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In vitro oxidized and glycated human low-density lipoprotein particles characterized by capillary zone electrophoresis

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ABSTRACT

A simple capillary zone electrophoresis (CZE) method was used to determine native, *in vitro* Cu^{2+} and glucose modified low-density lipoprotein (LDL) particles for four healthy subjects. The LDL electropherograms are highly reproducible with good precisions of effective mobility and peak area. The native LDL capillary electrophoresis (CE) profile shows a major peak with lower mobility and two minor peaks with higher mobilities. For three-hour Cu^{2+} oxidation, one major peak with mobility close to that of the native major peak, and one minor peak with mobility extending to -47×10^{-5} cm^2 V^{-1} s^{-1} appear. For eighteen-hour Cu^{2+} oxidation, one major peak with mobility much higher than that of the native major peak appears. As the reaction time for LDL and Cu^{2+} increases from 0 to 24 h, effective mobility of the LDL major peak increases, suggesting that LDL particles become more negatively charged and oxidized as the time increases. The *in vitro* glycated LDL particles are characterized by a major peak and two minor peaks. Mobility of the major peak is close to that of native major peak, but the second minor peak is much more negatively charged with mobility extending to -53×10^{-5} cm^2 V^{-1} s^{-1}. Native, oxidized and glycated LDL particles show distinctive differences in their CZE profiles. Agarose electrophoresis shows that the charge to mass ratios of native, three-hour Cu^{2+} and glucose modified LDL particles are similar, but that of eighteen-hour Cu^{2+} oxidized LDL particles is higher.

1. Introduction

In vivo oxidized and glycated LDL particles play important roles in the pathogenesis of cardiovascular diseases. In the human arterial plaques, there are evidences of aggregated ox-LDL particles. Plasma levels of ox-LDL also positively correlate with arterial inflammation. The mechanisms of how LDL oxidizes in the human body are not totally understood yet. However, it is accepted that it involves a free radical process. At the beginning of oxidation, minimally modified LDL (MM-LDL) is formed and stimulates endothelial cells to produce monocyte chemotactic protein-1 (MCP-1) and macrophage colony-stimulating factor (M-CSF). Then, monocytes are attracted to the endothelium and pass into the subendothelial space. M-CSF induces their differentiation to form macrophages. Meanwhile, macrophages are capable of promoting MM-LDL to ox-LDL. Ox-LDL is recognized by the scavenger receptor of macrophages and causes appreciable accumulation of cholesterol within macrophages and finally the formation of foam cells [1–4].

Non-enzymatic glycation occurs on both the apoprotein B (ApoB) and phospholipid moieties of LDL when it is modified by glucose. Elevated amounts of advanced glycation end-products (AGEs) modified LDL (AGE-LDL) are found in patients with diabetes mellitus or renal insufficiency. This leads to the production of reactive oxygen species (ROS) and up-regulates the expression of interlukin-6 (IL-6), vascular cell adhesion molecule-1 (VCAM-1) and MCP-1 by endothelial cells. AGEs strongly interfere with the clearance of LDL by its normal receptors, and increase the uptake of LDL by scavenger receptors of macrophages. Meanwhile, glycated LDL particles are found to be more easily oxidized. Therefore, mechanisms of glycation and oxidation of LDL seem closely related [5–9].

Previously, researchers have utilized various analytical methods to analyze human LDL samples. Several research groups have used ion-exchange chromatography to determine LDL fractions [10–14]. More recently, various forms of microchips were used to analyze LDL fractions [15–17].

Recently, Liu et al. has developed a CZE method for profiling circulating human LDL particles. LDL particles of healthy subjects and atherosclerotic patients were able to be distinguished by the

Abbreviations: LDL, low-density lipoprotein; UC, ultracentrifugation; UF, ultrafiltration; CZE, capillary zone electrophoresis; PBS, phosphate buffered saline; PB, sodium phosphate.

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difference of LDL isoforms and peak effective mobilities [18]. Liu et al. has also used CZE to separate and determine *in vitro* oxidized phosphatidylcholine (PC) since PC is the most important phospholipids on LDL particles [19]. Using capillary isotachophoresis (ITP), Bittolo-Bon and Cazzolato observed four LDL subfractions for a group of dyslipidemic patients [20]. Schlenck et al. observed two LDL subfractions for healthy subjects by ITP [21]. Zorn et al. also used ITP to determine LDL particles which were *in vitro* oxidized by copper sulfate, macrophages and smooth muscle cells. The authors observed six subfractions for LDL, with the oxidized LDL particles migrating faster than the native LDL particles [22]. Meanwhile, combining ITP and enzymatic methods, Schmitz et al. also observed seven LDL subfractions for patients of hypothyroidism. Schmitz et al. obtained three LDL subfractions in patients with arterial plaques using ITP [23–25]. Recently, Saku et al. found that ITP was suitable for monitoring Cu^{2+} *in vitro* oxidized LDL with various degrees of oxidation. The authors also used ITP for characterizing lipoprotein profiles in type III hyperlipoproteinemia (HLP) and insulin resistance as well as for evaluating the treatment with fenofibrate for type IV hyperlipoproteinemia patients [26–31]. Stocks et al. studied Cu^{2+} *in vitro* oxidized LDL using capillary zone electrophoresis (CZE). The LDL effective mobility increased with oxidation time. The background electrolyte chosen was 40 mM methylglucamine-Tricine, pH 9.0 [32,33].

CE has many advantages over other analytical methods for analyzing biological molecules including high sensitivity, high separation efficiency, minute volumes of sample and buffer needed and measuring of effective mobility which represents charge to volume ratio for a molecule. Compared to CE method, the ion-exchange chromatography and microchip methods have lower sensitivity and separation efficiency. Most of them are not able to separate LDL particles into distinct subfractions. They are also more laborious or time-consuming.

The objective of this study was to determine *in vitro* oxidized and glycated LDL particles by a simple CZE method which was a modification of our previous study [18]. *In vitro* oxidized and glycated LDL particles were used to monitor the *in vivo* modification of LDL particles in patients. Our CE buffer system (5 mM sodium phosphate, pH 7.4) has low ionic strength to reduce the ionic atmosphere effects around LDL particles. LDL particles are also stabilized by the physiological pH used in our CE buffer. To the best of our knowledge, this is the first report for determining *in vitro* glycated LDL particles using CZE. Meanwhile, this simple CZE method is unique for being able to analyze both *in vitro* oxidized and glycated human LDL particles, with the potential to characterize LDL subfractions for atherosclerotic and diabetic patients.

2. Experimental

2.1. Chemicals

The chemicals used were potassium bromide crystal (KBr; J.T. Baker, Phillipsburg, NJ, USA), phosphate buffered saline (PBS; Sigma Chemical, St. Louis, MO, USA), sodium phosphate monobasic (NaH_2PO_4; Sigma Chemical), sodium phosphate dibasic (Na_2HPO_4; Sigma Chemical), ethylenediaminetetraacetic acid (EDTA; Sigma Chemical), D-(+)-glucose (Sigma Chemical), copper(II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$; Riedel-de Haën, Seelze, Germany), phosphoric acid 85% (H_3PO_4; Riedel-de Haën), sodium hydroxide (NaOH; Riedel-de Haën) and deionized water (Millipore Simplicity; Millipore, Billerica, MA, USA).

2.2. Healthy subjects

The four healthy human blood plasma samples were generously provided by Taichung Blood Donation Center. This study was approved by National Changhua University of Education and Taichung Blood Center. Informed consent was obtained from each participant.

2.3. Separation of human low-density lipoprotein fractions by ultracentrifugation

Fresh healthy human plasmas were frozen at −80 °C until used for isolation of lipoprotein fractions. Purification of lipoprotein fractions was carried out using a Beckman Coulter Optima™ XL-100K following a standard procedure [34]. Plasma densities between 1.019 and 1.063 g/ml were collected for LDL fractions. Briefly, KBr was used to adjust the density of plasma to 1.019 g/ml, and the plasma was then subjected to ultracentrifugation at 45,000 rpm and 5 °C for 18 h. The floating layer was collected as very low density lipoprotein (VLDL) fraction. Density of the remained plasma

Table 1
Native LDL.

	μ_{eff} ($\times10^{-5}$ cm^2 V^{-1} s^{-1})	CV (%)	Area (214) ($\times10^4$)	CV (%)	CPA ratio (A_{234}/A_{214}) ($\times10^{-2}$)	CPA ratio (A_{280}/A_{214}) ($\times10^{-2}$)
Peak N-I						
Donor A	− 15.68 ± 0.24	1.53	19.25 ± 1.68	8.72	33.19 ± 0.88	14.44 ± 0.08
Donor B	−15.35 ± 0.19	1.26	35.47 ± 2.09	5.89	34.76 ± 2.20	11.78 ± 0.18
Donor C	−16.75 ± 0.26	1.57	13.74 ± 1.32	9.58	37.12 ± 0.90	9.97 ± 0.12
Donor D	−16.82 ± 0.48	2.83	11.75 ± 0.56	4.76	36.77 ± 2.68	12.60 ± 0.27
Peak N-II						
Donor A	−27.55 ± 0.67	2.44	1.21 ± 0.14	11.30	53.72 ± 0.39	35.54 ± 0.27
Donor B	−30.91 ± 0.19	0.63	0.62 ± 0.06	9.23	82.26 ± 1.41	25.81 ± 0.38
Donor C	−28.71 ± 0.88	3.07	0.57 ± 0.08	14.86	70.18 ± 0.28	29.82 ± 0.30
Donor D	−28.14 ± 0.36	1.27	1.34 ± 0.02	13.19	81.34 ± 0.82	44.03 ± 0.37
Peak N-III						
Donor A	−33.78 ± 0.63	1.85	*Notmeasurable*		*Notmeasurable*	*Notmeasurable*
Donor B	−38.15 ± 0.26	0.67	*Notmeasurable*		*Notmeasurable*	*Notmeasurable*
Donor C	−34.04 ± 0.97	2.85	*Notmeasurable*		*Notmeasurable*	*Notmeasurable*
Donor D	−34.12 ± 0.38	1.13	*Notmeasurable*		*Notmeasurable*	*Notmeasurable*

μ_{eff}: Effective mobility.
Peak N-I: Low-mobility native LDL subspecies.
Peak N-II: High-mobility native LDL subspecies.
Peak N-III: Much higher mobility native LDL subspecies.
CPA ratio: Corrected peak area ratio.

was then adjusted to 1.063 g/ml using KBr. The adjusted plasma was subsequently subjected to ultracentrifugation at 45,000 rpm and 5 °C for 18 h. The floating layer was collected as LDL fraction. The collected LDL fractions were immediately used for reaction and separation, otherwise kept at −80 °C until used.

2.4. Preparation of native LDL samples for CE analysis

A 200 μl LDL fraction from the above UC procedure was exchanged buffer from KBr solution to 2.5 mM sodium phosphate solution for four times using a 100 kDa ultrafiltration filter (Amicon, Micron Centrifugal Filter Devices, Ultracel YM-100; MW cut-off: 100,000, Millipore, Bedford, MA, USA). The final volume of LDL solution was reconstituted to 200 μl.

To prepare for 2.5 mM PB buffer, a 1.09 g Na_2HPO_4 was mixed with 0.32 g NaH_2PO_4, and dissolved in 80 ml of d.i. water. A 0.1N NaOH and 0.1N H_3PO_4 solutions were used to adjust the pH of solution to 7.40. Subsequently, the solution was reconstituted to 100 ml with d.i. water. The concentration of sodium phosphate was 100 mM, and then 2.5 mM sodium phosphate was obtained by appropriate dilution with d.i. water.

2.5. In vitro oxidation of LDL by Cu^{2+}

A 200 μl LDL fraction from the above ultracentrifugation procedure was exchanged buffer from KBr solution to 1× PBS solution using a 100 kDa ultrafiltration filter. The final volume of LDL solution was adjusted to 200 μl, and then mixed with 200 μl, 5 μM $CuSO_4$ solution by gently inverting the microcentrifuge tube several times. The solution mixture was incubated in 37 °C water bath and shaken at 80 rpm for various time intervals. At the end of incubation, a 0.0009 g of EDTA was added into the LDL solution mixture to quench the oxidation reaction. Finally, a 30 kDa ultrafiltration filter (Amicon, Micron Centrifugal Filter Devices, Ultracel YM-30; MW cut-off: 30,000) was used to exchange buffer for LDL to 2.5 mM sodium phosphate solution for four times, and then CE analysis was performed.

2.6. In vitro glycation of LDL by glucose

A 200 μl LDL fraction in 1× PBS was prepared as the above section. Then, it was mixed with 200 μl, 120 mM glucose solution (in 1× PBS) and 0.0009 g EDTA by gently inverting the microcentrifuge tube several times. The LDL solution mixture which has a final glucose concentration of 60 mM was then incubated in 37 °C oven for 7 days. Finally, the buffer of LDL sample was exchanged to 2.5 mM sodium phosphate as described in the above section, and subsequently CE analysis was performed.

2.7. CE analysis of LDL particles

A Beckman P/ACE MDQ capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA) equipped with a diode-array detector and capillary cartridge coolant tubing was used for CE analysis. The CE instrument was connected to an IBM Pentium 4 computer. Electropherograms were analyzed using 32 Karat software (version 7.0, Beckman). The capillaries used for CE analysis were uncoated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 75 μm i.d. and 375 μm o.d. with a total length of 60.2 cm and an effective length of 50.0 cm. A new capillary was sequentially rinsed with 1N NaOH for 5 min, 0.1N NaOH for 10 min, and deionized water for 10 min. The capillary was also rinsed with 1N NaOH for 1 min, 0.1N NaOH for 5 min, deionized water for 5 min, and CE buffer for 5 min every day before sample analysis. CE separation was carried out at the normal polarity (i.e., from anode to cathode). A voltage of 16 kV was applied and then the capillary was maintained at 25 °C. The sodium phosphate buffer was prepared by mixing 1.09 g Na_2HPO_4 (MW 142) with 0.32 g NaH_2PO_4 (MW 120). Then, the mixture was reconstituted in 100 ml d.i. H_2O, and the desired pH was adjusted with H_3PO_4 and NaOH. The total concentration of sodium phosphate is now 100 mM, subsequently it was diluted to 5 mM with d.i. H_2O. For

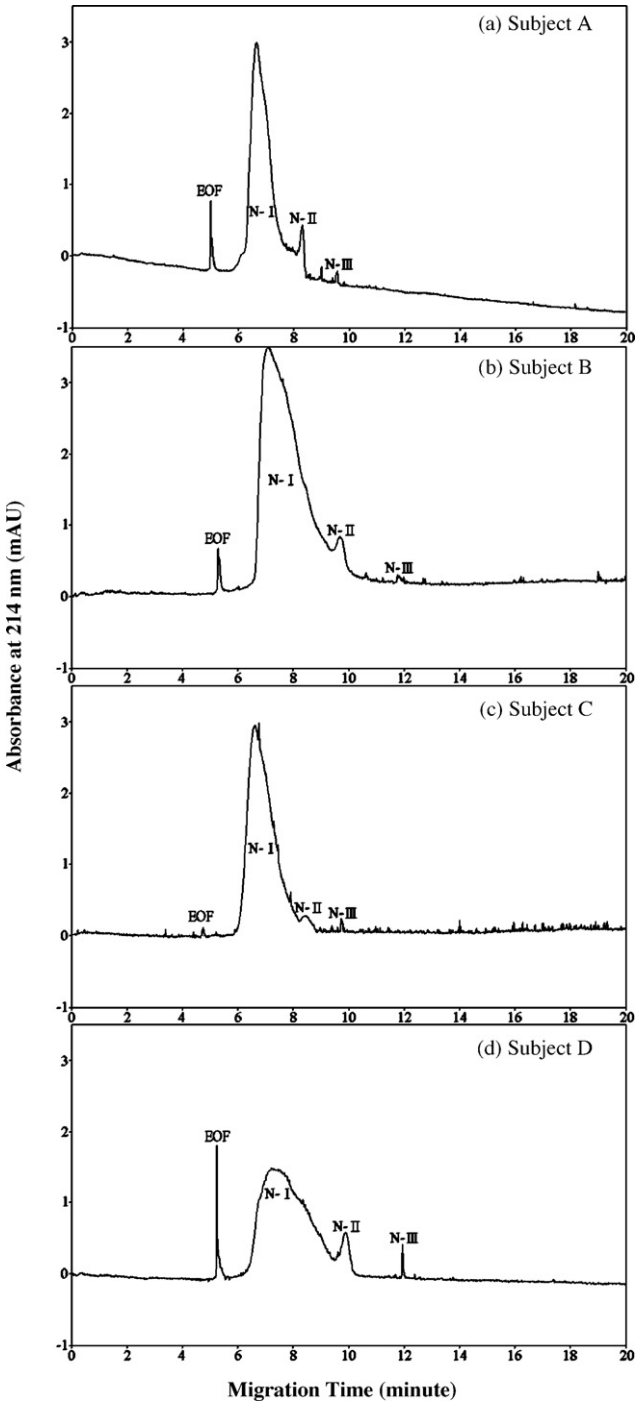

Fig. 1. Electropherograms of native LDL particles of four healthy subjects. A 200 μl LDL fraction from the UC procedure was exchanged buffer from KBr solution to 2.5 mM sodium phosphate solution four times using a UF filter. The CE voltage used was 16 kV. A pressure of 0.5 psi and 4-s injection were applied for LDL sample. CE buffer: 5 mM sodium phosphate, pH 7.40.

LDL sample introduction, a 4-s pressure injection of d.i. water was followed by a 4-s pressure injection of LDL, concluding with a 4-s pressure injection of CE buffer. A pressure of 0.5 psi was applied for sample injection. Between runs, the capillary was routinely conditioned with 0.1N NaOH for 2 min and deionized water for 2 min.

2.8. Agarose electrophoresis of Cu^{2+} and glucose in vitro modified LDL

A 0.5% agarose gel was used to analyze *in vitro* modified LDL particles. The running buffer was 1× Tris-glycine, pH 8.6. A 10 μl LDL sample was mixed with 10 μl sample buffer. The sample buffer was prepared by mixing 2 ml glycerol, 1 ml, 0.1% Bromophenol Blue and

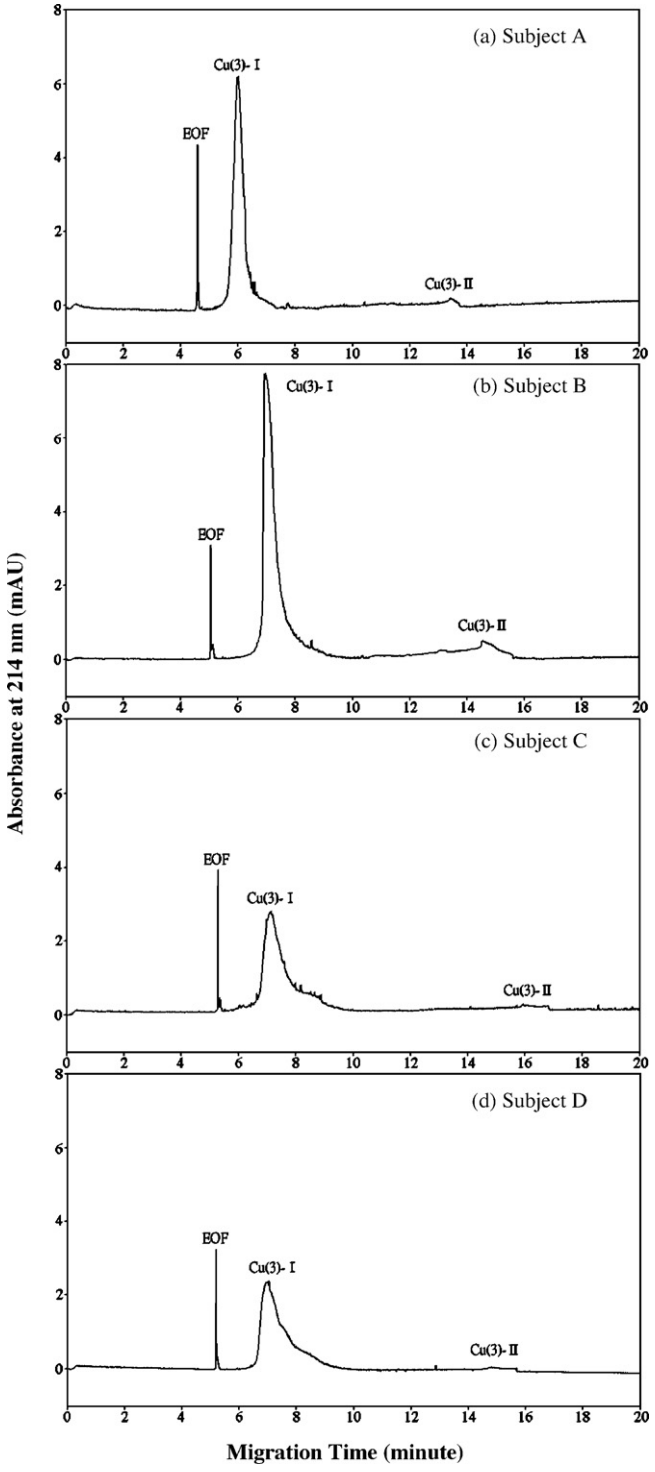

Fig. 2. Electropherograms of three-hour Cu^{2+} oxidized LDL particles of four healthy subjects. LDL was oxidized by 5 μM Cu^{2+} in 1× PBS buffer at 37 °C for 3 h. LDL was then exchanged buffer from 1× PBS to 2.5 mM sodium phosphate four times using a UF filter. The CE voltage used was 16 kV. A pressure of 0.5 psi and 4-s injection were applied for LDL sample. CE buffer: 5 mM sodium phosphate, pH 7.40.

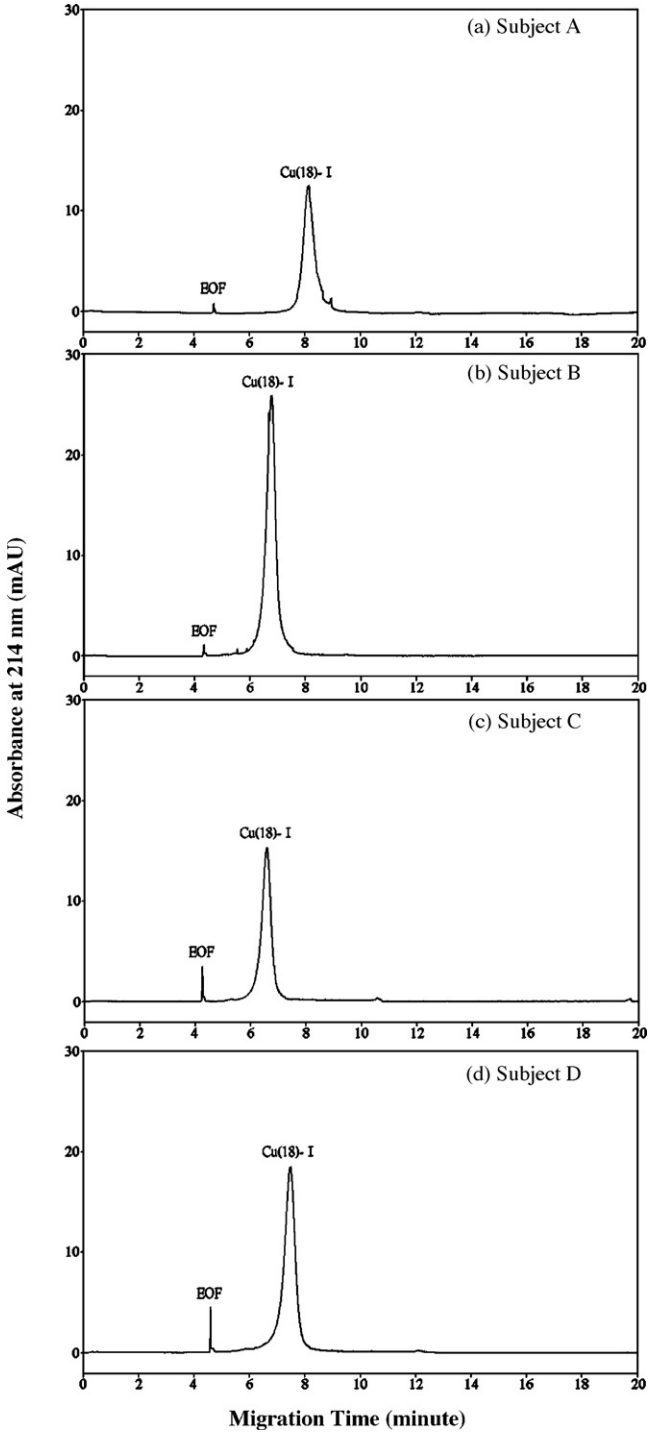

Fig. 3. Electropherograms of eighteen-hour Cu^{2+}oxidized LDL particles of four healthy subjects. LDL was oxidized by 5 μM Cu^{2+} in 1× PBS buffer at 37 °C for 18 h. LDL was then exchanged buffer from 1× PBS to 2.5 mM sodium phosphate four times using a UF filter. The CE voltage used was 16 kV. A pressure of 0.5 psi and 4-s injection were applied for LDL sample. CE buffer: 5 mM sodium phosphate, pH 7.40.

Table 2
Eighteen-hour Cu^{2+} oxidized LDL.

	μ_{eff} ($\times 10^{-5}$ cm^2 V^{-1} s^{-1})	CV (%)	Area (214) ($\times 10^4$)	CV (%)	CPA ratio (A_{234}/A_{214}) ($\times 10^{-2}$)	CPA ratio (A_{280}/A_{214}) ($\times 10^{-2}$)
Peak Cu(18)-I						
Donor A	−29.27 ± 0.36	1.24	64.14 ± 3.40	5.29	63.53 ± 0.77	24.31 ± 0.26
Donor B	−28.14 ± 0.27	0.96	72.50 ± 1.28	1.77	55.59 ± 1.20	16.97 ± 0.62
Donor C	−27.88 ± 0.26	0.93	45.62 ± 1.27	2.78	55.30 ± 0.78	16.33 ± 0.67
Donor D	−27.60 ± 0.27	0.99	62.09 ± 2.03	3.27	52.31 ± 0.63	12.79 ± 0.64

μ_{eff}: Effective mobility.
Peak Cu(18)-I: Low-mobility eighteen-hour Cu^{2+} oxidation LDL subspecies.
CPA ratio: Corrected peak area ratio.

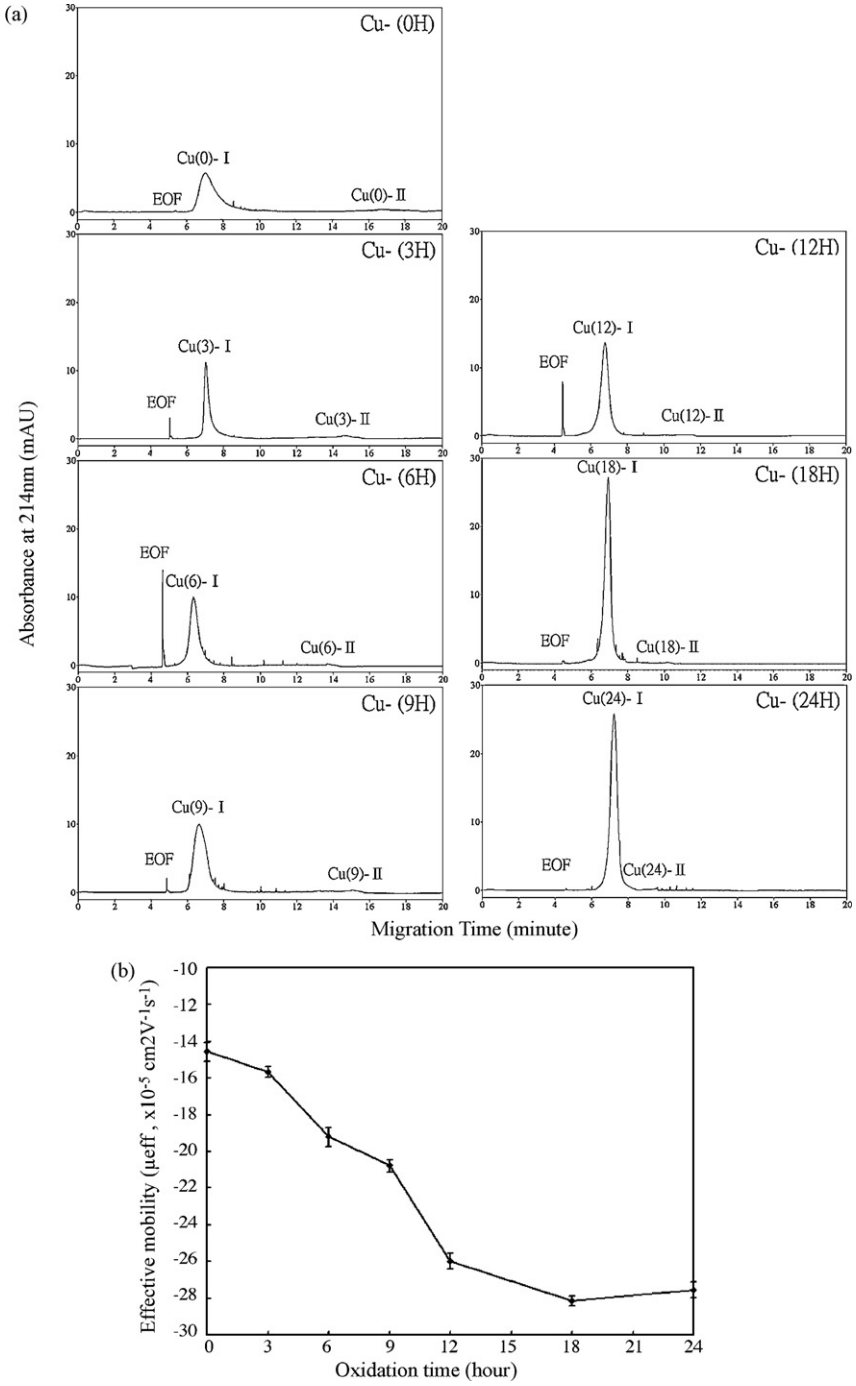

Fig. 4. (a) Electropherograms of 0, 3, 6, 9, 12, 18 and 24-h Cu^{2+} oxidized LDL particles of subject B, and (b) time dependence of the effective mobilities of oxidized LDL particles of subject B. LDL was oxidized by 5 μM Cu^{2+} in 1× PBS buffer at 37 °C for 0, 3, 6, 9, 12, 18 and 24 h. LDL was then exchanged buffer from 1× PBS to 2.5 mM sodium phosphate four times using a UF filter. The CE voltage used was 16 kV. A pressure of 0.5 psi and 4-s injection were applied for LDL sample. CE buffer: 5 mM sodium phosphate, pH 7.40.

then was reconstituted in 10 ml d.i. water. Each well was loaded with 10 μl LDL sample, and the gel was run at 65 V for 1 h and 10 min. At the end of electrophoresis, the gel was first soaked in d.i. water briefly and then stained for 0.5 h. The staining reagent was prepared by mixing 0.25% (w/v) Coomassie Brilliant Blue R250, 40% (v/v) methanol and 7% (v/v) acetic acid, and then was reconstituted in 1 l d.i. water. Finally, the gel was destained in 125/35 (v/v) methanol/acetic acid solution for 0.5 h.

3. Results and discussion

3.1. Search of the optimal conditions for LDL sample preparation and CE analysis

Since the healthy human plasmas used for this study were provided by Taichung Blood Center and anti-coagulants were added during their plasma preparations, purification of LDL is very important before *in vitro* modification and CE analysis. In addition, KBr was used to adjust for the densities of ultracentrifugation, and various chemical reagents were added for oxidation and glycation reactions. Therefore, extensive ultrafiltration procedures were used for LDL purification during various stages. At each stage, ultrafiltration procedures for repeating 1, 2, 3, 4 and 5 times were tested, and finally a total of 4 times were chosen as the most suitable one. It was observed that ultrafiltration for 4 times is able to clean the impurities of LDL samples and prevent long time exposure of LDL particles in air. In addition, it was also observed that without ultrafiltration to clean the anticoagulants in LDL samples, the oxidation and glycation reactions were not successful. The optimal CE buffer chosen was 5 mM PB, pH 7.4, which was similar to that chosen in our previous study [18]. However, the mobilities and CE profiles observed in this study for native LDL particles are not exactly the same as that observed in the previous study. It is possible due to different LDL preparation and purification conditions. The ultracentrifugation and ultrafiltration procedures for purifying LDL fractions have been changed in this study.

In the previous study, the native LDL profile for each healthy subject shows a major peak (LDL) and a minor peak (LDL^-), but the minor peak has a small shoulder. Mobilities of the major peak range from -13.52 to -14.10×10^{-5} cm^2 V^{-1} s^{-1}, and mobilities of the minor peak range from -19.79 to -20.37×10^{-5} cm^2 V^{-1} s^{-1}. In this study, the native LDL profile for each healthy subject shows a major peak (N-I) and two minor peaks (N-II and N-III). Mobilities of the major peak range from -15.35 to -16.82×10^{-5} cm^2 V^{-1} s^{-1}, mobilities of the first minor peak (N-II) range from -27.55 to -30.91×10^{-5} cm^2 V^{-1} s^{-1}, and mobilities of the second minor peak (N-III) range from -33.78 to -38.15×10^{-5} cm^2 V^{-1} s^{-1}.

3.2. Native LDL CE profiles for four healthy subjects

Highly reproducible LDL electropherograms are crucial in order to compare the difference between native and *in vitro* oxidized LDL particles. To test the overall reproducibility of LDL electropherograms, native LDL fractions were isolated from four healthy subjects and analyzed by the optimal CE method. The whole procedure including ultracentrifugation isolation, ultrafiltration purification and CE analysis for LDL fractions was performed for a total of six times for each plasma sample.

Fig. 1 shows the results of native LDL profiles for four healthy subjects. Each donor shows a major LDL peak (N-I) with lower mobility and two minor LDL peaks (N-II and N-III) with higher mobilities. It indicates that N-II and N-III particles are more negatively charged than N-I particles. The features of LDL profiles are similar, but the peak area ratio of N-I:N-II:NIII varies for each donor. Table 1 shows the average effective mobility μ_{eff}, peak area (A_{214}), peak area ratios (A_{234}/A_{214} and A_{280}/A_{214}) and CV (%) for LDL particles. For each donor, the whole procedure including ultracentrifugation, ultrafiltration and CE analysis was repeated for at least six times to obtain the measurements. Simultaneous analysis of absorbance at 214, 234 and 280 nm were carried out to measure lipids, conjugated dienes and protein contents in LDL

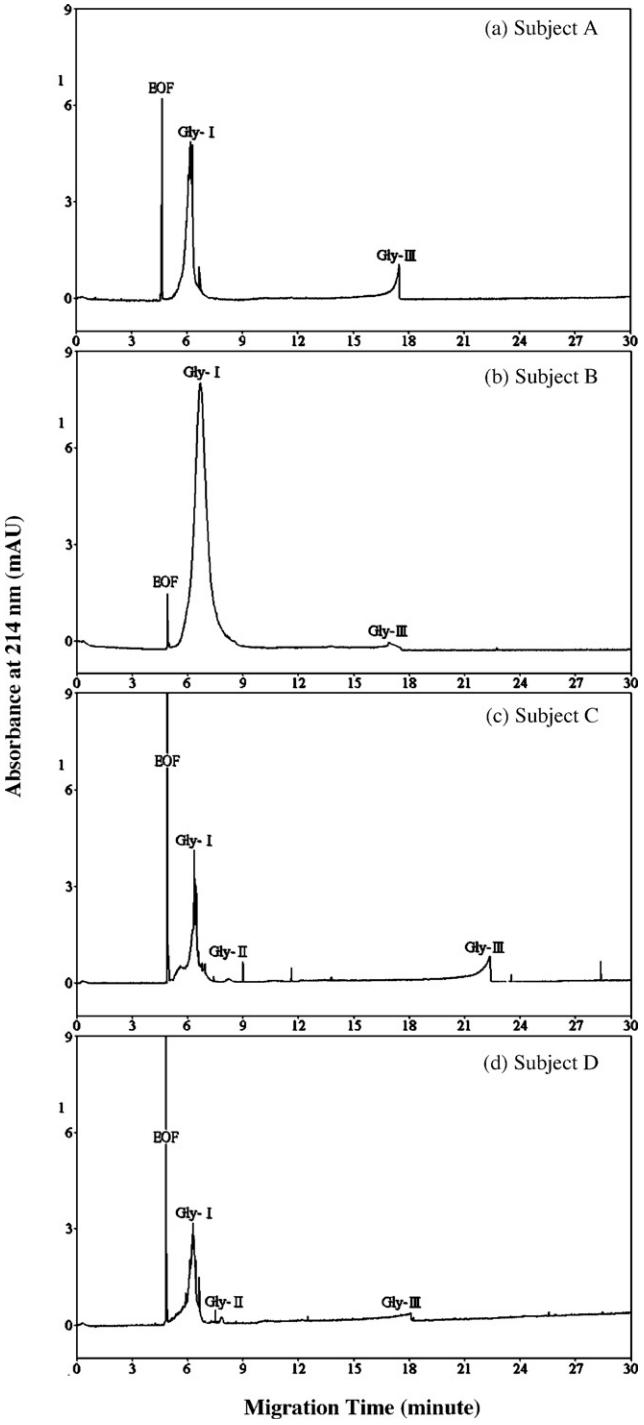

Fig. 5. Electropherograms of glycated LDL particles of four healthy subjects. LDL was modified by 60 mM glucose in 1× PBS buffer at 37 °C for 7 days. LDL was then exchanged buffer from 1× PBS to 2.5 mM sodium phosphate four times using a UF filter. The CE voltage used was 16 kV. A pressure of 0.5 psi and 4-s injection were applied for LDL sample. CE buffer: 5 mM sodium phosphate, pH 7.40.

particles, respectively. The average area ratio (A_{234}/A_{214}) of peak N-II is higher than peak N-I, suggesting the conjugated diene concentration is higher in N-II particles than that in N-I particles. Similarly, the average area ratio (A_{280}/A_{214}) of peak N-II is higher than that of peak N-I, suggesting the protein/lipid ratio is higher in N-II particles than that in N-I particles. Table 1 suggests that the native LDL electropherograms are highly reproducible with good precision of effective mobility and peak area.

3.3. Cu^{2+} in vitro oxidized LDL CE profiles

In order to further understand *in vivo* oxidized LDL particles associated with cardiovascular diseases, LDL particles of the four healthy donors were first *in vitro* oxidized by 5 μM Cu^{2+} to monitor the *in vivo* LDL oxidation, and subsequently CE analysis was carried out. The LDL electropherograms of Cu^{2+} oxidation for 3 and 18 h are shown in Figs. 2 and 3, respectively. Table 2 shows the average effective mobility μ_{eff}, peak area (A_{214}), peak area ratios (A_{234}/A_{214} and A_{280}/A_{214}) and CV (%) for eighteen-hour oxidized LDL particles. For each donor, the whole procedure including ultracentrifugation, ultrafiltration, oxidation reaction and CE analysis was repeated for at least six times to obtain the measurements. For three-hour oxidation, one major peak (Cu(3)-I) with lower mobility and one minor peak (Cu(3)-II) with higher mobility appear. Mobility of the major peak is close to that of native LDL major peak (N-I), but the minor peak appears as a broad and complex region with mobilities extending to -47×10^{-5} cm^2 V^{-1} s^{-1}. Since mobility of the minor peak is higher than that of the two native LDL minor peaks (N-II and N-III), it suggests that the minor peak of three-hour oxidized LDL is more negatively charged than the two minor peaks of native LDL.

For eighteen-hour oxidation, one major peak (Cu(18)-I) appears with the diminish of the higher mobility peaks. Mobility of the major peak (Cu(18)-I) is much higher than that of native LDL major peak (N-I), indicating it is much more negatively charged. Actually, mobility of the major peak (Cu(18)-I) is close to that of the minor peak (N-II) of native LDL, suggesting that N-I particles of native LDL have been oxidized and their structures or charges are close to that of N-II particles. Diminish of the minor peaks in native LDL after eighteen-hour oxidation is possibly due to the break down of the particles.

The average peak area ratio (A_{234}/A_{214}) of peak Cu(18)-I is higher than that of native LDL major peaks, suggesting that it has a higher level of conjugated dienes due to longer hours of oxidation.

The LDL electropherograms of Cu^{2+} oxidation for 0, 3, 6, 9, 12, 18 and 24 h are shown in Fig. 4a. It appears that shape of the LDL major peak becomes sharper as time increases. It is possible that as oxidation time increases, the charge to mass ratios of oxidized LDL particles become more homogeneously distributed. Meanwhile, it appears that minor peaks gradually diminish as oxidation time increases, possibly due to breaking down of the minor LDL particles. Fig. 4b shows time course of the change of LDL major peak for donor B. Effective mobility of the LDL major peak increases with the oxidation time. Peak area ratio (A_{234}/A_{214}) (data not shown) of the major peak also increases as time increases, suggesting that LDL particles become more negatively charged and oxidized as time increases.

3.4. Glucose in vitro modified LDL CE profiles

To understand *in vivo* glycation reaction of LDL particles for diabetic patients who often show high levels of blood glucose, LDL particles of the four healthy donors were *in vitro* modified by 60 mM glucose and then CE analysis was performed. Since EDTA was included in the reaction to eliminate the possible metal ions present, the modification of LDL particles was glycation rather than oxidation. The CE profiles of glucose modified LDL are shown in Fig. 5. Table 3 shows the average effective mobility μ_{eff}, peak area (A_{214}), peak area ratios (A_{234}/A_{214} and A_{280}/A_{214}) and CV (%) for glucose modified LDL particles. For each donor, the whole procedure including ultracentrifugation, ultrafiltration, glycation reaction and CE analysis was repeated at least six times to obtain the measurements. The average effective mobilities of the major peak (Gly-I) are generally close to that of the native major peak (N-I), but mobilities of donors A and B are a little higher than that of their native

Table 3
Glycated LDL.

	μ_{eff} ($\times 10^{-5}$ cm^2 V^{-1} s^{-1})	CV (%)	Area (214) ($\times 10^4$)	CV (%)	CPA ratio (A_{234}/A_{214}) ($\times 10^{-2}$)	CPA ratio (A_{280}/A_{214}) ($\times 10^{-2}$)
Peak Gly-I						
Donor A	−18.77 ± 0.65	3.47	13.99 ± 0.60	4.30	45.39 ± 0.97	15.73 ± 0.51
Donor B	−18.30 ± 0.51	0.51	42.53 ± 2.68	6.30	39.27 ± 0.55	15.68 ± 0.35
Donor C	−16.50 ± 0.44	1.57	8.69 ± 0.77	8.90	47.53 ± 0.41	19.91 ± 0.32
Donor D	−16.27 ± 0.46	2.87	9.54 ± 1.14	11.97	46.33 ± 0.49	18.76 ± 0.23
Peak Gly-II						
Donor A	Not measurable		Not measurable		Not measurable	Not measurable
Donor B	Not measurable		Not measurable		Not measurable	Not measurable
Donor C	−28.56 ± 0.74	2.60	0.18 ± 0.02	10.55	61.11 ± 1.13	66.67 ± 1.18
Donor D	−27.00 ± 0.22	0.93	0.26 ± 0.03	11.76	50.00 ± 0.35	26.92 ± 0.28
Peak Gly-III						
Donor A	−35.42 ± 0.11	0.32	5.43 ± 0.74	13.60	47.88 ± 0.46	Not measurable
	∼ −52.99 ± 0.38	0.72				
Donor B	−36.98 ± 0.47	0.47	2.37 ± 0.33	13.87	32.48 ± 0.34	Not measurable
	∼ −49.16 ± 1.20	0.12				
Donor C	−35.93 ± 0.44	1.23	4.95 ± 0.86	17.43	76.57 ± 0.64	Not measurable
	∼ −52.47 ± 0.71	1.35				
Donor D	−35.06 ± 0.29	0.85	4.67 ± 0.50	10.75	29.98 ± 0.38	Not measurable
	∼ −50.12 ± 1.11	2.22				

μ_{eff}: Effective mobility.
Peak Gly-I: Low-mobility glucose modified LDL subspecies.
Peak Gly-II: High-mobility glucose modified LDL subspecies.
Peak Gly-III: Much higher mobility glucose modified LDL subspecies.
CPA ratio: Corrected peak area ratio.

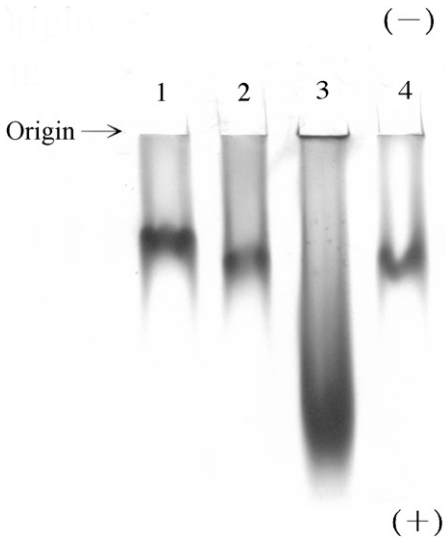

Fig. 6. Agarose electrophoresis of Cu^{2+} and glucose modified LDL. Lanes 1, 2, 3 and 4 represent native, three-hour Cu^{2+} oxidized, eighteen-hour Cu^{2+} oxidized and glucose modified LDL samples, respectively.

major peaks. The peak area ratio (A_{234}/A_{214}) of Gly-I is higher than that of N-I, suggesting that glucose modified LDL particles show higher content of conjugated dienes. Similarly, the peak area ratio (A_{280}/A_{214}) of Gly-I is higher than that of N-I, suggesting that glucose modified LDL particles have higher protein to lipid ratio. Mobility of the first minor peak (Gly-II) of modified LDL is close to that of the first minor peak (N-II) of native LDL. However, the second minor peak (Gly-III) appears as a broad region and is much more negatively charged than peak N-III with mobilities extending to $-53 \times 10^{-5}\,cm^2\,V^{-1}\,s^{-1}$. The peak areas ($A_{214}$) of Gly-I and Gly-II are generally lower than that of N-I and N-II, although their mobilities are similar. It suggests that part of N-I and N-II particles of native LDL have been modified and become Gly-III particles. N-III particles of native LDL disappear possibly due to break down or modification by glucose.

3.5. Agarose electrophoresis of Cu^{2+} and glucose in vitro modified LDL particles

Agarose gel electrophoresis was used to analyze Cu^{2+} and glucose *in vitro* modified LDL particles for subject D, and the results are shown in Fig. 6. Lanes 1, 2, 3 and 4 represent native, three-hour Cu^{2+} oxidized, eighteen-hour Cu^{2+} oxidized and glycated LDL particles, respectively. Native, three-hour Cu^{2+} oxidized and glycated LDL particles migrate at similar speeds, but eighteen-hour Cu^{2+} oxidized LDL particles migrate much faster, suggesting that they are the most negatively charged. The bands seen on the agarose gel should be corresponding to the major peaks of CE profiles. For the major peaks, the results of gel electrophoresis are comparable to that of CE. However, the minor peaks of the CE profiles are not seen on the agarose gel, suggesting that CE is a much more sensitive method than agarose gel for LDL analysis.

4. Conclusions

A simple CZE method was used to determine native, *in vitro* Cu^{2+} oxidized and glucose modified LDL particles. The native LDL CE profile shows a major LDL peak (N-I) with lower mobility and two minor LDL peaks (N-II and N-III) with higher mobilities for each donor. The native LDL electropherograms are highly reproducible with good precision of effective mobility and peak area.

For Cu^{2+} three-hour oxidation, one major peak (Cu(3)-I) with lower mobility and one minor peak (Cu(3)-II) with higher mobility appear. However, for eighteen-hour oxidation, one major peak (Cu(18)-I) appears with the diminish of the higher mobility peaks. Mobility of the major peak (Cu(18)-I) is close to that of native LDL minor peak (N-II). As the oxidation time increases from 0 to 24 h, effective mobility μ_{eff} and peak area ratio (A_{234}/A_{214}) of the major peak increase, suggesting that LDL particles become more negatively charged and oxidized as time increases.

The *in vitro* glycated LDL particles are characterized by a major peak and two minor peaks. The effective mobility of the major peak (Gly-I) is generally close to that of the native major peak (N-I). The second minor peak (Gly-III) is much more negatively charged with mobility extending to $-53 \times 10^{-5}\,cm^2\,V^{-1}\,s^{-1}$.

Agarose gel electrophoresis shows that the charge to mass ratios of native, three-hour Cu^{2+} oxidized and glucose modified LDL particles are similar, but that of eighteen-hour Cu^{2+} oxidized LDL particles is much higher. The result of agarose electrophoresis is comparable to that of the LDL major peaks in CZE profiles, but the minor peaks of CZE are not seen on agarose gel. It appears that CZE is more sensitive than agarose electrophoresis for the analysis of LDL particles.

This simple CZE method is unique for being able to monitor and compare native, *in vitro* Cu^{2+} oxidized and glucose modified LDL particles. The native, Cu^{2+} and glucose modified LDL particles show significant differences in their CZE profiles. The next study could be using this method to compare *in vivo* circulating LDL particles for various patient groups such as atherosclerotic and diabetic patients. It is feasible to apply this CZE method for the diagnosis, prevention and treatment of patients.

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