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Single-particle Tracking of Human Lipoproteins

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Abstract

Lipoproteins, such as high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very-low density lipoprotein (VLDL), play a critical role in heart disease. Lipoproteins vary in size and shape as well as in their apolipoprotein content. Here, we developed a new experimental framework to study freely diffusing lipoproteins from human blood, allowing analysis of even the smallest HDL with radius of 5 nm. In an easily constructed confinement chamber, individual HDL, LDL, and VLDL particles labeled with three distinct fluorophores were simultaneously tracked by wide-field fluorescence microscopy and their sizes were determined by their motion. This technique enables studies of individual lipoproteins in solution and allows characterization of the heterogeneous properties of lipoproteins which affect their biological function but are difficult to discern in bulk studies.

Graphical Abstract

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Supporting Information

Experimental details, data analysis, Protocols S1–S6, Table S1, Figures S1–S13 and videos, each with a detailed subset of particle tracks. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Cholesterol is transported in plasma on lipoproteins, which are very heterogeneous in size and in protein and lipid composition, affecting their biological properties. Lipoproteins have distinct size regimes with mean radius ($\langle R \rangle$) of 5 nm for HDL and $\langle R \rangle$ of 10 nm for LDL, while VLDL are larger and have a more polydisperse size distribution ($R \sim 15\text{--}45$ nm). The cholesterol content of the different lipoproteins species is known to be closely related to cardiovascular disease (CVD) risk, but the particle count of the different types of lipoproteins or the count of certain subfractions may be a better marker.^{1–5} For example, a recent study suggested that the number of LDL particles is a better predictor of risk than the cholesterol content of LDL.⁶ Furthermore, it has been proposed that different subclasses of HDL particles can impart CVD risk such as the observed inverse correlation between the number of larger HDL particles and disease.^{3–5} Heterogeneous protein distributions on lipoproteins such as apolipoprotein C-III (ApoCIII) on VLDL, can also potentially serve as strong predictors of CVD.^{7,8} However, there are limited methods for accurately characterizing the different physical and chemical properties of individual lipoprotein particles as current techniques largely measure only bulk properties.

In this work, we developed an experimental method using a confinement chamber for characterizing freely-diffusing individual lipoproteins using fluorescence microscopy. We chose to perform measurements on diffusing particles to avoid the use of surface attachments which have been previously used to study particles attached to a surface.^{9–12} One experimental concern was that attachment labels may not incorporate evenly and may create artifacts due to unknown heterogeneous properties of the lipoproteins. Moreover, we sought to have a more direct measurement of size through particle tracking (*i.e.* diffusion coefficient) rather than by fluorescence intensity calibration, which is assumed to be proportional to the surface area and impractical to implement for native lipoproteins. To validate our methodology, we simultaneously measured individual HDL, LDL, and VLDL and directly compared their size distributions.

The general approach was to confine the particles in a thin fluid layer of thickness ~ 100 nm so they were approximately in focus at all times. There are many precedents for tracking single particles using confinement^{13–15} and we are extending this method to track lipoproteins from human blood. Our confinement chamber design is a variation of the CLIC method¹⁶ but does not require a mechanically-mounted lens to compress the chamber. The chamber was constructed from a PEG-coated glass slide, a PEG-coated coverslip, and double-sided tape (Figure 1 and Protocol S1). The chamber width is wider than typical flow-cell designs, allowing the coverslip to flexibly bend inward.

After flowing in a sample, a clamp was placed across the center axis of the chamber and liquid was pulled from the edges, creating two air gaps on either end of the chamber. After removing the clamp, we found the central region would remain collapsed to a thin layer of liquid which increased in thickness as we moved progressively away from the central axis towards the sides of the chamber (Figure S1). An interference band at the center of the chamber provided visual confirmation that the chamber was in a collapsed state. Filling the air gaps with fluid caused the chamber to revert to the original state. The stability of the chamber appeared dependent on the use of PEG-coated slides and we hypothesize this may be due to a uniform surface tension which forms at the edges of the chamber. Compared to other methods, our confinement chamber design is easy to implement but there is no direct control over exact chamber thickness.

Blood plasma was separated by ultracentrifugation into three fractions, HDL, LDL, and VLDL. HDL ($\langle R \rangle \sim 5$ nm) and LDL ($\langle R \rangle \sim 10$ nm) have fairly narrow size distributions and therefore serve as effective controls. VLDL has a more polydisperse size distribution ($R \sim 15\text{--}45$ nm), making it an interesting target of study since we can use diffusion to sort VLDL particles according to size and potentially consider distributions of bound apolipoproteins on their surfaces. Primary amine groups on HDL, LDL, and VLDL were labeled with Alexa Fluor[®] 594 (*succinimidyl ester*), 488 (*tetrafluorophenyl ester*), and 647 (*succinimidyl ester*) and separated from free dye using desalting columns (Protocol S2). Free protein may be present, which can also be labeled, and these populations, especially if aggregated, may appear in the same size regime as the particles being studied. UV absorption measurements (Figure S2) were used to estimate appropriate dilutions (Table S1) for simultaneous tracking experiments at 200 frames per second. Example videos simultaneously tracking all three lipoproteins are provided in the Supporting Information and the first frame of one video is shown in Figure 2 as well as representative transmission electron microscopy (TEM) images of lipoproteins. A calibration slide with a grid was used to determine an alignment correction between the three cameras and applied to the images. All analysis was conducted using custom software (Protocol S3). Background subtraction was applied, taking advantage of the fact that the particles of interest are moving (some lipoproteins were stuck to PEG-coated glass surfaces). Each particle position was localized using a Gaussian fit to the dominant fluorescent signal. A semi-automated routine was used to facilitate linking particles between successive frames, but all tracks were verified through manual inspection. Frames where the particle appeared stuck to the surface or overlapped with another particle were not included in the analysis. The lipoprotein type was determined

by the dominant fluorescent channel and a few cases of fusion between different particle types were apparent, possibly induced by the PEG surfaces (Figures S3–S8).

The effective radius was determined by diffusion using the relationship¹⁷ $\langle x^2 \rangle = 2Dt$ where $D = k_B T / (6\pi\eta R)$ k_B is the Boltzmann constant, T is the temperature, $\langle x^2 \rangle$ is the mean squared displacement in the x - or y -direction, t is the time interval for one frame (5 ms), D is the diffusion constant, η is the fluid dynamic viscosity (1 mPa s)¹⁸, and R is the radius of the particle. We approximated viscosity as 1 mPa s but the precise viscosity is unknown, due to drag effects from the surface and other interactions. Step sizes may be fit to histograms (See SI), but here, we used the simplest conversion with $R = 2tk_B T / (6\pi\eta \langle x^2 \rangle)$. Reported radii are the averages from the x - and y -directions in the image plane.¹⁹ A set of example particles from all three donors as well as simulations are provided in the SI. The distributions of radii for three blood donors measured by both particle tracking and TEM are shown in Figure 3 and quantified in Table 1. See SI for details on analysis of TEM images (Protocol S4 and Figures S9–S11) as well as dynamic light scattering (DLS) measurements (Figure S12). DLS and other label-free methods, such as NMR,²⁰ can complement this technique but may average out features, such as heterogeneous binding of apolipoproteins. DLS is also biased towards larger particles making interpretation difficult for polydisperse sizes. Confinement chambers also create bias as larger particles experience more drag from the confining surface which slows down their diffusion,²¹ causing an effective increase in diffusion radius. Sticking to the surface and blurring of the particle²² during the finite exposure time will also cause an apparent increase in the radius. Blurring could cause as much as ~ 30% of the increase (Figure S13), but in practice this contribution is expected to be smaller since other effects reduce speed and therefore, blurring as well. Decoupling these effects precisely is not possible with our current setup. The mean effective diffusion rates for HDL, LDL, and VLDL were 30, 12, and 8 $\mu\text{m}^2/\text{s}$, respectively. In general, VLDL and LDL are tracked fairly easily while HDL is close to the limit of our resolution.

In conclusion, we demonstrated a simple method to confine small native particles, allowing them to be tracked by fluorescence. We applied this method to human lipoproteins and simultaneously tracked HDL, LDL, and VLDL. We measured an effective radius for each particle, which was larger than the expected radius, attributed to effects from the confining surfaces.²³ The distribution of radii for each lipoprotein type is a potential CVD biomarker. For example, this method can be used to compare radii distributions of VLDL collected before and after a high-fat meal. Some measurements are probably not possible, such as resolving small size differences between subclasses of HDL. Of particular interest would be the quantification of physiological concentrations of individual apolipoprotein associated with CVD such as ApoCIII⁷ on individual lipoproteins though clearly more work is needed to accomplish this goal. Another application would be to study the simultaneous, competitive binding of two apolipoproteins, such as ApoCIII and ApoE. The method could also be extended to studies of other biological particles, such as synaptic vesicles or exosomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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19. The particles are constrained in the *z*-direction (axial direction) by the height of the chamber and may traverse this distance in a single frame, so the *z*-position, if measured, would not be useful for determining the particle size. However, diffusion along the *x*- and *y*-directions is independent of *z*-motion, though it will be affected by sticking and drag from the surfaces. There was no measurable anisotropy for radii determined using steps in the *x*- and *y*-directions. The chamber thickness changes subtly in the *y*-direction but there was no measurable dependence of radii on the particle's mean *y*-position.
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23. If HDL, for example, had a stronger non-specific interaction with the surface (sticking), it may have resulted in a larger effective radii. There is no method to anticipate these effects and they may be undetectable if they occur on time scales shorter than the measurement window of 5 ms. The distribution of radii for the three lipoprotein types when measured simultaneously correlates well with the TEM values suggesting that the measured diffusion rates are meaningful.

Figure 1.

Design for confinement chamber construction. A PEG-coated glass slide and coverslip form a chamber using double-sided tape (~ 100 nm thick) and a clamp compresses the central axis (red dashed line). After absorbing liquid from both sides of the chamber, which produces an air gap on either side, the clamp is removed and the central region remains collapsed. The ideal region for imaging lies between the center region (too thin) and the outer regions (too thick).

Figure 2.

Simultaneous fluorescence tracking of human lipoproteins. HDL (red), LDL (green), and VLDL (blue) diffuse in the confinement chamber and are imaged simultaneously at 200 frames per second (5 ms exposure time) using excitation from three lasers (top left). Representative TEM images of the lipoproteins are shown (right). Each particle is tracked using a Gaussian fit to the fluorescent signal and an example time series of the first 15 frames of each lipoprotein type is shown (bottom). The yellow boxes represent the same size in the top and bottom panels.

Figure 3. Radius (R) distribution of lipoproteins determined by particle tracking (left) and TEM (right) for HDL (red), LDL (green), and VLDL (blue) from three different blood donors A, B, and C.

Table 1

Mean radii, $\langle R \rangle$, obtained from analyses of particle tracking and TEM images.^[a]

Donor	Lipoprotein (Count)	$\langle R \rangle_{\text{particle tracking}}$ (nm)	$\langle R \rangle_{\text{TEM}}$ (nm)
A	HDL (34)	8 ± 3	5 ± 2
	LDL (129)	21 ± 7	12 ± 1.2
	VLDL (133)	36 ± 16	19 ± 6
B	HDL (48)	7 ± 2	6 ± 1.5
	LDL (24)	17 ± 6	12 ± 2
	VLDL (105)	25 ± 10	16 ± 3
C	HDL (16)	8 ± 2	6 ± 1.3
	LDL (89)	17 ± 4	$12 \pm 3^{[b]}$
	VLDL (54)	25 ± 10	15 ± 4

^[a] For HDL or LDL, $\langle R \rangle$ were determined using Gaussian fits and for VLDL, $\langle R \rangle$ were calculated as a simple mean (standard deviation of the distributions are given).

^[b] Donor C TEM distribution of LDL was fit to two Gaussians (the larger value is reported) as small particles which may indicate the presence of HDL were observed (Figures 3 and S11).