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Microchip-based human serum atherogenic lipoprotein profile analysis

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ABSTRACT

Owing to the mounting evidence of serum lipid changes in atherosclerosis, there has been increasing interest in developing new methods for analyzing atherogenic lipoprotein profiles. The separation of lipoprotein and lipoprotein subclasses has been demonstrated using a microchip capillary electrophoresis (CE) system [Chromatographia 74 (2011) 799–805]. In contrast to this previous study, the current report demonstrates that sdLDL peak efficiencies can be improved dramatically by adding gold nanoparticles (AuNPs) to the sample. Moreover, NBD C_6-ceramide was identified as a satisfactory dye for specific labeling and quantitation of individual serum lipoproteins. The accuracy of the method was evaluated by comparison with ultracentrifuge separated small, dense, low-density lipoprotein (sdLDL). A high correlation was observed between these two methods for sdLDL cholesterol. Lipid levels were investigated between atherosclerotic patients and healthy controls. The variation of serum atherogenic lipoprotein profiles for atherosclerotic patients pre- and post-treatment was assessed by microchip CE. This method has potential for the rapid and sensitive detection of different lipoprotein classes as well as their subclasses and, therefore, is suitable for routine clinical applications. Microchip-based atherogenic lipoprotein profile assays will greatly improve the analysis of risk factors in atherosclerosis and will provide useful information for monitoring the effect of therapies on atherosclerotic disease.

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Atherosclerosis is a chronic inflammatory disease of the large arteries driven by lipids [1]. The term "atherogenic lipoprotein profile" has been introduced to describe a common form of dyslipidemia characterized by three lipid abnormalities: increased triglyceride-rich very low-density lipoprotein (VLDL)[1] levels, decreased high-density lipoprotein cholesterol (HDL-C) concentrations, and the presence of small, dense low-density lipoprotein (sdLDL) particles [2]. Determination of the circulating levels of plasma of the LDL subclass profile as well as plasma HDL and VLDL is important in the diagnosis of primary and secondary lipid transport disorders and in the risk assessment for atherosclerosis and coronary heart disease [3]. LDL particles are heterogeneous with respect to size and density of lipid composition. Two distinct phenotypes based on LDL particles have been recognized: pattern A, with a higher proportion of large buoyant LDL particles (lLDL), and pattern B, with a predominance of small, dense LDL particles (sdLDL). Intense clinical interest in the measurement of LDL subclasses stems from a strong and consistent association between a predominance of sdLDL and increased risk of coronary heart disease [4]. In addition, VLDL tends to promote atherosclerosis [5], and VLDL levels are more highly correlated with atherosclerosis lesion area in the aortic root than LDL [6]. Many epidemiological studies have confirmed the early observation of Barr and coworkers that the concentration of HDL cholesterol is inversely correlated with the risk of premature cardiovascular disease [7]. The main methods for separation and analysis of plasma lipoprotein levels are based on differences in physical properties and include ultracentrifugation, electrophoresis, and differential precipitation. However, these methods are labor-intensive, time-consuming, and costly. Nuclear magnetic resonance (NMR) is the most rapid and convenient method for determining LDL size and subfraction concentration [8]. However, it is limited by lack of

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[1] *Abbreviations used:* VLDL, very low-density lipoprotein; HDL-C, high-density lipoprotein cholesterol; sdLDL, small, dense low-density lipoprotein; lLDL, large buoyant LDL particles; CE, capillary electrophoresis; AuNP, gold nanoparticle; LPDS, lipoprotein-deficient serum; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; TC, total cholesterol; LDL-C, LDL cholesterol; sdLDL-C, sdLDL cholesterol; HDL-C, HDL cholesterol; CA–IMT, carotid artery intima media thickness; RSD, relative standard deviation.

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published data on detailed procedures, calibration, and validation, which are expected when novel methods are established. A method for the separation of the predominant LDL subclasses, HDL, and VLDL using a single-step iodixanol gradient has also been described [9]. However, its routine use is still limited to the analysis of serum lipoprotein.

Free zone electrophoresis in capillaries and microfluidic channels has emerged as an important separation technique because of its highly efficient separation, small injection volumes, and short analysis times [10]. Liu and coworkers demonstrated that capillary electrophoresis (CE) can be used to separate two LDL particles with significantly different charge/volume ratios given that the different ratios result in different electrophoretic mobilities [11]. Schmitz and coworkers developed a capillary isotachophoresis procedure for the separation of lipoproteins into 14 subclasses [12]. However, the addition of up to nine spacers to the running buffers was required, making this method complicated and less practical. Weiller and coworkers first used microchip CE to separate HDL and LDL [13,14]. However, HDL and LDL were not baseline resolved, and reproducibility was poor. Ping and coworkers used a polymethylmethacrylate (PMMA) chip for lipoprotein separation [15]. Baseline separation of standard lipoproteins, including HDL, LDL, and VLDL, were achieved with different selectivities. However, Ping and coworkers did not determine the subclasses of lipoprotein.

We have previously shown that the lipoprotein and lipoprotein subclasses can be determined by microchip CE [16,17]. However, it is still difficult to completely separate all of the lipoprotein classes and subclasses synchronously. Recently, we demonstrated that microchip CE could be used to analyze subclasses of LDL, VLDL, and HDL [18]. However, poor sdLDL peak efficiencies were not sufficient for quantitative analysis and, thus, need to be improved. In contrast to this previous study, the current report demonstrates that sdLDL peak efficiencies can be improved dramatically by simply adding gold nanoparticles (AuNPs) to the sample buffer at a concentration of 80 nmol/L. In microchip CE analysis, lipoproteins are stained with the lipophilic dye NBD C_6-ceramide and monitored by laser-induced fluorescence detection, allowing for lipoprotein analysis without prior separation from other serum proteins. However, it is unclear whether lipoprotein fractions separated by microchip CE are equivalent to those in lipoprotein fractions separated by ultracentrifugation. Although microchip CE separates serum lipoproteins into four fractions, there is still no direct evidence that these fractions represent lipoproteins rather than other serum proteins. Therefore, in the current study, we demonstrated the utility of NBD C_6-ceramide as a specific stain for serum lipoproteins on microchip CE. We also confirmed that NBD C_6-ceramide shows a saturation limit for lipoprotein labeling. In addition, we examined the linearity of the relation between levels of lipoprotein fractions and lipoprotein cholesterol to evaluate the utility of microchip-based lipoprotein analysis. This microchip-based method is able to detect the atherogenic lipoprotein phenotype that is most strongly correlated to atherosclerosis progression in patients.

Materials and methods

Microdevices

The design of the microchip was described in a previous study [17]. The chip's microchannels were fabricated in a quartz glass substrate ($63.5 \times 31.7 \times 2\ mm^3$) by a standard photolithography and chemical wet etching process. The microchannel dimensions were 21 ± 2 μm in depth and 100 ± 2 μm in width at half of the depth. Holes 2 mm in diameter were drilled at microchannel terminals and used as reservoirs. The sample injection channel length was 28 mm, the total separation channel length was 76.9 mm, and the effective separation length was 42.5 mm. Details of the integrated microchip capillary electrophoresis detection microsystems were described previously [19].

Reagents and buffer solutions

Lipoprotein-deficient serum (LPDS) was obtained from EMD Millipore (Billerica, MA, USA). According to product specifications, the original concentration of LPDS was 35 mg/ml. Standard solutions of HDL, LDL, and VLDL were obtained from Sigma Chemical (St. Louis, MO, USA). According to product specifications, the original concentrations of HDL, LDL, and VLDL were 5.9, 7.6, and 1.92 mg/ml, respectively. Fluorescent dye, NBD C_6-ceramide, was purchased from Molecular Probes (Eugene, OR, USA). Ethylene glycol and dimethyl sulfoxide (DMSO) were purchased from Sigma. Citrate-stabilized colloidal gold nanoparticles (5 nm diameter, 0.01% concentration as $HAuCl_4$, CAS no. 7440-57-5) were purchased from Sigma and served as a stock solution. The separation buffer used in this experiment was a mixture of 40 mmol/L Tricine and 40 mmol/L methylglucamine (both obtained from Sigma). The pH value of the separation buffer was adjusted to 9.8 by adding NaOH. Before electrophoresis separation, 0.1 mmol/L sodium dodecyl sulfate (SDS) and 80 nmol/L AuNPs were added to the sample buffer and 0.02 mmol/L SDS and 20 nmol/L AuNPs were added to the running buffer.

Serum collection and biochemical analysis

The study included 60 control subjects and 60 patients. This study was approved the ethics committees of Fudan University Huashan Hospital, and samples were collected only after the participants had given their informed consent. Fasting blood samples were collected from healthy volunteers and patients attending the university hospital. The 60 healthy subjects (32 men and 28 women) between 41 and 65 years old had no history of dyslipidemia, obesity, diabetes, or any known disease, were not taking drugs known to affect plasma lipids (e.g., hormone therapy, hypolipidemic drugs), and had only moderate alcohol and tobacco consumption. The 60 patients (35 men and 25 women), ranging from 45 to 66 years old, had carotid atherosclerosis and hypercholesterolemia. Atorvastatin was administered at 10 mg/day for 3 months, and the serum lipoprotein profiles and carotid atherosclerotic plaque were monitored pre- and post-treatment. Serum was prepared by low-speed centrifugation at 4 °C. Samples for analysis of microchip CE were stored at 4 °C, and analysis was completed 4 h after blood extraction. Aliquots of unused sample were stored at −80 °C. lLDL (d = 1.019–1.044 g/ml) and sdLDL (d = 1.044–1.063 g/ml) were separated from serum by sequential flotation in an ultracentrifuge (Himac CS120GX) with an S100AT6 rotor (both obtained from Hitachi Koki, Tokyo, Japan) according to the method of Havel and coworkers [20]. Briefly, the density of serum was raised by the addition of concentrated salt solution. The stock salt solution contained 153.0 g sodium chloride and 354.0 g potassium bromide per liter (density: 1.346). Solutions of lower density were prepared by dilution of the stock solution with 0.15 M sodium chloride solution (density: 1.005) according to the formula reported by Havel and coworkers [20]. Ultracentrifugation was carried out in the S100AT6 rotor. Serum (4 ml) was delivered into a capped plastic tube from a calibrated syringe. The appropriate volume of salt solution was added, and the tube was filled with a small amount of salt solution of the same density as the mixture. After centrifugation at 105,000g for 16 h at 12 °C, lipoproteins of less than solvent density were concentrated in a layer at the top of the tube. Beneath this was a clear colorless region occupying approximately one-fourth of the length of the tube; the remainder of the serum was stratified below

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