methods

Analysis and quantitation of biotinylated apoB-containing lipoproteins with streptavidin-Cy3

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Abstract Non denaturing gradient gel electrophoresis (GGE) is commonly used to analyze the size distribution of lipoprotein particles. Its relatively low sensitivity and linear dynamic range limit use of GGE to quantify protein content of lipoproteins. We demonstrate a new high sensitivity method for analysis and quantitation of biotinylated apolipoprotein B (apoB)-containing lipoproteins using a fluorescent streptavidin-Cy3 conjugate and non covalent preelectrophoretic binding. Forty-four lipoprotein subfractions spanning the VLDL and LDL particle spectrum subfractions (11 each from four human subjects) were prepared by density gradient ultracentrifugation. An aliquot of each sample was biotinylated and GGE was performed. Gels also were stained for lipid with Oil Red O (32 samples) and for protein with Coomassie Brilliant Blue (30 samples). There was a significant relationship between the Cy3 fluorescent label area under the curve and the mass of apoB ($P < 0.02$–0.004) and total cholesterol ($P < 0.03$–0.004). Particle diameters of each absorbence/fluorescent peak were comparable between Oil-Red O and streptavidin-Cy3 treated biotinylated lipoproteins (±3.54 Å, $P = 0.3$). Biotinylation and prestaining of lipoprotein particle with streptavidin-Cy3 provides a new fluorescence-based method for detection and quantitative analysis of lipoprotein subspecies by gradient gel electrophoresis.—Berneis, K., M. La Belle, P. J. Blanche, and R. M. Krauss. **Analysis and quantitation of biotinylated apoB-containing lipoproteins with Cy3-streptavidin. *J. Lipid Res.* 2002.** 43: **1155–1159.**

Supplementary key words fluorophor • LDL • apolipoprotein B • gradient gel electrophoresis

Apolipoprotein B (apoB) containing lipoproteins comprise multiple subclasses differing in size, density, and chemical composition (1–8). The size distribution of these subclasses is commonly analyzed by non-denaturing GGE using stains for protein (Coomassie Brilliant Blue) or lipids (Sudan black, Oil Red O).

Coomassie Brilliant Blue (CBB) R-250 dye is capable of detecting microgram to sub-microgram amounts of protein (9). The linear dynamic range of the stain (~100–1,000 ng of protein) is limited (10) in relation to the range of protein mass in apoB containing lipoprotein fractions. Silver staining of proteins in polyacrylamide gels is at least 100× more sensitive than CBB staining (11). However, this method is limited by the need for numerous solution changes and carefully timed steps. Biotinylation of lipoproteins with detection using fluorescently labeled streptavidin offers the possibility of an improved method for detecting and quantitating lipoprotein protein mass with high sensitivity and linear dynamic range. Lipoprotein biotinylation has been described previously using D-biotin-*N*-hydroxysuccinimide ester (12) or modification with biotin hydrazide of periodate-oxidized lipoprotein sugar residues (13), which did not affect LDL binding to the LDL receptor. SDS-PAGE electrophoresis of biotinylated lipoprotein lipase and transfer to a nitrocellulose membrane has successfully been performed previously (14). Also, LDL receptors have been identified on nitrocellulose membranes after transfer from SDS/polyacrylamide gels by ligand blotting with biotin-modified LDL (13). However, these methods included a blotting step of samples and later incubation with a biotin detection system.

Here we demonstrate a new, convenient, and simple method for analysis and quantitation of biotinylated lipoproteins by preelectrophoretic binding of biotinylated lipoproteins with streptavidin-Cy3.

METHODS

Fasting plasma from four healthy subjects was separated from whole blood in the presence of a preservative cocktail at 4°C (plasma lipid levels: total triglycerides 269 ± 122, range: 94–624 mg/ml; total cholesterol 232 ± 16, range: 192–264 mg/ml; HDL-C 56 ± 8, range: 40–70 mg/ml). All plasma processing and lipoprotein fractionation was carried out in the presence of 2.7 mM EDTA and 10 μM Trolox. To obtain apoB-containing lipoprotein subfractions, 2 ml plasma was diluted to a final volume of 6 ml

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with density adjusted to 1.075 g/ml by the addition of NaCl (108 g/l) and centrifuged at 40,000 rpm for 20 h at 10°C. The top 1 ml was pipetted to obtain a lipoprotein fraction with d < 1.072 g/ml. This fraction was then mixed with 0.5 ml of deuterium oxide (D_2O) (d = 1.07 gm/ml) and the resultant 1.5 ml (d = 1.084 g/ml) was added to the bottom of a 12 ml ultracentrifuge tube. With tube angled at ~45°, a continuous, linear density gradient (d = 1.054–1.005 g/ml in 9.5 ml) was then added to the tube at 1 ml/min using two programmable high precision pumps (Pharmacia FPLC System, Piscataway, NJ) for delivery and mixing of two solutions: *A*) 0.15 mM NaCl in D_2O, pH 7.8, d = 1.11 g/ml; and *B*) 0.15 mM NaCl in H_2O, pH 7.8, d = 1.006 g/ml. The tube was then adjusted to a vertical position and 1 ml H_2O was layered on top. Tubes were centrifuged at 22,500 rpm, 16 h at 10°C in an SW41 swinging bucket rotor (Beckman Instruments, Palo Alto, CA). After stopping the rotor with controlled mechanical braking, non-equilibrium density fractions were pipetted from the top of the tube at the air-liquid interface volumetrically to obtain 12 1 ml fractions. Fraction 12 has only very low protein mass and is usually not visible with Coomassie stain. Therefore, this fraction could not be used for comparison between the methods.

Protein concentrations were determined by the Lowry method modified to include sodium dodecyl sulfate (15), and apoB concentrations were determined using sandwich style ELISA (16). Total cholesterol was measured by an enzymatic method with a Gilford Impact 400 E analyzer (Gilford Instruments, Oberlin, OH) (17). Non denaturing GGE of fraction 1–11 was performed at 10°C in 2–14% polyacrylamide gradient gels for 24 h at 125 V in Tris (0.09 M)-boric acid (0.08 M)-Na2 EDTA (0.003 M) buffer (pH 8.3) as described elsewhere (1, 18), except that the gradient gels were prepared in our laboratory essentially as described by Rainwater et al. (19).

Aliquots of the ultracentrifuged fractions were biotinylated using EZ-Link™ p-aminobenzoyl biocytin from Pierce (Rockford, IL) and Dr. Meir Wilchek (Department of Biological Chemistry, Weizmann Institute of Science, Rehovot, Israel) (20). The stable precursor for p-diazobenzoyl biocytin (DBB) is p-aminobenzoyl biocytin (20, 21). DBB is prepared from p-aminobenzoyl biocytin by treatment with HCL and sodium nitrite. This diazotizes the precursor to the reactive DBB compounds. DBB is reactive toward the phenolic group of tyrosine and the imidazole group of histidine (20, 21). Two milligrams of p-aminobenzoyl biocytin were mixed with 147 μl of 1 M HCL and allowed to dissolve for 40 min. One hundred forty-seven microliters of ice-cold Na NO2 (7.7 mg NaNO2/ml distilled water) were added and incubated for 5 min. One hundred twenty-six microliters of 1 M NaOH was added to stop the reaction, after which 100 μl of 0.2 M sodium borate pH 8.4, 2.7 μl of 0.5 M EDTA (pH 8.5), and 5 μl of 1 mM Trolox were added. Of this reagent, 10 μl (corresponding to 37.9 μg of p-aminobenzoyl biocytin), was added per 100 μl of lipoprotein sample. All samples were incubated for 2 h on ice. Thereafter they were dialyzed overnight in PBS pH 7.4.

The effect of increasing amounts of reagent ranging from 0.087 to 1.74 μg of p-aminobenzoyl biocytin per μl of a well defined lipoprotein sample (used in our laboratory as calibrator) was tested to assess the effect of different degrees of biotinylation on particle diameters and signal intensity.

Biotinylated lipoproteins were incubated with streptavidin-Cy3 (1 μl/40 μl sample) for 1 h and then directly added to gels (10–15 μl each lane). Samples with the highest mass reduction of streptavidin-Cy3 to 0.4 μl/40 μl sometimes resulted in better comparability with Coomassie, as the higher concentrations of streptavidin-Cy3 rarely resulted in double bands. Non-biotinylated aliquots of the samples were also analyzed by GGE and stained for lipids and protein respectively with Oil Red O (32 samples) in 60% ethanol at 55% and 0.1% Coomassie Brilliant Blue R-250, 50% ethanol, and 9% acetic acid (v/v) (30 samples). Gels stained with Oil Red O and Coomassie were scanned with a Molecular Dynamics Personal Densitometer. Fluorescence stained gels were scanned with a FX laser scanner (BioRad, Richmond, CA). Migration distance for each absorbence peak was determined and the molecular diameter corresponding to each peak was calculated from a calibration curve generated from the migration distance of size standards of known diameter, which included carboxylated latex beads (Duke Scientific, Palo Alto, CA), thyroglobulin, and apoferritin (HMW Std, Pharmacia, Piscataway, NJ) having molecular diameters of 380 Å, 170 Å and 122 Å, respectively (high molecular weight calibrators), and lipoprotein calibrators of previously determined particle size. Thyroglobulin and apoferritin were labeled with Cy3 using a FluoroLink™ Cy3 reactive dye 5-pack from Amersham Pharmacia Biotech.

Lipoprotein calibrators were also biotinylated and treated as described above. Scanned images were analyzed using NIH Image version 1.60/ppc.

Statistical analysis

Statistical analysis was performed using Stat View II (SAS Institute, Cary, NC 27513) software. Paired *t*-tests were performed to compare particle diameters as assessed by Coomassie, Oil Red O, and streptavidin-Cy3 stain. Spearman rank correlations were per-

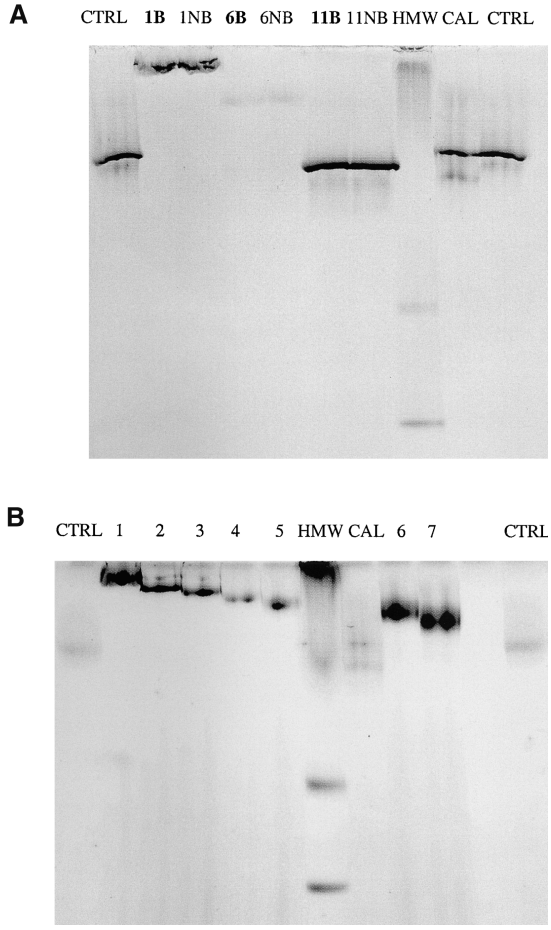

Fig 1. A: Comparison between biotinylated (B) and nonbiotinylated samples (NB) using Coomassie stain on a 2–14% gel. B: Example of a 2–14% gel using biotinylated VLDL, IDL, and LDL samples stained with streptavidin-Cy3. CTRL, Control LDL sample; HMW, High molecular weight standards; CAL, Calibrator LDL samples.

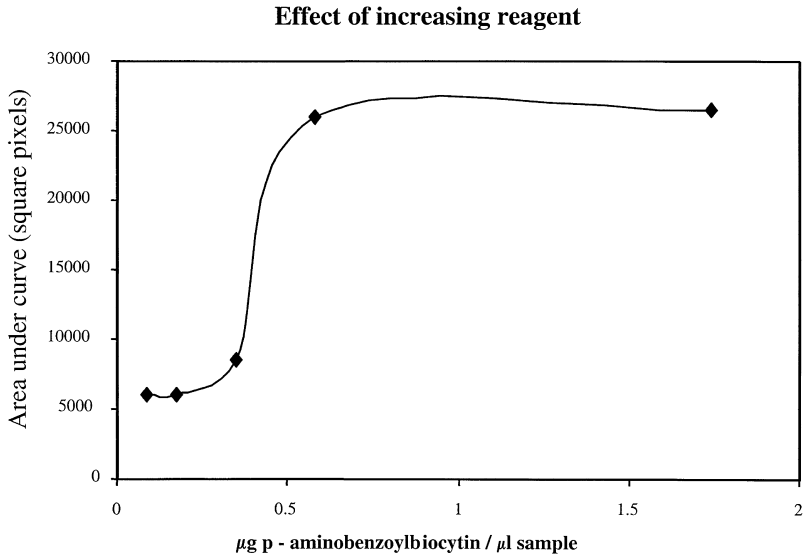

Fig 2. Effect of increasing amounts of p-aminobenzoyl per microliter of sample on measurements of the area under the curve of fluorescence of biotin-streptavidin-Cy3.

formed to compared areas under the curve and apoB or protein mass. Simple regression analyses were performed to compare the mass-area relationships. Values given are mean ± SEM.

RESULTS

Laser scanning of non-biotinylated lipoprotein did not result in a detectable signal when compared with biotinylated Cy3 labeled lipoproteins loaded onto the same gel (result not shown). Biotinylated and non biotinylated lipoprotein samples at the extremes and the middle of the density ranges (fraction 1, 6, and 11) resulted in identical results when stained with the same method (Coomassie), thereby excluding significant aggregation by the biotinylation process **(Fig. 1A)**. Increasing degrees of biotinylation led to an increase in the area under the curve reaching a plateau when 0.58 µg of p-aminobenzoyl biocytin were added to l µl of sample **(Fig. 2)**. A significant relationship was demonstrated between the area under the curve (square pixels) of major peaks and apoB mass ($P < 0.004$, 0.0058, 0.02, and 0.01, respectively, in all four subjects) and total cholesterol ($P < 0.004$, 0.02, 0.02, and 0.03, respectively, in all four subjects) and protein mass ($P <$ 0.008 in one subject). A linear relationship was also demonstrated when a series of increasing volumes ranging from 1, 3, 5, 7, 9, 11, 13, and 15 µl of the original sample was applied to the gel and compared with the area under the curve ($R^2 = 0.97$, $P < 0.0001$) **(Fig. 3)**. Particle diameters of lipoproteins over a broad range from large VLDL to small LDL were similar for staining with Oil Red O and Cy3 (−1.2 ± 0.8 Å, Cy3 vs. Oil Red O, respectively n = 32, $P = 0.3$) but were slightly but significantly different between Coomassie and Cy3 (−2.69 ± 0.6 Å, Cy3 vs. Coomassie, n = 30, $P = 0.005$). Biotinylated lipoprotein fractions resulted in distinct fluorescent peaks for all of the examined subfractions (Fig. 1B). Fluorescent peaks were easily detectable over a wide range of apoB and protein mass of 0.067–5.91 µg and 0.31–17.9 µg, respectively), whereas samples with the lowest apoB mass were either very faint or not detectable with Coomassie stain (Fig. 1A, fraction 6).

Particle diameters remained unchanged when increasing sample volumes were loaded on the gel (±1.2 Å, n = 8) (result not shown).

Figure 4 displays scan of two individual LDL samples (calibrator X and Y) and the resulting scan of the combined sample. Combination of two LDL samples, each of which had two distinct peaks, resulted in good resolution of the combined samples where all four peaks were visible at the expected size. Furthermore, the percentages of the seven LDL subclasses of the combined two samples were compared with the calculated percentage from the added individual samples. The mean difference between the percentage of the calculated and measured subclasses was 2.2 ± 0.6% (P = ns).

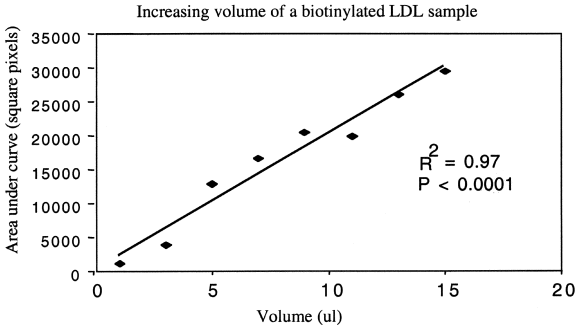

Fig 3. Correlation analysis between area under the curve of fluorescence of biotin-streptavidin-Cy3 and increasing sample volume of a biotinylated LDL sample (1, 3, 5, 7, 9, 11, 13, and 15 µl).

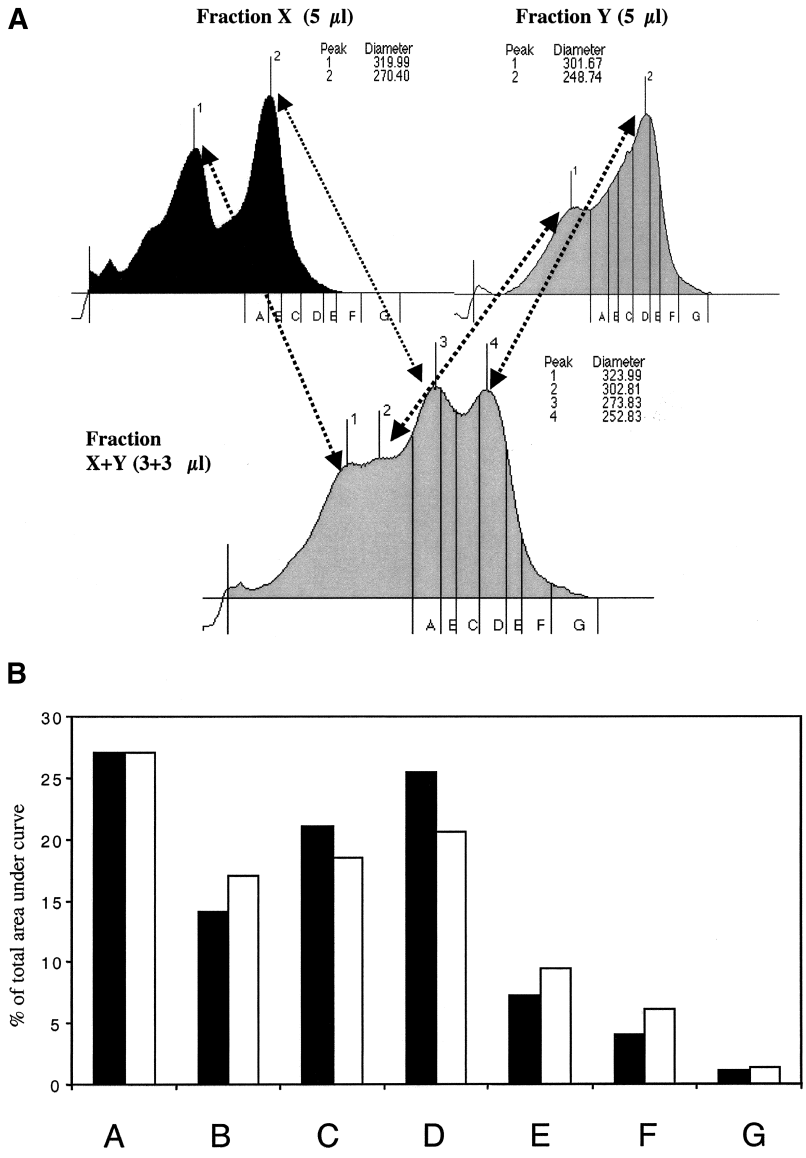

Fig 4. A: Cy3 scans of d < 1.063 fractions (5 μl) from two subjects (X and Y) and combined sample (X + Y; each 3 μl). Peak diameters are given in Å. B: Distribution of LDL subclasses in combined lipid fraction X and Y as calculated from individual measurements [closed bar (B), upper panel (A)] and compared with measurements of combined samples [open bar (B), lower panel (A)].

DISCUSSION

The present study has shown for the first time that electrophoretic analysis of lipoproteins can be performed using non-covalent binding of a fluorescent dye to biotinylated lipoproteins. The Cy3 label resulted in a wide linear dynamic range of signal for protein mass allowing quantitation of the electrophoresed samples. This is an advantage for lipoprotein samples with low protein content (e.g., <0.1 μg per lane), where bands may be below the limit for with Coomassie stain.

An intrinsic advantage of fluorescence technology is that the detection of the primary signal provides a linear response with respect to the amount of protein over a much wider range than is found for the nonfluorescent alternatives. Among the nonfluorescent detection technologies, only radiolabeling provides comparable performance. Preelectrophoretic covalent derivatization of proteins with fluorophores invariably leads to altered protein mobility (22–24). Biotin, a 144 Da vitamin found in tissue and blood, binds with high affinity to avidin. Since biotin is a relatively small molecule, it can be conjugated to many proteins without altering the biological activity of the protein. The avidin-biotin interaction is the strongest known noncovalent biological recognition. The bond formation between biotin and avidin is very rapid and once formed is unaffected by most extremes of pH, organic solvents, and other denaturing agents.

Streptavidin, a nonglycosylated 60,000 Da protein isolated from *streptomyces avidinii* with a near-neutral isoelectric point reportedly exhibits less nonspecific binding than avidin. Purified streptavidin covalently conjugated to Cy3 dye (25) (a fluorophor) is commercially available.

The common method for detecting biotinylated pro-

teins on gels (including lipoproteins) with the avidin/streptavidin system usually includes blotting of gels to a nitrocellulose membrane with blocking and washing procedures. Here we have used electrophoresis to both separate biotinylated lipoprotein fractions and wash unbound streptavidin-Cy3, thus avoiding the blotting and washing procedures. Due to its lower detection limits compared with the well-established Coomassie stain, we suggest this new method may be useful for analysis and quantitation of lipoproteins with low protein mass. In addition, this technique is very simple and rapid compared with other high sensitivity protein stains (e.g., silver stain) and the reagents applied are relatively inexpensive. In comparison with radiolabeling, this new system also has the aspect of waste reduction and advantages in handling.

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