A comparison of LDL size determination using gradient gel electrophoresis and light-scattering methods

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Abstract **This study compared gradient gel electrophoresis (GGE) and light-scattering (LS) methods of determining low density lipoprotein (LDL) particle size. LDL was isolated from 27 fasting subjects. Peak particle size was determined by GGE on 3–13% gradient gels (Gradipore, Sydney, Australia) and by LS using a Zetasizer 3000 (Malvern Instruments, Malvern, UK). Repeated measurements on a single specimen indicated a coefficient of variation (CV) of 0.3%. A correlation was noted ($P < 0.0001$; $r = 0.78$) when comparing LDL particle size determined by LS methodology and GGE. Particle diameter results obtained by LS were smaller than those obtained by GGE (23.1 ± 0.1 vs. 26.1 ± 0.1 nm; $P < 0.0001$). LDL particle size determined by LS methodology correlated inversely with the log of triglyceride level ($P < 0.0001$; $r = -0.77$) and positively with high density lipoprotein (HDL) cholesterol level ($P < 0.002$; $r = 0.57$).**—O'Neal, D., P. Harrip, G. Dragicevic, D. Rae, and J. D. Best. **A comparison of LDL size determination using gradient gel electrophoresis and light-scattering methods.** ***J. Lipid Res.*** **1998.** 39: **2086–2090.**

Supplementary key words LDL size • light scattering • photon correlation spectroscopy • gradient gel electrophoresis

Low density lipoprotein (LDL) particles display a heterogeneity in size and density which has previously been well documented (1, 2). This heterogeneity has clinical relevance, in that small, dense LDL particles have been demonstrated to be associated with an increased risk of coronary artery disease (3). A number of tools are available to assess heterogeneity of LDL particles. The methods in most common use include gradient gel electrophoresis (GGE) (3) and density gradient ultracentrifugation (4).

Another potential method for measurement of LDL particle size relies on the random movement of particles suspended in a liquid or gas due to collision with the molecules of the suspending medium (Brownian motion). Scattering of incident light occurs in all directions, but because the particles are in random motion, the intensity of the scattered light fluctuates in relation to the diffusion speed of the particles. The diffusion coefficient, D, is related to the particle diameter, S, by the Stokes-Einstein Equation:

$$D\alpha\frac{kT}{3\pi\eta S}$$

where k is Boltzman's constant, T is the temperature, and η is the viscosity of the sample (5). If the diffusion coefficient D can be measured for a population of particles in suspension, the diameter S may be calculated.

Light scattering (LS) methodology was used by De Blois et al. (6) and Packard et al. (7) 20 years ago to determine the size of LDL and very low density lipoprotein (VLDL) particles. While no direct comparison was made, the authors stated that the results were in agreement with those obtained in earlier studies which made use of electron microscopy and negative staining techniques (8, 9). The method did not come into widespread use at the time, possibly because the technology required was highly specialized and was not readily accessible.

In the last 5 years a series of compact and relatively inexpensive instruments has become available which use LS in the sizing of submicron particles. It was our aim to determine LDL particle size using LS methodology and to compare the results to those obtained by GGE.

MATERIALS AND METHODS

Subjects

Twenty-seven subjects (13 men, 14 women; mean age ± SEM 48 ± 3 years) were enrolled for the study. In order to provide a broad range in the size of the LDL particles, 7 of the subjects were patients with Type 2 diabetes mellitus. All subjects were clinically stable and no one was taking lipid modifying medication.

Abbreviations: GGE, gradient gel electrophoresis; LS, light scattering; PCS, photon correlation spectroscopy; LDL, low density lipoprotein; IDL, intermediate density lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein; CV, coefficient of variation.

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Informed consent was obtained from all subjects and the study was approved by the St. Vincent's Hospital Human Research Ethics Committee.

Methods

Plasma from 40 ml of blood collected in EDTA after a 12-h fast was separated by centrifugation at 1300 *g* for 15 min at 4°C. Total plasma cholesterol, total plasma triglyceride, and high density lipoprotein (HDL) cholesterol were measured as previously described (10). LDL cholesterol was calculated using the equation of Friedewald, Levy, and Fredrickson (11) when the total triglyceride level was ≤4.5 mmol/l. For higher levels, VLDL was isolated by ultracentrifugation and cholesterol was measured separately.

For size determination, the preparation of LDL was based upon an adaptation of the method of Chung et al. (12) using vertical density gradient ultracentrifugation. The ultracentrifuge tube was punctured by a needle attached to a syringe and the LDL was aspirated.

Determination of the particle diameter of the isolated LDL by GGE was performed using commercially available 3–13% nondenaturing polyacrylamide native gels (Gradipore Ltd., Sydney, Australia). A single batch of gels was used for this study. LDL particle size was calculated using a regression plot derived from standards of known diameter (28 nm latex beads [Duke Scientific, Palo Alto CA], thyroglobulin and ferritin [Pharmacia high molecular weight standards, Pharmacia, Piscataway, NJ]). Isolated LDL of a known diameter, previously aliquoted and frozen at −80°C was thawed and run on the gel as a quality control (QC). Each aliquot was used once only. The diameter of this quality control was 26.5 nm and the inter-gel coefficient of variation (CV) was 0.7% (2, 13).

LS instruments use photon correlation spectroscopy (PCS) in order to derive particle size data from the differential velocities exhibited by particles of different sizes undergoing Brownian motion. A correlator is used to analyze the variable intensity of light scattered from a sample in the time domain. The correlator consists of a large number of digital channels, such that each adjacent correlator channel measures scattered light fluctuations offset by a delay time. For short delay times, the change in light intensity may be small, indicating the particles have not had time to move far, so the correlation level is high. For longer delay times, the positions of particles will have altered significantly, so the correlation will be low. Small particles move more quickly than larger particles, so the rate of fluctuation of the scattered light is also greater (14). The actual delay times, slope of the correlation function, and deviations in the correlation function can be analyzed to provide particle size information (15).

For LDL size determination by LS methodology, LDL isolated after vertical density ultracentrifugation was passed through a 0.1-μm filter (Millipore Products Division, Bedford, MA) while being injected into a disposable 10 × 10 × 48 mm cuvette (Sarstedt, Rommelsdorfer, Germany) to exclude dust particles. Viscosity of three representative samples was determined at 15°C using a Cannon-Fenske viscometer (Cannon Instrument Company, State College, PA; quoted precision ±0.2%) and found to be identical (16). The viscosity of pure water (17) was modified, using the correction factor determined by the viscosity measurements and applied to all samples. A Zetasizer 3000 (Malvern Instruments, Malvern, UK) was used for the study. The instrument uses a 10 mW helium-neon laser at 632.8 nm to excite the samples. Scattered light is collected at an angle of 90° by a photon counting photomultiplier tube that is then directed to a correlator. The software of the instrument derives particle size from the correlator function. Results are expressed as the Z-average mean which is the harmonic intensity averaged particle diameter (15). For the study, all LS measurements were performed at 15°C (unless otherwise stated), in triplicate, with 10-min runs, using a 400-μm aperture. Prior to commencing the study, commercially available latex beads (Duke Scientific, Palo Alto, CA) of known diameter (220 nm, 38 nm, and 28 nm) were subjected to size determination by the instrument under the operating conditions described above. The results obtained matched the diameters quoted by the manufacturer.

In order to determine the reproducibility of measurements obtained on a single sample of isolated LDL, hourly readings were made over a period of 12 h. To determine variability resulting from repeated sampling, one of the investigators (D. O'N) was bled at 8 separate time points over a period of 34 weeks. Samples of isolated LDL obtained on each occasion were subjected to LS measurements and GGE.

The effect of temperature was studied by repeated measurements made upon the same sample at 10, 12.5, 15, 17.5, and 20°C, with viscosity adjusted appropriately for the change in temperature. The effect of sample dilution was also examined. The diameter of a single sample of isolated LDL was determined by LS on an undiluted sample (2.8 mmol/l cholesterol) and after dilution 2- and 4-fold. The diluent was prepared in order to match the density and salt concentration of the solution in which the isolated LDL was suspended so that viscosity and particle–particle interaction were not altered.

The effect of freezing plasma was also examined. Aliquots of plasma (2 × 8 ml) were obtained from 7 subjects. One aliquot was frozen for 24 h at −70°C and the other was stored at 5°C. The frozen sample was then thawed and LDL was isolated from both aliquots. LDL size was then determined on frozen and refrigerated samples by LS and GGE.

Comparisons were made in the fasting and post-prandial states in order to examine the effect upon results obtained using the two methods. In 8 subjects samples were obtained in the fasting state and 2 h after a standard meal. The diameter of LDL in both fasting and post-prandial samples was determined by LS and GGE. In addition, samples of plasma and isolated LDL obtained in the fasting and post-prandial states were subjected to agarose gel electrophoresis (Paragon Lipoprotein Electrophoresis System, Beckman Instruments Inc., Fullerton, CA) in order to detect possible contamination by other lipoprotein species in the aspirated sample.

LDL samples isolated from unfrozen plasma, obtained in the fasting state from all 27 subjects, had particle size determined by LS methodology and GGE.

Statistical analysis was performed with Statview (Abacus Concepts, Berkeley, CA) statistical package on a Macintosh computer. A simple linear regression analysis was used to examine the relationship between LDL diameter as determined by the two methods and with triglyceride and HDL cholesterol levels. Triglyceride levels were log-transformed in order to normalize their distribution. The other parameters were included without modification. A paired Student's *t* test was used to compare LDL diameters obtained by the two methods with each other, results obtained fasting and post-prandially, and results from frozen and unfrozen samples.

RESULTS

The CV of results obtained by LS from multiple measurements performed upon a single LDL sample over a period of 12 h was 0.3%. On fasting samples obtained from 8 venesections of the same subject performed over a period of 34 weeks the CV for GGE was 0.9% and for LS was 0.8%.

Varying the temperature between 10 and 20°C with vis-

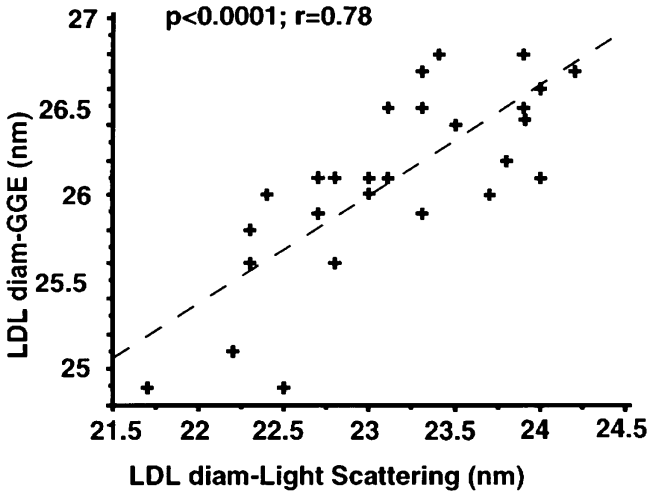

Fig. 1. Plot of LDL diameter, determined by light-scattering methodology versus that determined by gradient gel electrophoresis (GGE).

cosity adjusted appropriately and diluting the samples up to 4 times had no effect on the results obtained. LDL particle size measured by LS methodology on plasma samples that had been frozen was significantly larger compared to measurements performed on unfrozen samples (23.5 ± 0.2 vs. 22.9 ± 0.2 nm; $P < 0.005$). In contrast, no effect was noted between fresh and frozen samples when size determination was performed using GGE (25.9 ± 0.2 vs. 25.9 ± 0.2 nm; P = NS). While the difference in LDL particle size determined by LS methodology did not reach significance, a trend was observed towards larger diameters in the post-prandial state (23.9 ± 0.2 vs. 23.5 ± 0.2 nm; $P = 0.09$). Post-prandial samples where this difference in particle size was most marked, revealed contamination by intermediate density lipoprotein (IDL) and chylomicrons when subjected to agarose gel electrophoresis. No difference in size was noted in the post-prandial and fasting state when LDL diameter was determined by GGE (26.2 ± 0.1 vs. 26.3 ± 0.1 nm; P = NS).

A strong correlation was noted ($P < 0.0001$; $r = 0.78$) when comparing LDL particle size determined by LS methodology and GGE (**Fig. 1**). However, results obtained by LS were significantly smaller (23.1 ± 0.1 vs. 26.1 ± 0.1 nm; $P < 0.0001$) than those obtained by GGE. A comparison of the distribution of LDL particle size in two representative subjects is shown in **Fig. 2** using GGE and LS methods.

LDL particle size, determined by GGE and LS methodology, correlated inversely with the log of triglyceride level ($P < 0.0001$; $r = -0.74$ and $P < 0.0001$; $r = -0.77$, respectively) and positively with HDL cholesterol level ($P < 0.0001$; $r = 0.70$ and $P < 0.002$; $r = 0.57$, respectively). These relationships are illustrated in **Fig. 3**.

DISCUSSION

We have compared two methods for LDL size determination, that are radically different in approach. Each of

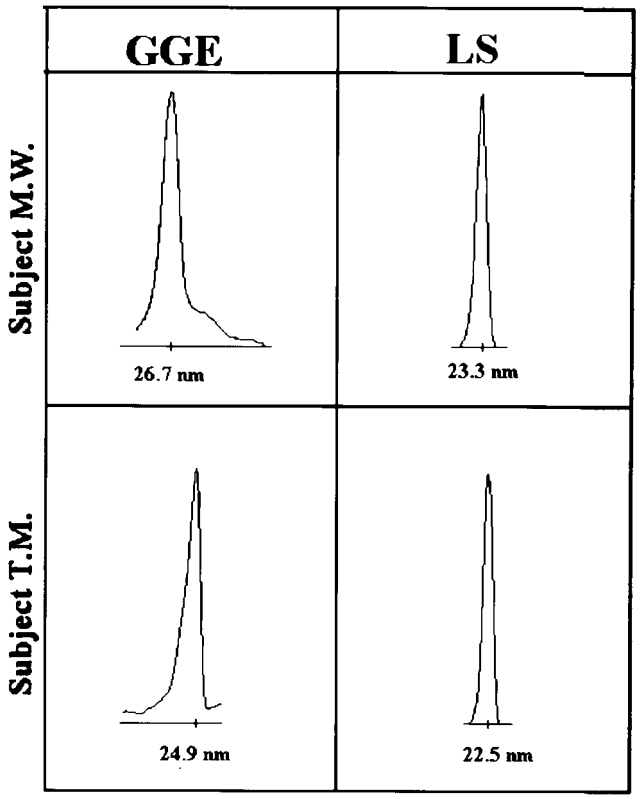

Fig. 2. A comparison of the densitometric scans taken from gradient gels with corresponding printouts after light-scattering (LS) measurements. The densitometric scan of the LDL band from subject M. W. indicates a peak of 26.7 nm with the distribution skewed toward the smaller LDL particles, while subject T. M. has an LDL peak of 24.9 nm with the distribution skewed toward larger LDL particles, corresponding to phenotypes A and B, respectively (3). In contrast, printouts from LS measurements show a single symmetric peak only, indistinguishable in contour from one another.

the methods observes an effect that is translated into particle size data. LS observes Brownian motion and calculates particle size from the diffusion coefficient (14). GGE observes the point of entrapment of particles undergoing motion in a non-Newtonian fluid, due to the influence of an applied electric field, and derives particle size from the point of entrapment of standards with a known diameter (18). LS is a measurement based on scientific first principles, so there is no need to calibrate the instrument, whereas GGE does require calibration. Our findings indicate that the results obtained by LS methodology are reproducible and correlate satisfactorily with nondenaturing GGE, currently the most widely accepted method for LDL sizing.

The absolute values of the results obtained by LS were 10% smaller than the corresponding results obtained by GGE. This finding was unexpected, given that the size of the latex beads, as determined by LS, corresponded with the sizes quoted by the manufacturer and that the 28-nm latex beads were incorporated as a standard on the gradient gels. However, the mean LDL diameter of 23.1 ± 0.1 nm as measured by LS in our study was very similar to the mean diameter of 22.9 ± 0.1 nm previously reported by

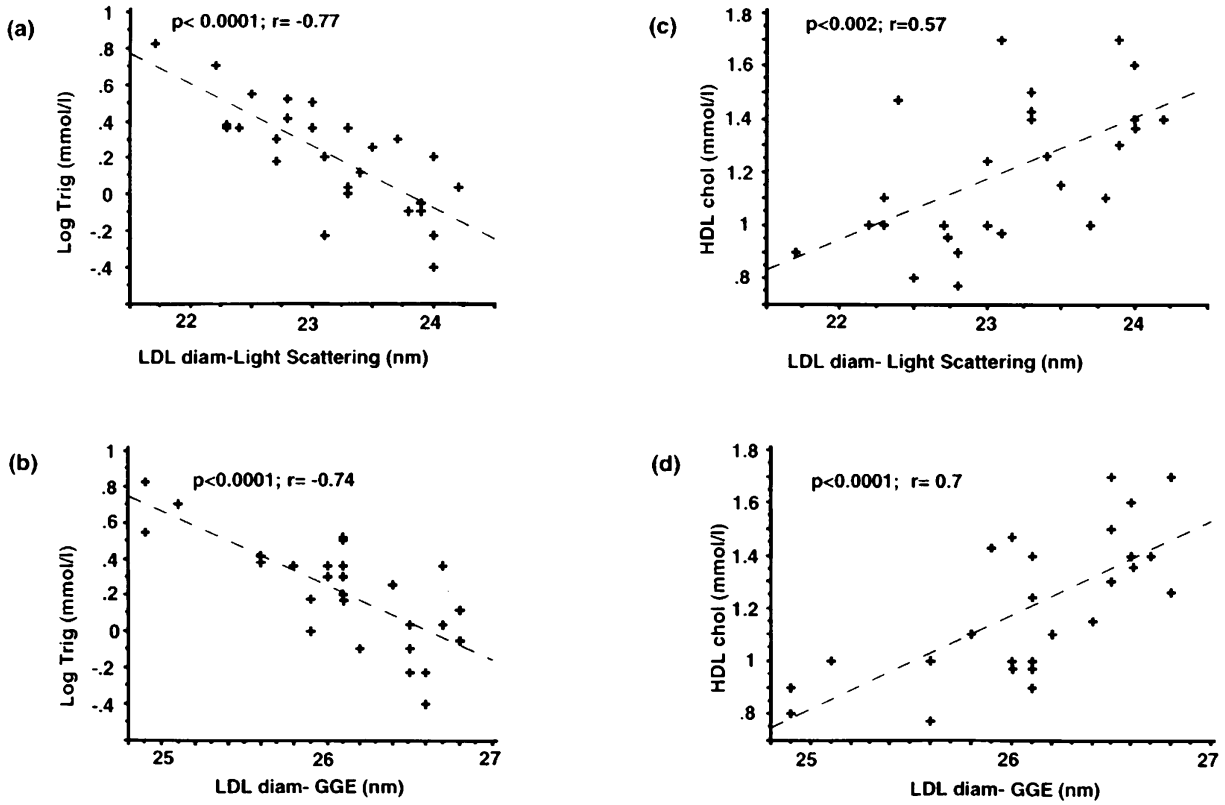

Fig. 3. (a) Plot of LDL diameter determined by light-scattering methodology versus log of total plasma triglycerides. (b) Plot of LDL diameter determined by gradient gel electrophoresis (GGE) versus log of total plasma triglycerides. (c) Plot of LDL diameter determined by light-scattering methodology versus plasma HDL cholesterol. (d) Plot of LDL diameter determined by GGE versus plasma HDL cholesterol.

De Blois et al. (6) using the same methodology. It is possible that polystyrene latex particles do not act the same way as LDL in the gel media, and so calibration can only be an indicative measure, with determinations of particle size resulting in comparative data. It is also possible that the charged double layer (19) around LDL particles interacts differently with the differing suspending media of each technique.

Packard et al. (7), measured the diameter of different LDL subclasses in individual subjects, but these subclasses were isolated prior to size determination. While LS techniques have sufficient sensitivity to allow determination of peak particle size, the technology does not currently have the resolution to enable the evaluation of the size distribution of LDL particles in individual subjects. As illustrated in Fig. 2, the asymmetric distribution of particle size observed on the scan of the gradient gel was not resolved by the Zetasizer. This difference is relevant because Austin et al. (3) have incorporated the distribution characteristic into their definition of LDL phenotype. However, it is not clear whether assessment of the morphology of the scan of the gel confers any added sensitivity in identifying those individuals at greatest risk of atherosclerotic vascular disease, and some investigators have used peak particle size alone (20).

Our data indicate that particle diameter determined by LS methodology was greater when post-prandial samples were compared with samples obtained after a 12-h fast. This difference was not found with LDL size determination using GGE. While compositional changes in LDL post-prandially may have made a contribution to the difference in size, we hypothesize that these observations were predominantly due to contamination of the aspirate with other lipoproteins such as chylomicrons. This interpretation is supported by our findings when aspirate samples obtained from post-prandial plasma were run on agarose, producing bands consistent with the presence of contaminating IDL and chylomicrons in addition to LDL. This observation may be peculiar to the use of vertical density ultracentrifugation in the isolation of LDL where gradient compression and mixing occurs during the reorientation period. Use of a swinging-bucket rotor may result in better separation of lipoprotein species by minimizing the contamination of LDL isolated from post-prandial samples, though this benefit would be off-set by a longer ultracentrifugation time. GGE is not affected by such contamination, as it allows separation of particles according to size by sieving with increasing polyacrylamide concentration. The Zetasizer, however, measures light scattered by all particles in suspension. As the intensity of the light scattered varies to the sixth power of the radius (21), a small number of larger particles may exert a disproportionate effect on particle size estimation. While this effect is most apparent with contaminating dust particles, it could also provide an explanation for our observations when comparing fasting and post-prandial samples. Simi-

larly, aggregates formed after freezing plasma may account for the difference in size determination observed when comparing fresh plasma with that which had been frozen.

In summary, GGE and LS methods used to estimate size of LDL particles provide results that correlate satisfactorily. GGE offers advantages in that it delineates the distribution of LDL, allowing classification into phenotype. In addition, although isolated LDL was used in this study, GGE can be performed using plasma samples, and results are not subject to perturbations by contamination with dust, lipoprotein aggregates, or larger lipoprotein particles. Conversely, sample preparation is crucial when using LS, as all particles in suspension are assessed and any contaminating particles or aggregates will affect results. LS methodology does offer advantages, in that it is not subject to inconsistencies in the gradient and quality of the gels, which can vary from batch to batch. There are potential savings in time and labor as the peak particle size of an LDL sample can be determined in approximately 30 min, and the method has the potential for automation. Also, while different lipoprotein classes require gels with different gradients, machines using LS principles are theoretically able to determine the size of a wide range of lipoprotein particles. Finally, because repeated measurements can be made upon the same sample, there is the possibility for kinetic studies.

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