi_2$, and/or Δ (due to changes in the chemical composition, structure, orientational order, etc.).

The lipoprotein samples for the ^{1}H NMR measurements were obtained from blood samples of a healthy male volunteer. They were drawn into tubes containing ethylene-diaminetetraacetic acid (EDTA) after an overnight fast and plasma was separated by centrifugation at 1200g for 15 min (+4 °C). The main lipoprotein categories, very-low-, intermediate-, low-, and high-density lipoproteins (VLDL, IDL, LDL, and HDL), were isolated from 3.6 ml of plasma by sequential ultracentrifugation. After isolation the lipoprotein fractions were dialyzed against 0.15M NaCl solution containing 0.01% EDTA, pH 7.4 (EDTA-saline). In order to obtain highly size-specific NMR data, homogenous lipoprotein samples of narrow particle size range were prepared by combining ultracentrifugation and gel filtration chromatography. The lipoprotein density range was first separated from plasma by a single ultracentrifugation step of 48 h. 10 ml of the lipoprotein sample was then applied to a Sepharose 4B column (Pharmacia LKB Biotechnology, Uppsala, Sweden) with total bed volume of 180 ml (900×16 mm) and eluted with EDTA-saline at a flow rate of 27.4 ml/h. To stabilize the pH, 1M sodium phosphate buffer, pH 7.4, was added to a final concentration of $10^{-2}M$. The radii of the particles eluted to different fractions were determined by calibrating the column by a series of protein standards of known size. The elution curves of the lipoprotein sample and protein standards (showing the variation of solute concentration in the eluent with the fraction number) were recorded by monitoring ultraviolet

absorbance at the wavelength of 280 nm. The resulting information on the size distribution function of the lipoprotein particles and on the characteristics of the response function of the column (i.e., on the width and shape of the elution peak of a standard) was used to correct the calibration for the effects of the instrumental broadening of the elution curves.

The ^{1}H NMR spectra of all the isolated lipoprotein fractions were recorded at +37 °C on a Jeol JNM-GX400 FT NMR spectrometer at $\nu_0 = 399.8$ MHz. A double tube system was used; the sealed external reference tube (5 mm) containing the reference and locking substances (sodium 3-trimethylsilyl[2,2,3,3-D_4]propionate (TSP) 4 mmol/l, $MnSO_4$ 0.3 mmol/l in 99.8% D_2O) was placed coaxially into the NMR sample tube (10 mm) containing 2.5 ml of a sample. In each experiment 256 free induction decay signals were accumulated using the binomial $1-\bar{1}$ pulse sequence to suppress the water signal.

The low frequency parts of the aliphatic regions (190 to 840 Hz from the external TSP-based reference) of the ^{1}H NMR spectra of the VLDL, IDL, LDL, and HDL samples are shown in Fig. 2. As noticed earlier [2–6], the methyl and methylene resonances shift towards lower frequencies with the decreasing size of the particles. Note that the frequencies of the lactate doublets and ethanol triplets (from the solvent outside the lipoproteins) remain constant. As a matter of fact, similar size-related shift behavior is observed for *all* the lipid resonances of the lipoprotein particles. This indicates that the shifts are not due to changes in the chemical composition of the particles (which should have different effects on different resonances) but there must be a common mechanism behind them.

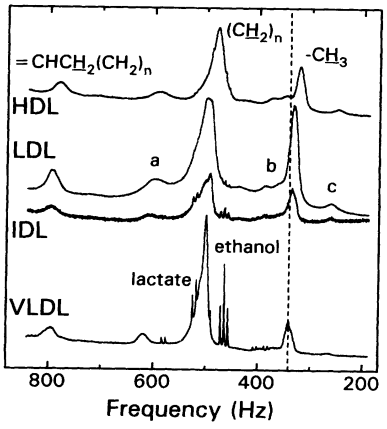

FIG. 2. The low frequency parts of the aliphatic regions of the experimental ^{1}H NMR spectra of the ultracentrifuged VLDL (radius $R_2 = 150$–400 Å), IDL (125–175 Å), LDL (90–140 Å), and HDL (25–60 Å) fractions. The frequency scale is from the external TSP-based reference. Note the frequency shifts of the six marked lipid resonances. Peak *a* is from the methylene protons $-CH_2CH_2COOC-$ and peaks *b* and *c* are from methyl protons of the cholesterol backbone.

The ^{1}H NMR spectra of the size-specific gel filtrated lipoprotein samples made it possible to study the size-dependent frequency shifts quantitatively. The three most intensive resonances in the NMR spectra, arising from different parts of the hydrocarbon chains of the lipids, were selected for a detailed analysis. These were the signals from the lipid hydrocarbon methyl $-CH_3$ and methylene protons $(CH_2)_n$, and the methylene protons next to the olefinic groups $=CHCH_2(CH_2)_n$, around 330, 490, and 790 Hz, respectively. In order to obtain accurate frequency values, the envelopes of these signals were subjected to line shape analyses by fitting one to three individual Lorentzian components to each resonance by using the program FITPLA [6], which performs Levenberg-Marquardt-method based modeling in the frequency domain. In the final analysis, the shift behavior of each resonance was investigated by examining the behavior of its most distinctive Lorentzian component.

Figure 3 displays the frequencies of the three Lorentzians as functions of the radius of the lipoprotein particle. The theoretical expressions for the frequencies, Eqs. (6), were fitted to these points by a single least-squares refinement. The three curves were determined by four adjustable parameters: Their positions on the frequency axis were specified by the asymptotic frequencies ν_1^0, ν_2^0,

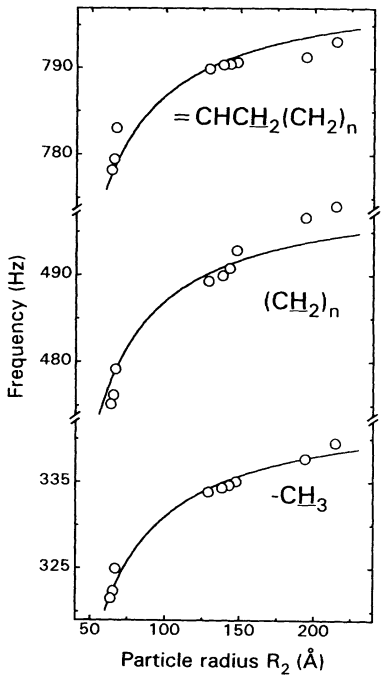

FIG. 3. The experimental NMR frequencies (circles) of the lipoprotein lipid hydrocarbon $-CH_3$, $(CH_2)_n$, and $=CHCH_2(CH_2)_n$ protons as functions of the lipoprotein radius R_2. The solid curves represent a least-squares fit to the points using Eq. (6). The resulting values of the magnetic susceptibility anisotropy and asymptotic frequencies are $\Delta\chi_2 = -0.223$ ppm, $\nu_1^0 = 344.2$ Hz, $\nu_2^0 = 500.2$ Hz, and $\nu_3^0 = 800.0$ Hz.

and ν_3^0, and their curvature was specified by the susceptibility anisotropy $\Delta\chi_2$ (actually there is only a one single curve shifted vertically to different positions). The thickness of the shell was constrained to the value $\Delta = 20$ Å. The results of the fit are shown in Fig. 3.

Figure 3 reveals that the dependence of the frequencies on the particle size is a strikingly general phenomenon. All the studied resonances exhibit a shift of similar magnitude and functional form. The theoretical expression for the shift, Eq. (6), is in good agreement with this form. The theoretical curves reproduce the observed behavior, if the magnetic susceptibility anisotropy is $\Delta\chi_2 \approx -0.2$ ppm. This value can be compared with the local susceptibility anisotropy $\Delta\chi_N = \chi_\parallel^N - \chi_\perp^N$ of the nonspherical micelles making up lyotropic liquid crystals, where $\chi_\parallel^N$ and $\chi_\perp^N$ are the susceptibilities parallel and perpendicular to the local normal to the micelle surface, respectively. In a lyotropic nematic phase $\Delta\chi_N$ can be determined from the macroscopic susceptibility anisotropy in the laboratory frame, $\Delta\chi_D = \chi_\parallel^D - \chi_\perp^D$, where $\chi_\parallel^D$ and $\chi_\perp^D$ are the susceptibilities along and perpendicular to the liquid crystal director, as $\Delta\chi_D = S_{DN}\Delta\chi_N$ [7]. The order parameter S_{DN} is the average value of $P_2(\cos\theta_{DN})$ where θ_{DN} is the angle between the director and the local surface normal. By combining representative experimental values for $\Delta\chi_D$ [8] and S_{DN} [7], the resulting $\Delta\chi_N$ is of the order of -0.3 ppm (in SI units). It has the same sign and magnitude as the present $\Delta\chi_2$. This is an important result, as it means that application of the magnetic susceptibility attributes of simple micelles to lipoproteins leads directly (without any adjustable parameters) to the prediction of a size-dependent NMR frequency shift of correct sign, magnitude, and functional form.

To conclude, the present results demonstrate that there exists a mechanism by which the internal orientational order of the lipoprotein particles can affect the NMR line positions of the lipids. The order is measured by the susceptibility anisotropy $\Delta\chi_2$, which may be determined from the particle size dependence of the line positions. Studies of the temperature dependence of $\Delta\chi_2$ may make it possible to detect, e.g., liquid-crystal phase transitions inside the lipoproteins. Another interesting application of the shift function, Eq. (6), may be found in the determination of the size distribution of the lipoproteins directly from the composite NMR spectrum of whole blood plasma [2,3,5,6].

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