

DIFFERENTIAL DENSITY LIPOPROTEIN PROFILING FOR THE
CHARACTERIZATION OF LIPOPROTEIN(a)

A Dissertation

by

IRMA LETICIA ESPINOSA GARCIA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2006

Major Subject: Chemistry

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Major Subject: Chemistry

ABSTRACT

Differential Density Lipoprotein Profiling for the Characterization of Lipoprotein(a).

(August 2006)

Irma Leticia Espinosa Garcia, B.A., Monterrey Institute of Technology, Mexico

Chair of Advisory Committee: Dr. Ronald D. Macfarlane

Lipoprotein(a) (Lp(a)) has been described as an emerging risk factor for cardiovascular disease. The complexity of the Lp(a) molecule sets a challenge for the determination of the risk it represents for the cardiovascular system. The objective of the present study was to develop a rapid method for the separation, purification, density measurement, and characterization of Lp(a) from serum using a procedure that is isoform independent.

The objective was met by linking ultracentrifugation with affinity separations for the specific separation of Lp(a) from other lipoproteins. The mean density distribution of Lp(a) was determined by a differential density lipoprotein profile (DDLDP). For DDLDP, the lipoprotein density distribution of a serum sample with elevated Lp(a) levels was determined by ultracentrifugation using NaBiEDTA complex as a density gradient. Lp(a) was removed from a second aliquot of the same serum sample by carbohydrate affinity using wheat germ agglutinin (WGA). WGA was demonstrated to have high specificity for Lp(a) in serum. The Lp(a)-depleted sample was ultracentrifuged to obtain a lipoprotein density distribution in the absence of Lp(a). A DDLDP was obtained after subtracting the Lp(a)-depleted lipoprotein density profile from the untreated lipoprotein density profile. DDLDP gives relevant information of the lipoproteins in serum such as density, Lp(a) isoform, and subclass characteristics.

Lp(a) was quantitatively removed from serum with a recovery efficiency of more

than 80%. Lp(a) was purified by ultracentrifugation. Lp(a) obtained in this way retained its inherent density and immunoreactivity. Lp(a) was further characterized by gel electrophoresis and Western blot as well as by capillary electrophoresis. Capillary electrophoresis demonstrated to be a powerful analytical technique for the characterization of Lp(a) and apoprotein(a) isoforms.

The major outcome of this research was the effectiveness of using affinity separations coupled with density ultracentrifugation for the isolation of pure Lp(a) from serum and its isoform characterization based on density and electromobility. The methodology developed and described here are relevant in a clinical setting for the analysis of Lp(a).

To my parents and siblings for all their unconditional love.

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CHAPTER I

INTRODUCTION

1. Lipoproteins and Cardiovascular Disease

The 2003 report on cardiovascular disease statistics by the American Heart Association declared coronary heart disease as the leading cause of mortality in America. Coronary heart disease is caused by atherosclerosis. Atherosclerosis is the narrowing of the coronary arteries due to fatty build up of plaque. During the last ten years, the death rate from coronary heart disease in America declined by 30.2 percent. In 2003, coronary heart disease death rates per 100,000 people were 209.2 for white males, 241.1 for black males; 125.1 for white females, and 160.3 for black females [1].

In 2001 the National Cholesterol Education Program (NCEP) published a report on the detection, evaluation and treatment of high blood cholesterol in adults [1]. According to this report, an elevated level of Low Density Lipoprotein, (LDL), cholesterol is a major cause of Coronary Heart Disease (CHD). The first step in risk assessment requires measurement of LDL cholesterol as part of lipoprotein analysis and identification of accompanying risk determinants [1]. NCEP guidelines suggest that adults aged 20 years or older should be tested for a fasting lipoprotein profile once every 5 years.

According to NCEP, major risk factors for coronary heart disease are:

- LDL cholesterol higher than 160 mg/dL
- Total cholesterol higher than 200 mg/dl

This dissertation follows the style of *Biochimica et Biophysica Acta*.

- High-Density Lipoprotein (HDL) cholesterol lower than 60 mg/dl

Other risk determinants in addition to elevated LDL-cholesterol include:

- Cigarette smoking
- Hypertension
- Low HDL cholesterol
- Family History of premature CHD
- Age (men \geq 45 years; women \geq 55 years)

The same report by NCEP elaborates on the evaluation of risk factors such as Lipoprotein(a) (Lp(a)), and homocysteine as emerging risk factors for cardiovascular disease [1]. A better understanding of these emerging risk factors for CHD is needed in order to determine the steps toward its control.

The objective of the study reported here was to develop a rapid method for the separation, purification, density measurement, and characterization of Lp(a) from serum using a procedure that is isoform independent. The complexity of the Lp(a) molecule sets a challenge for the determination of the risk it represents for the cardiovascular system. The objective the study was met by taking advantage of Lp(a)'s glycosylation and using a novel density gradient ultracentrifugation method for lipoprotein separation.

2. Background on Lipoproteins

Blood triglycerides, cholesterol and cholesterol esters travel through the human body in nanoparticle size packages called lipoproteins. Lipoproteins are a vital part of the study and prevention of cardiovascular disease and are classified as chylomicrons, very

low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), lipoprotein(a) (Lp(a)), and high density lipoproteins (HDL) [2].

A schematic of the general structure of a lipoprotein is shown in Fig. 1. Serum lipoproteins consist of a lipid core (triglycerides and cholesterol esters) contained in a phospholipid layer. Apolipoproteins are located on the surface of the structure and are attached to the lipids in the core. Lipoproteins differ in protein/lipid distribution, from chylomicrons containing 98% lipids to mature HDL containing 50 % lipids [3]. Lipoproteins also differ in the identity of the apolipoprotein or apolipoproteins attached to the lipid core. Table 1 summarizes heterogeneity in size, density and function resulting from the different levels of lipid and protein content in the lipoprotein classes [4].

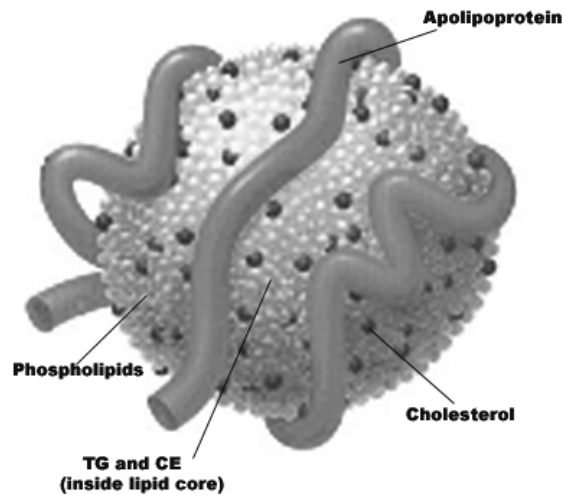


Fig. 1. General structure of a serum lipoprotein [5]

Table 1. Differences between lipoproteins directly involved in CHD [4]

Lipoprotein	Size (nm)	Density (g/mL)	Apolipoprotein	Function
Chylomicron	75-1200	<0.930	B48,C, E	Triglyceride carrier (diet)
VLDL	30-80	0.95-1.006	B100,C,E	Triglyceride carrier
IDL	25-35	1.006-1.019	B100,E	Triglyceride carrier
LDL	18-25	1.019-1.063	B100	Cholesterol carrier
Lp(a)	25-35	1.050-1.100	B100, apo(a)	Unknown
HDL	8-11	1.063-1.21	A, C, D	Cholesterol transfer carrier

3. Lipoprotein(a)

Lipoprotein(a), discovered by Berg in 1963 [6], has recently been assessed as an emerging risk factor for CHD [7, 8, 9, 10]. Since then, much progress has been made in the study of its structural and functional properties. Little is known of the function and metabolism of Lp(a) [11]. Understanding the role of Lp(a) in the development of atherosclerotic cardiovascular disease is necessary for the eventual development of clinical therapies.

Lp(a) is an LDL-like particle formed by the covalent link of a unique glycoprotein called apolipoprotein(a), (apo(a)), to one molecule of LDL's apolipoprotein B-100 (apoB-100) (Fig. 2) [11, 12]. Lp(a) is found only in humans, in Old World nonhuman primates, and in the European hedgehog [13].

The major constituents of Lp(a) are cholesterol (47.2%), phospholipid (24.5%), protein (21.2%), and triglyceride (4.0%) [14]. Apolipoprotein(a) is characterized by a considerable content of proline, tryptophan, tyrosine, arginine, threonine and a low amount of lysine, phenylalanine, and isoleucine. Apo(a) is highly glycosylated (28% carbohydrate [15]), and the most represented carbohydrates in apo(a) are mannose, galactose, galactosamine, glucosamine and sialic acid, in an approximate molar ratio of 3:7:5:4:7 [16].

A. Lipoprotein(a) and Atherosclerosis

Several studies have found an association between Lp(a) and cardiovascular disease. Most commonly a combination of known risk factors such as high LDL concentration and low HDL concentration with C-reactive protein plus homocysteine and serum Lp(a) concentrations in higher than 30 mg/dL are considered of high risk factor for CHD [4, 18]. Lp(a) concentration in serum remains relatively constant over a long

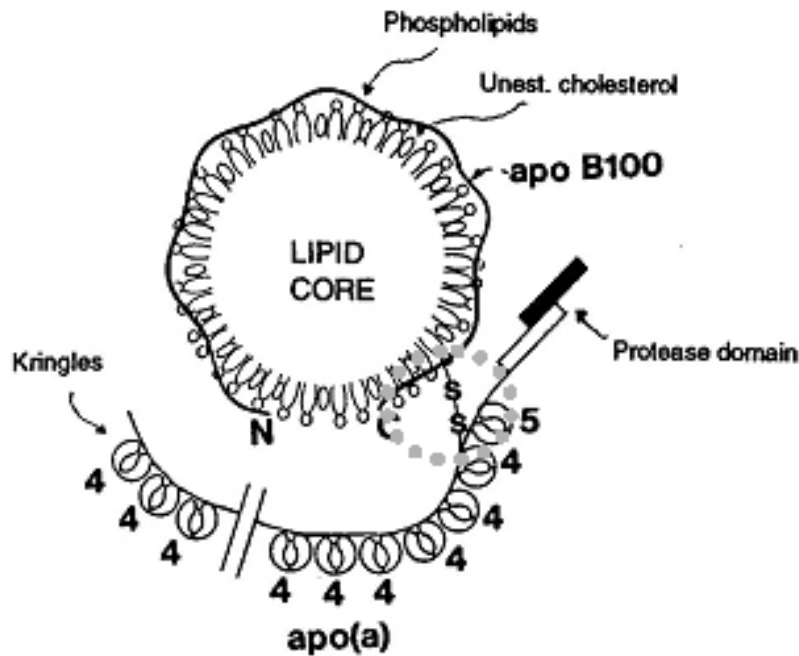


Fig. 2. Model of lipoprotein(a) [17]

period of time and is not effectively influenced by lipid-lowering drugs, diet or exercise [19].

The catabolism of Lp(a) is still under debate. Several studies demonstrate that the liver and the kidney play an important role in the clearance of Lp(a) from serum [15]. It has also been suggested that Lp(a) is cleared from the blood by many lipoprotein-specific receptors such as LDL-R, LRP, VLDL-R and GP-330/megalin [20]. Not more than 1% of Lp(a) is removed from circulation by proteolytical cleavage [21]. The concentration and size of Lp(a) in serum are genetically determined and inversely related; the smaller isoforms being the more atherogenic [9].

The atherogenicity of Lp(a) is attributed to its large cholesteryl esters content, to its deposition in atherosclerotic plaque and, to a competitive inhibition of plasminogen action. Lp(a) may bind to fibrin by the kringles in apo(a) and deliver cholesterol to sites of recent injury and wound healing [15]. This would facilitate wound healing

and may be beneficial, but as a side effect, it may also trigger deposition of cholesterol in growing atherosclerotic plaques and inhibit fibrinolysis.

Another means by which lipoprotein(a) might enter the vessel wall is inside macrophage cells [5]. Macrophages, which are part of the immunologic defense against pathogens, dispose of microscopic debris in the body. In the presence of too much oxidized-LDL, macrophages fail as scavengers and become lipid foam cells. These cells become struck in blood vessels and release growth factors that promote multiplication, which leads to thickening of the artery wall. Lipoprotein(a), being an LDL-like particle, could be oxidized and stick to macrophages in the same way as LDL does.

Lipoprotein(a) may also promote CHD by helping blood clots to persist. Clots in blood vessels are the ultimate trigger of a heart attack and contribute to the gradual thickening for the artery wall that precedes a heart attack. Any factor responsible for increasing the formation of blood clots or impeding their dissolution could play a role in causing heart attacks. Apolipoprotein(a) cannot dissolve fibrin, as plasminogen can. Fibrin is the main protein component of blood clots and it is cleaved by plasminogen. In certain conditions, apolipoprotein(a) can compete with plasminogen for access to fibrin. This keeps plasminogen from cleaving fibrin, upsetting the balance between blood clot formation and disintegration.

Thus, the cardiovascular risk associated with Lp(a) could be due to atherogenic properties because of its similarity to LDL, its similarity to plasminogen due to its thrombogenic properties, or both.

B. Apolipoprotein(a)

Fig. 3 is a general representation of apo(a). The structure of apo(a) closely resembles that of plasminogen, the precursor of the fibrinolytic enzyme plasmin [22].

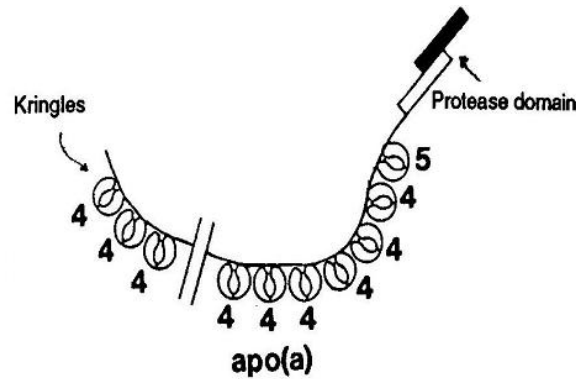


Fig. 3. Apolipoprotein(a). Apolipoprotein(a) is formed by an inactive protease domain, one copy of kringle V and multiple copies of kringle IV [25]

Plasminogen is formed with an active protease domain and five domains called kringles (KI to KV) (Fig. 4). A kringle is a highly conserved tri-loop polypeptide with six conserved cysteine residues stabilizing the structure by three intrakringle disulfide bridges [23]. The name kringle comes from its structural similarity the pastry danish. Kringles are also found in other proteins involved in hemostasis and fibrinolysis such as prothrombin, factor XII, t-PA, urokinase, macrophage-stimulating protein and hepatocyte growth factor [4, 24].

Apolipoprotein(a) contains an inactive protease domain, one copy of kringle V that shares 94% homology with that of plasminogen, and multiple copies of kringle IV (KIV) sharing 65 to 71% homology with plasminogen KIV [26]. Not all KIV of apo(a) are alike; they are classified as ten different types: KIV-type 1 (KIV-1) to KIV-type 10 (KIV-10).

The specific function of some kringles in apo(a) has been described [15]. KIV-9 contains an extra, unpaired cysteine residue that bonds via a disulfide bond with apoB-100. KIV-10 has high homology with plasminogen KIV suggesting that this kringle has lysine binding capacity. Both KIV-3 and KIV-10 are suspected to have a role in the complex formation with LDL. The assembly of Lp(a) from apo(a) and

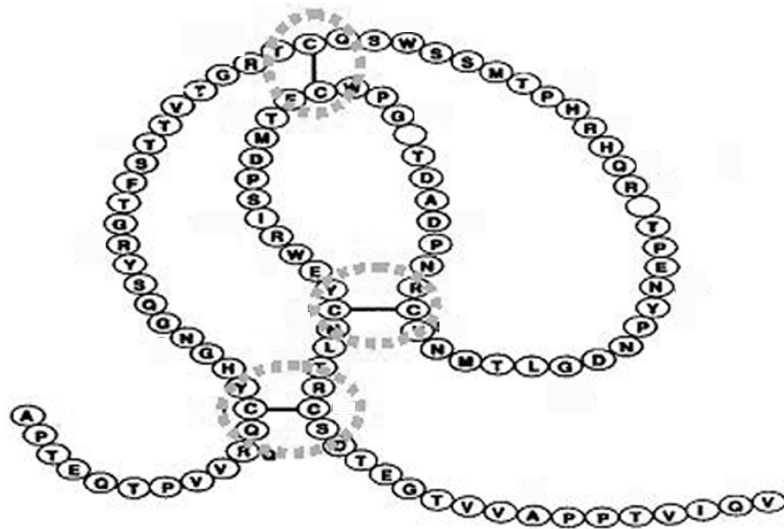


Fig. 4. General structure of a kringle from plasminogen and Lp(a). Disulfide bonds stabilizing the kringle are indicated by dotted circles. [25]

LDL is a two-step process. The first step is a non-covalent interaction of apo(a) with LDL. The second step is the disulfide binding between a cysteine in KIV-9 of apo(a) and apoB cysteine 4326.

C. Lp(a) Size and Density Heterogeneity

Every apo(a) contains a single copy of these KIV types except for KIV-2 which is present in up to 51 copies per Lp(a) particle. As a consequence, apo(a) is an apolipoprotein with a very significant size heterogeneity. Apo(a) mass varies between 300 and 800 kDa, and approximately 30% of this mass is due to carbohydrates. Serum Lp(a) concentration and size are genetically determined. Up to 34 different apo(a) isoforms have been described [27]. This size heterogeneity makes it difficult to isolate Lp(a) from other serum lipoproteins based on density criteria only [17].

Most of the Lp(a) in serum is found within the 1.05 to 1.10 g/mL density range, but it is possible to find small amounts for Lp(a) anywhere within 1.00 to 1.21 g/mL

density range [28]. Table 2 shows the density distribution of serum lipoproteins. Lp(a) density heterogeneity results in an overlap of densities between Lp(a), HDL and LDL. Lp(a) particle densities can be associated with variation in both HDL and apoB-containing lipoproteins, however apo(a) size is responsible for 81% of variation in Lp(a) density [29]. Less than 3% of total Lp(a) concentration in serum is present as free apo(a) [19]. Lp(a) density heterogeneity can be also attributable to metabolic changes such as triglyceride levels in serum and LDL levels [30]. This metabolic Lp(a) density heterogeneity is best observed when the same subject is studied over a long period of time.

Table 2. Serum lipoproteins density distribution [4]

Lipoprotein	Density (g/mL)
Chylomicron	<0.930
VLDL	0.95-1.006
IDL	1.006-1.019
LDL	1.019-1.063
Lp(a)	1.050-1.100
HDL	1.063-1.21

Standardized methods for Lp(a) isolation are not available. The most common method for the isolation of Lp(a) from plasma is sequential ultracentrifugation [31, 32, 33]. Plasma is adjusted to 1.050 g/mL with either KBr or NaBr and centrifuged at $\geq 100,000$ rpm and 10°C for more than 10 hours. The floating lipoproteins: chylomicrons, VLDL, and LDL are removed and the infranatant readjusted to 1.10 g/mL with the same solute. The sample (HDL and Lp(a)) is centrifuged again under similar conditions and Lp(a) is recovered and purified by gel filtration. Since

Lp(a) can also be found in LDL related densities, plasma is sometimes adjusted to 1.02 g/mL, ultracentrifuged and Lp(a) recovered along with the LDL fraction when plasma is readjusted to 1.05 g/mL and ultracentrifuged again.

Gel filtration and affinity chromatography on heparin-sepharose [34] or CNBr-activated lysine-sepharose [35, 36, 37] are two methods commonly used to purify isolated Lp(a). In affinity chromatography, pure Lp(a) is eluted with ϵ -aminocaproic acid (ϵ -ACA), dialyzed, sterilized, and stored for future use [38].

D. Lp(a) Fatty Acid Composition

Plasma cholesterol and triglycerides, along with phospholipids are carried on lipoprotein particles. The lipid composition of all lipoproteins differs considerably as well as its influence in CHD. The fatty acid composition of LDL has been related to its atherogenic role. For example, linoleic acid content of LDL is related to plasma cholesterol [39]. Oxidized LDL is considered to be highly atherogenic. The fatty acid composition of a lipoprotein dictates its ability to undergo oxidation, thus its atherogenicity [40].

The overall lipid content of Lp(a) and LDL are statistically identical. However, there are differences in individual lipid classes in both lipoproteins [41]. Table 3 enumerates the differences in lipid content for LDL and Lp(a). Significant differences were encountered in the triglyceride content by the presence of 20:5 n -3 in LDL and its absence in Lp(a). On the contrary, choline-containing phospholipids of the type 20:0, 20:1 and 20:2 were detected in Lp(a) but not in LDL. LDL has a higher proportion of saturated long chain fatty acids compared to Lp(a). According to Barre, this may represent that LDL has a more rigid surface monolayer compared to Lp(a) [41]. The most significant difference was detected in the phospholipid fractions. Apparently Lp(a) has a larger number of longer chain unsaturated and monosaturated fatty acids.

In general, the differences encountered by Barre in his study of fatty acid composition of LDL and Lp(a) were minor (less than 5 mol %), but they may be significant for the assessment of Lp(a)'s cardiovascular risk.

Table 3. Representative fatty acid composition (mol %) of different lipid fractions of Lp(a) and LDL[41]

Fatty Acid	Lp(a)	LDL
CHOLESTERYL ESTERS		
16:0	9.1±1.4	14.3±1.5
18:1 n -9	22.7±4.2	14.7±1.6
18:3 n -6	0.6±0.1	2.7±0.4
TRIGLYCERIDES		
14:0	1.8±0.3	3.1±0.4
16:0	30.5±2.4	25.6±3.5
18:3 n -3	1.5±0.4	0.5±0.1
20:5 n -3	0.0	3.4±0.4
ETHANOLAMINE-PHOSHPOLIPIDS		
18:1 n -9	13.9±0.2	7.4±0.4
18:2 n -6	19.2±1.2	15.7±1.3
20:4 n -6	21.2±1.5	26.2±1.5
SPHINGOMYELIN		
18:1 n -9	18.0±1.2	7.8±0.7
24:1 n -9	5.1±0.4	7.9±1.2

4. Characterization of Apolipoprotein(a) from Purified Lipoprotein(a)

The process of isolating Lp(a) by ultracentrifugation and further purification is tedious and time consuming. A more efficient way for Lp(a) isolation from serum is needed. Once Lp(a) has been isolated and purified, free apo(a) can be obtained by reducing the disulfide bond between apo(a) and apoB-100. In one method for the isolation of apo(a), Lp(a) was reduced with dithioeritol (DTE), followed by carboximethylation with ϵ -ACA. This method relies on the relatively higher sensitivity to reducing agents of the apo(a)-apoB-100 disulfide bond.

Free apo(a) is recovered by rate zonal ultracentrifugation. After being reduced and dialyzed, the mixture containing apo(a) is adjusted to 1.150 g/mL, centrifuged at 80,000 rpm for 20 hours and 15°C. After ultracentrifugation, LDL free of apo(a) and unreacted Lp(a) are found in the top fraction meanwhile free apo(a) is recovered from the bottom fraction [33, 36, 42]. This recovered apo(a) is stable in storage, retains the same electrophoretic properties as when apo(a) is bound to LDL, exhibits lysine binding properties and binds to VLDL and LDL *in vitro* [33].

In a common method for the phenotyping of apo(a), the sample is prepared for electrophoresis after reducing the bond between apo(a) and apoB-100 with β -mercaptoethanol (2-ME). For this method, the Lp(a) sample is mixed with reducing buffer, incubated at 100°C and applied to a sodium dodecyl sulfate (SDS) gel for apo(a) phenotyping [43]. Once the bond has been reductively cleaved, free apo(a) is separated from apoB-100 by SDS-polyacrylamide gradient gel electrophoresis (SDS-PAGE) followed by immunoblotting using antibodies against Lp(a) [28, 44]. Gel electrophoresis is performed in 1.5% or 4-12% SDS gradient gels under reducing conditions. Up to 34 apo(a) isoforms have been observed byin SDS-PAGE analysis. A single individual may exhibit one or two apo(a) isoforms. A standard nomencla-

ture has been established based on the respective number of KIV repeats (KIV₁₀ - KIV₅₁) [27] or more commonly, based on the isoform mobility compared to apoB-100 (molecular weight 500,000 da) as a molecular weight standard [28]. According to this nomenclature, phenotype F (400 kDa) migrates faster than apoB-100; B (460 kDa) is similar to apoB-100; S1 (520 kDa), S2 (580 kDa), S3 (640 kDa) and S4 (700 kDa) are slower to different degrees than apoB-100, S1 being the slowest apo(a) phenotype observed by SDS-PAGE [38].

5. Limited Proteolysis of Apo(a): Elastase and Thermolysin

Due to its large size, it is difficult to study apo(a) as a whole by other analytical techniques such as mass spectrometry. Limited proteolysis of apo(a) will help to better define the properties and study of its structure and function. In one study, apo(a) was subjected to limited proteolysis using enzymes of the elastase family. These enzymes cleave the interkringle region of the five kringle-containing plasminogen [45]. This approach was re-applied for limited proteolysis of apo(a) resulting in the generation of seven main fragments: F1 (KIV-1 to KIV-4); F2 (KIV-5 to the protease region); F3 (KIV-5 to KIV-10); F4 (KIV-8 to the protease region); F5 (KIV-8 to KIV-10); F6 (KIV-5 to KIV-7) and F7 (KV to the protease region) [46]. The sites where elastase cleaves apo(a) are depicted in Fig. 5. These elastase cleavages are independent of the apo(a) isoform. Moreover, elastase cleavage on Lp(a) generates an Lp(a) particle in which apoB-100 is still linked to truncated apo(a), the size of which depends on the site of elastase cleavage [46].

Limited proteolysis of Lp(a) using thermolysin, a serine protease, results in the generation of two fragments. One of the fragments generated exhibits a constant size of 170 kDa. This domain is the C-terminal domain, and it is linked to apoB-100.

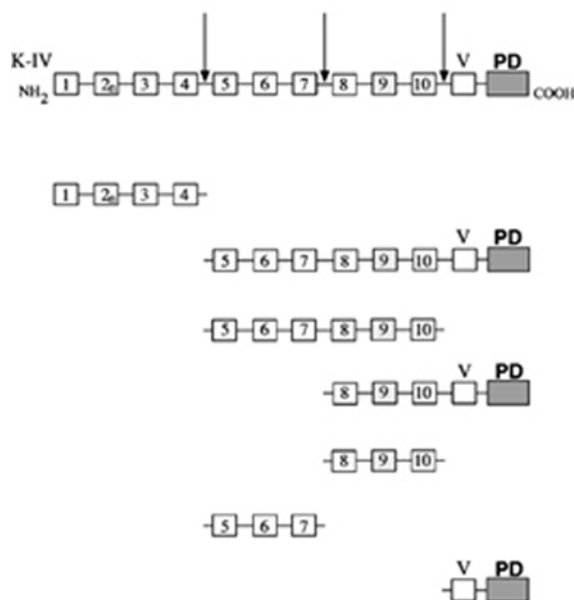


Fig. 5. Limited proteolysis of apo(a) by elastase. Cleavage sites are indicated by an arrow. Apo(a) is conformed by KIV repeats (1-10) one KV a protease domain (PD); KIV-2 reflects different repeats possible as $2n$ [47]

The second fragment is the N-terminal domain and varies in size depending on the number of KIV-2 repeats (see Fig. 6) [48]. The C-terminal fragment contains KIV-5 to KIV-10, KV and the protease domain, while the N-terminal fragment contains a copy of KIV-1, KIV-3, KIV-4, and variable numbers of KIV-2 depending on the apo(a) isoform [48].

Limited proteolysis of apo(a) and Lp(a) will allow for a better understanding of their role in cardiovascular disease. Fragments obtained after proteolysis have to be purified in order to use them for further protein characterization. Purification of these fragments can take place by traditional methods such as lysine-sepharose chromatography or SDS-PAGE. Purification of apo(a) fragments has to be performed under non-reducing conditions due to the propensity of apo(a) to denature upon reduction [48]

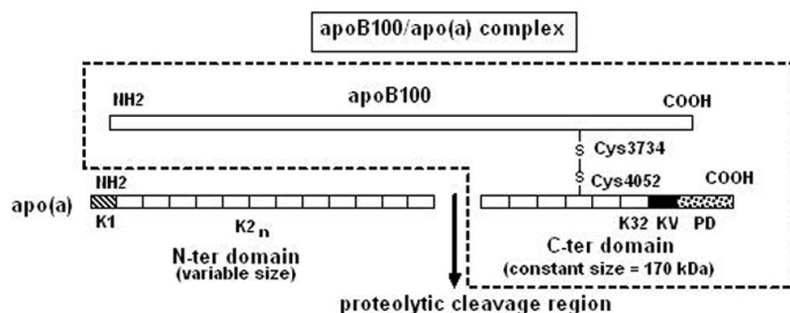


Fig. 6. Limited proteolysis of apo(a) by thermolysin. Cleavage sites are indicated by an arrow. Apo(a) is conformed by KIV repeats (1-10) one KV a protease domain (PD); KIV-2 reflects different repeats possible as $2n$ [48]

6. Sialylation in Lipoproteins

Lipoprotein glycosylation is a complex process that is under control of factors that are not fully understood. Sialic acid has a role in the affinity of some apolipoproteins for lipoproteins, apolipoprotein secretion into plasma and further clearance. Sialic acid on plasma lipoproteins is thought to influence the development of atherosclerosis [49].

Sialic acids are formed by the union of *N*- and *O*-substituted derivatives of neuraminic acid. Neuraminic acid is an amino sugar [49]. The functions of sialic acids include conformational stabilization, protease resistance, charge, enhancement of water binding capacity, cellular recognition, protein targeting and developmental regulation [50]. The most common sialic acid found on human plasma glycoproteins is *N*-acetylneuraminic acid. Sialic acid is most commonly linked to sugars such as galactose, *N*-acetyl galactosamine or another molecule of sialic acid [51].

The sialic acid content of plasma lipoproteins varies considerably among different lipoprotein fractions. Table 4 is a summary of the molar contribution of each apolipoprotein to the total sialic acid content of the major apoB-100 containing lipoproteins. ApoB-100, C-II, C-III, and E are the main contributors to the sialic

acid content of VLDL. Sialic acid increases VLDL's charge and thus its electrophoretic mobility. This charge effect was also noted in LDL [52, 53]. It is believed that sialic acid enhances the solubility of LDL, and it can also be involved in cellular recognition of LDL [54].

Lp(a) is the most highly sialylated serum lipoprotein (see Table 4). ApoB-100 derived from Lp(a) has the same carbohydrate content as apoB-100 from LDL [55]. The association of apo(a) with LDL to form apo(a) is independent of the sialylation of apo(a) [56].

Table 4. Molar contribution of each sialic acid-containing constituent to the total sialic acid content of apoB-100 containing lipoprotein fractions [49]

Apolipoprotein	VLDL	LDL	Lp(a)
ApoA-II	0	0	0
ApoB-100	13.0	13.0	13.0
ApoC-II	7.3	0	0
ApoC-III	110.8	1.2	0
ApoD	0	0	0
ApoE	12.1	0.1	0
Apo(a)	0	0	119.7-290.7
Gangliosides	19.5	5.3	5.3
Total sialic acid (mol to mole)	162.7	19.6	138.0-309.0

7. Sialylation in Apolipoproteins

Of all the plasma apolipoproteins, apo A-II, B-48, B-100, C-II, C-III, D, E, J and (a) have been reported to be sialylated [49]. Many of these apolipoproteins are found in plasma as a mixture of glycosylated and non-glycosylated forms, with the exception of apoB-100. A summary of the sialylation of apolipoproteins of some plasma lipoproteins is presented in Table 5 [49]. In this table, the sialylation numbers for apo(a) are estimated per kringle IV repeat. As it can be seen, of all the sialylated apolipoproteins, apo(a) is the most highly sialylated.

Table 5. Summary of the sialylation of apolipoproteins from plasma lipoproteins [49]

Apolipoprotein	Number of sialic acid	Average sialic acids per mole protein	Glycation linkage
A-II	0-2	0.001	<i>O</i>
B-100	12-14	13	<i>N</i>
B-48	Unknown	1.7	<i>N</i>
C-II	0-2	0.23	Unknown
C-III	0-2	1.27	<i>O</i>
D	0-6	5.4	<i>N</i>
E	0-2	1.29	<i>O</i>
(a)	5.7	5.7	<i>N,O</i>

The majority of sialic acid is protein- and lipid bound. An increase of protein-bound sialic acids in plasma have been related to inflammatory disease. One reason for this effect could be that the levels of acute phase proteins, many of which are sialylated are also increased [57, 58]. Other studies demonstrate that individuals with low levels of lipoprotein-associated sialic acid are at increased risk of developing CHD

[59, 60, 61].

The glycosylated form of apo A-II has shown influence on the lipid binding of the lipoprotein and binds preferably to HDL3 [62]. ApoB-100 is multi-sialylated in addition to containing high-mannose structures. ApoB-100 polypeptides contain at least one sialic acid residue [63]. The significance of the sialylation of apo C-II is unknown. It is found in di-, mono- and non-sialylated forms in plasma [64]. On the other hand, apoC-III exists as three isoforms in plasma, having none, one or two sialic acid residues per polypeptide. Hypertriglyceridemic patients have increased levels of apoC-III with two sialic acid residues in VLDL [65, 66]. ApoD is primarily found in HDL and is highly glycosylated with carbohydrates contributing to 18% of its weight. The significance of sialic acid on apoD is unknown [67]. ApoE occurs as di-, mono- and non-sialylated in plasma. Sialylated apoE has a higher affinity for HDL than desialylated apo E *in vitro* [68].

Apolipoprotein(a) is highly glycosylated with approximately 28% of its molecular weight due to carbohydrates [15]. The contribution of glycosylation to lipoprotein(a) function *in vivo* remains unknown. A study using elastase to cleave apo(a) between KIV-4 and KIV-5 has demonstrated that the lack of glycans at this site results in an increased susceptibility to proteolytic cleavage [69]. Apo(a) contains a significant amount of *O*-glycosidic oligosaccharides rich in *N*-acetyl-D-neuraminic acid and *N*-acetyl-D-glucosamine [70, 71]. Apo(a) contains six times more of these oligosaccharides than any other apolipoprotein in serum. Several studies have been dedicated to the understanding of the role of *O*- and *N*-glycans in apo(a). These include a role for intracellular processing [72, 73], maintaining the tertiary structure of apo(a), and preventing aggregation [74]. As a result of containing a vast amount of *O*-glycans, Lp(a) and apo(a) are highly hydrophilic [75].

8. Current Methods for the Determination of Lipoprotein(a) Concentration

The main challenge in measuring Lp(a) is to overcome the structural homology of Lp(a) with plasminogen and obtaining specific antibodies that discriminate between the multiple apo(a) isoforms that can be present in a single sample. Current methodologies do not discriminate against Lp(a) size. Immunoassays are widely used for the quantitation of lipoprotein(a) of which the most common are immunoturbidimetric [76], nephelometric [77], and enzyme-linked immunosorbent assays (ELISA) [38, 78, 79]. These methods are fast, accurate and reproducible but rely on the specificity of the antibody used, are very sensitive to turbidity of the sample and are affected by high concentrations of triglycerides. In nephelometry, the inherent polymorphism of Lp(a) may induce errors in regard to the light scattered due to the different sizes of the lipoprotein itself [80].

The most commonly used immunoassay to measure Lp(a) concentration is a sandwich-type ELISA. In this assay, Lp(a) particles are captured by monoclonal or polyclonal anti-apo(a) antibodies. The complex is then sandwiched with an enzyme conjugated (typically with peroxidase or alkaline phosphatase) monoclonal or polyclonal antibodies against apoB. The change in color intensity is monitored at the appropriate wavelength and Lp(a) concentration is determined. This method is very specific because it includes two antibodies, is very sensitive to antigen concentrations and can be easily automated [4].

Antibodies generated for measuring apo(a) need not discriminate against different apo(a) isoforms. Monoclonal or polyclonal antibodies produced to recognize epitopes present in KIV-2 tend to under estimate apo(a) concentrations depending on the size of the assay calibrator. This problem can be overcome by producing

antibodies directed to apo(a) moieties not expressed in KIV-2.

9. Lipoprotein(a) and Affinity Separations

As mentioned previously, Lp(a) can be found in density ranges of 1.05 to 1.1 g/mL. This density range overlaps with that of LDL and HDL (see Table 1). It is because of this density overlap that Lp(a) cannot be isolated without including some LDL or HDL contamination. In the same way, Lp(a) separation based on size is compromised because of overlapping size distribution with LDL.

Different techniques have been developed to study the heterogeneity of Lp(a) density and size. Mainly, a Lp(a) fraction is obtained from sequential centrifugation and further Lp(a) purification from other lipoproteins contained in that density range by affinity separations. The most common affinity separations for Lp(a) purification include heparin-sepharose chromatography, lysine-Sepharose chromatography, and immunoaffinity.

A. Heparin-Sepharose Chromatography

Heparin-Sepharose chromatography is an affinity technique used to purify Lp(a) and LDL from serum by affinity to heparin before or after density centrifugation. Heparin interacts specifically with apoB and apoE. The mechanism of binding of apolipoproteins to heparin is not well understood. It is believed that interaction between positive groups of apolipoproteins and anionic groups of heparin are responsible for this selective binding .

In heparin-Sepharose chromatography, plasma is incubated with commercially available Sepharose beads coated with heparin in 10 mM sodium phosphate (pH 7.0). The samples are then centrifuged at 11,000 g for 15 seconds and the supernatant

is removed. The sepharose beads are then washed three times with 10 mM sodium phosphate buffer. The heparin-bound fraction, containing Lp(a) is eluted with 50 μ L of 1 M NaCl, 10 mM sodium phosphate buffer [81].

In another method, the heparin-Sepharose was equilibrated with 0.006 M barbital buffer, pH 7.4, containing 0.15 M NaCl, 0.01% Na₂EDTA, and NaN₃. About 2-5 mg of sample containing Lp(a) was loaded to the column and eluted with 30 ml of a linear 0.2-0.5 M NaCl gradient. Lp(a) was eluted first at 0.3-0.35 M NaCl while LDL was eluted at approximately 0.4 M NaCl [34].

B. Lysine-Sepharose Chromatography

Lysine-shepharose chromatography is an extremely useful tool for the isolation of all apo(a)-containing particles, free of LDL and HDL. This technique is used extensively for the purification of plasminogen from plasma. Plasminogen has two lysine binding domains in KI and KIV [45]. Due to its homology to plasminogen, apo(a) also contains these lysine binding properties [24].

Variables that can reduce the capacity of Lp(a) binding to lysine-Sepharose include buffer ionic strength, density of lysine groups on the gel and temperature [82]. The percentage of Lp(a) binding to lysine-Sepharose can be increased by lowering the ionic strength of the buffer medium. However, nonspecific binding increases also at lower ionic strength, and other lipoproteins such as LDL and HDL start binding. Commercially available lysine-Sepharose has 3 to 4 fold less lysine per milliliter gel than lysine-Sepharose prepared in the laboratory. Temperature is also a factor. Lipoprotein(a) particles with large apo(a) isoforms tend to self-associate in the cold, and this process is concentration independent [83].

In practice, CNBr-activated Sepharose 4B is coupled to the α -amino group of L-lysine following the instructions provided by the manufacturer. The amount of

L-lysine crosslinked to the beads was calculated to be between 16 and 21 μmol of L-lysine per mL bead suspension. Lysine-Sepharose chromatography is performed at 22°C. Columns are packed with lysine-Sepharose at a ratio of 5 mL of packing material to 1 mg of Lp(a) protein and equilibrated with PBS containing 1 mM EDTA and 0.02% NaN_3 . The column is then washed with 3 column volumes of equilibrating buffer followed by 3 column volumes of 500 mM NaCl in order to elute non-specifically absorbed material. Lp(a) is eluted from the column with 200 mM ϵ -ACA [35, 36, 37].

C. Immunoaffinity

Immunochemistry describes the reaction between an antibody and its complementary antigen. An immunoassay is a technique that utilizes an antibody or antigen to recognize and quantitate its complementary partner. The reaction between the antibody and antigen depends on an array of chemical interactions. Reagent specifications and optimization of the reaction conditions are vital to ensure a good analytical performance of the immunoreaction [84].

The region of the antigen that binds to the antibody is known as the epitope or antigenic-determinant region. In a macromolecule such as a protein, it constitutes an area and number of residues similar to those of the paratope. The antibody-antigen complex binding depends on factors such as chemistry (thermodynamics) and spatial orientation of the epitope and paratope [84].

Immunoprecipitation is widely used in homogeneous immunoassays for the quantitation of the antibody-antigen reaction. The principle of immunoprecipitation is based on antibody molecules binding first to one antigen molecule to form a dimer, and then binding to a second antigen resulting in the formation of a complex with increases in size. When the antibody and antigen concentrations are high enough a visible immunoprecipitate is formed. This was described by Heidelberger and Kendall

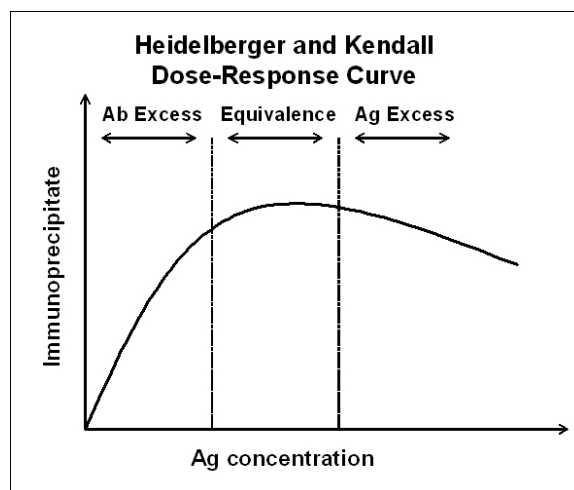


Fig. 7. Heidelberger and Kendall immunoprecipitin curve [85]

in 1935 and is defined by the precipitin curve (Fig. 7) [85]. In the presence of an excess antigen the immunocomplex would not form and free antigens would remain in solution. If the antibody is present in excess, they would not complex with the antigen and would not precipitate. It is always desirable for all the determinations to take place in the antibody excess zone.

Immunoaffinity has been used to purify Lp(a) extracted from serum after ultracentrifugation. In this technique, anti-Lp(a) immunoglobulins were coupled to CNBr-Sepharose 4B. The beads were then packed onto a column and the column equilibrated with 0.5 M Tris, pH 7.4, 0.15 M NaCl, 1 mM EDTA. After loading the Lp(a) containing sample, the column was washed with buffer. Lp(a) was eluted from the column with 3 M KSCN followed by the column buffer. Lp(a) was then desalted by dialysis [86].

All the affinity separation techniques covered in this section have been widely applied for the purification of Lp(a) after separation from other lipoproteins in serum by ultracentrifugation. Standardized methods for Lp(a) isolation are not available. These processes of isolating Lp(a) by ultracentrifugation and further purification is

tedious and time-consuming. A more efficient way for Lp(a) isolation from serum is much needed. A different approach for affinity separations based on carbohydrate affinity will be discussed in the next section.

10. Lipoprotein(a) Affinity Separation with Lectins

Taking advantage of the high degree of glycosylation of Lp(a) and the lectin wheat germ agglutinin (WGA) specificity for sialic acid, Seman *et al* achieved a rapid separation of Lp(a) from other plasma lipoproteins [87]. In this study, Seman states that because of the large number of WGA binding sites per Lp(a) molecule, Lp(a) competes strongly with other plasma glycoproteins that are present in plasma in much greater molar concentrations than Lp(a). This section will elaborate on the principles of carbohydrate affinity and different types of lectins and its application to Lp(a) isolation and purification.

A lectin is defined as a carbohydrate-binding protein of non-immune origin which agglutinates cells and/or precipitates glycoproteins [88]. Lectins are derived from plants and bind to specific oligosaccharides on the surface of cells causing the cells to aggregate [89]. The binding between sugars and lectins is relatively weak, not resulting in the formation of covalent bonds. This binding is reversible, like the reaction of an antibody with an antigen or an enzyme with an inhibitor. Lectins are classified into specificity groups according to the monosaccharide that is the best hapten inhibitor for the lectin [89].

Individual glycoproteins in a complex mixture can be readily detected with the aid of lectins. These glycoproteins can be separated by gel electrophoresis and western blotted onto a porous nitrocellulose membrane. Lectins are used to reveal the glycoproteins on the blot. Affinity chromatography of glycoproteins on immobilized lectins

is widely used for preparative purpose and also as an analytical technique [89]. In this case, the solution to be separated is applied to a column of the immobilized lectin and washed to remove any unbound sample. The specifically bound glycoprotein is eluted with a solution of the sugar for which the immobilized lectin is specific. The high specificity of lectins allows for the separation of closely related compounds, such as molecular variants of a glycoprotein differing only in their carbohydrate composition or structure of carbohydrate chains [89].

A. Wheat Germ Agglutinin

Wheat germ agglutinin (WGA) is a plant lectin which agglutinates various types of animal cells, of malignant cells and of protease-treated cells. WGA is a dimeric protein with a subunit molecular mass of 17 KDa [90]. WGA belongs to the family of chitin-binding proteins, characterized by high cysteine and glycine content [91].

Fig. 8 shows a diagrammatic representation of WGA. WGA has specificity for *N*-acetyl-D-neuraminic acid (NeuNAc) and *N*-acetyl-D-glucosamine (GlcNAc) (Fig. 9). The molecular structure of WGA is highly stable due to the presence of 32 disulfide-linked cysteine residues distributed over four identically folded domains. The lectin-sugar complex is stable over a pH range of 4.5 to 10.0, but its formation is controlled by an ionizable group with an apparent pK of 3.9 [92].

Solution studies of the binding of simple sugars to WGA predict a chemical environment at the saccharide binding site which consists of a tryptophan, a tyrosine, disulfides and an acidic aminoacid (pK of 3.9). The binding domains of WGA are comprised of two independent non-cooperative sites per subunit. One of the domains is a *principal* domain and the other is a *helper* domain. These domains are stabilized by four disulfide bonds and have an aromatic amino acid residue rich region for sugar binding.

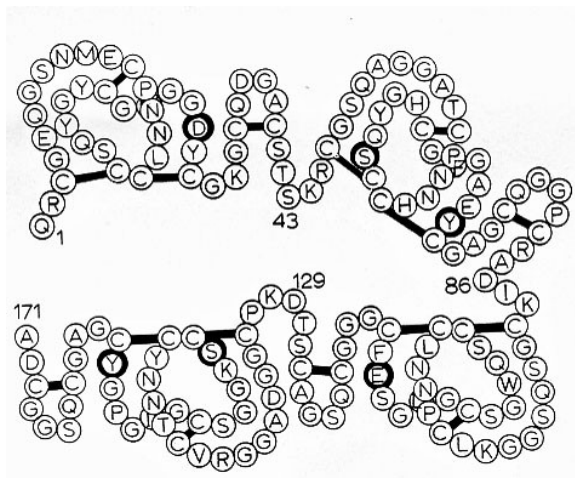


Fig. 8. WGA monomer [88]

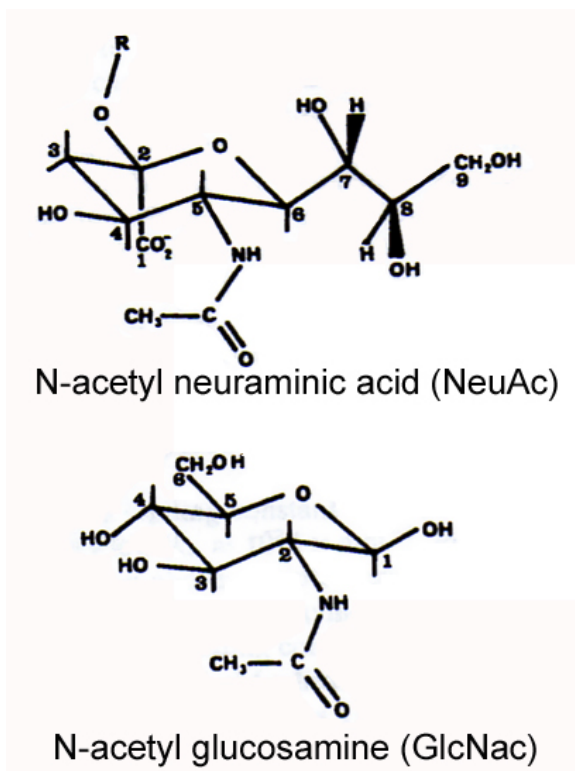


Fig. 9. WGA binds specifically to *N*-acetyl-D-neuraminic acid (NeuNac), and *N*-acetyl-D-glucosamine (GlcNac) [88]

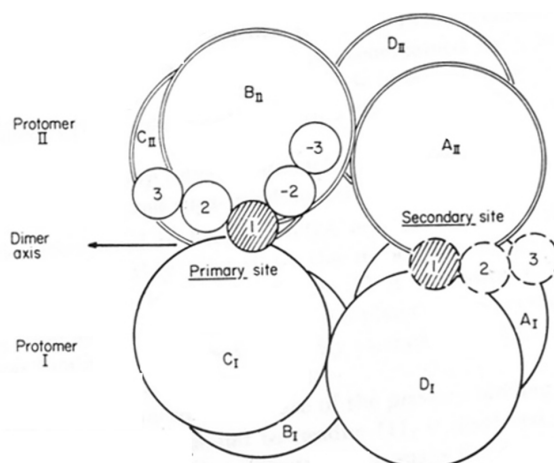


Fig. 10. WGA binding sites of the *primary* and *helper* (or *secondary*) domains. GLC-NAc oligomers bind at subsites 1,2, and 3 in both binding locations. NeuNAc binds to subsites 1, -2, and -3 in the *primary* binding location only. Domains in each of the two WGA promoters are labeled I and II [92]

The *helper* domains contribute with a carboxylate group [93]. The *principal* domain has been shown to bind NeuNAc and GluNAc while the *helper* domain binds poorly to NeuNAc. The reason of the poor binding of NeuNAc to the (*helper*) location is not well understood. However, it is believed that the proximity of neighboring WGA molecules causes an unfavorable charge environment for the binding [92]. Since each WGA monomeric subunit contains two identical and independent binding sites for GlcNAc, WGA binds two mol of GlcNAc per WGA binding domain [88]. NeuNAc binds to WGA with an affinity fourfold less than GlcNAc.

An schematic of WGA binding sites is presented in Fig 10. Both *primary* and *secondary* binding locations on the WGA dimer are represented. The domains for WGA are labeled a I and II for each promoter. Sugar binding locations are indicated by small circles. GlcNAc oligomers bind at subsites 1,2, and 3 in both binding locations. NeuNAC oligosaccharides bind at subsites 1, -2, and -3 in the *primary* binding location only. The strongest binding interaction location is indicated by a shaded circle.

NeuNAc and GlcNAc are pyranose sugars with an acetamido group ($\text{CH}_3\text{-CO-NH}$) and a neighboring hydroxyl group both in equatorial position and in an identical position with reference to the ring oxygen (see Fig 9). This configuration is required for the binding of these sugars to WGA [94]. Hydrogen bonding and Van der Waals interactions are the main forces responsible for sugar-lectin binding [95]. The aromatic side-chains of WGA provide Van der Waals interaction in three of the four domain binding sites. Tyrosine appears to be most common, possibly due to the additional hydrogen-bonding capability of the phenolic hydroxyl groups.

The acetamido group interacts with the binding domains in WGA as follows. The CH_3 group is in van der Waals' contact with an alanine and a tyrosine in WGA at a distance of about 3 amstrongs. The carbonyl oxygen lies within hydrogen-bonding distance of the backbone NH of a histidine residue, while the NH group is oriented towards the side chain of a glutamic acid to form a hydrogen bond. The OH group in in C-3 (GlcNAc) or C-4 (NeuNAc) is pointed in the direction of the side chain of a tyrosine and the backbone NH for potential hydrogen bonds [95]. It is possible that a charge effect has to be taken into consideration, since WGA is basic with a high isoelectric point [94].

11. Lipoprotein(a) Cholesterol Measurement

LDL-cholesterol (LDL-c), traditionally measured by the Friedewald formula, does not take into consideration the cholesterol content of Lp(a) (Lp(a)-c). The Friedewald formula is expressed as the result of the addition of HDL-cholesterol (HDL-c), VLDL-cholesterol (VLDL-c) subtracted from total cholesterol [4]. This equation assumes that the total mass of VLDL-c in serum is equivalent to one fifth of the mass of triglycerides in VLDL. One of the major differences between LDL and Lp(a) is

that LDL-c can be effectively lowered by statins, while Lp(a) concentrations remain unaffected after treatment with statins [9]. In this case, LDL cholesterol would be overestimated when Lp(a) levels are high.

Lp(a) has been traditionally measured by immunoassays and its concentration expressed as total mass of Lp(a) and not as Lp(a)-c as is with the other lipoproteins. Lp(a)-c contributes to less than 15% of the total serum cholesterol [96]. When LDL-c is calculated by the Friedewald formula calculating HDL-c after precipitating all apoB-100 containing lipoproteins, the result is not taking into account any cholesterol contribution from Lp(a). On the contrary, the result obtained by the Friedewald formula is a sum of the cholesterol contribution from both lipoproteins. A correction to the Friedewald formula has been suggested to include Lp(a)-c contribution to total cholesterol [96]. This correction simply adds Lp(a)-c to the formula and subtracts it from serum total cholesterol along with HDL-c and VLDL-c. When Lp(a) and triglycerides (TG), concentrations are known and are reported in mg/L then the following can be applied: $LDL-c = \text{Total Cholesterol} - (\text{HDL-c} + 0.2 \text{ Triglycerides} + 0.3 \text{ Lp(a)})$ [96]. This correction is taking into consideration that Lp(a)-c corresponds to 30% of Lp(a) mass [97]. A recent study corrected the Lp(a)-c mass percentage to 45% [9].

Efforts to calculate Lp(a)-c and its contribution to cardiovascular disease led to the commercialization of an Lp(a)-c kit: LipoPro Lp(a)-c assay (Genzyme Corporation, Cambridge, MA). This kit is no longer available. It was a non-immunological assay system based on lectin affinity chromatography to isolate intact Lp(a) particles followed by a cholesterol measurement of these particles [98]. Plasma was loaded on to a centrifuge filter unit to which a lectin-coupled resin reagent was added. The lectin binds the plasma Lp(a) and then intact Lp(a) eluted. Lp(a)-c was measured using a routine enzymatic cholesterol assay [99].

It was reported that the LipoPro Lp(a)-c assay is sensitive enough to detect

Lp(a)-c concentrations as low as 15 mg/dL [87]. One of the advantages of this technique was that the assay was not affected by apo(a) size polymorphism. One study concluded that this Lp(a)-c method showed an inverse and linear relation with apo(a) size, but that it did not have an added clinical value compared to Lp(a) mass measurements [100]. The use of lectin affinity chromatography to isolate Lp(a) particles and measure Lp(a)-c is a robust and easy-to-use assay, but it has reduced throughput compared to automated immunoturbidimetric assays [98].

Another approach for the measurement of Lp(a)-c uses agarose gel electrophoresis with enzymatic staining for cholesterol [101, 102]. In this method, VLDL was removed by ultracentrifugation and agarose gel electrophoresis was used to separate Lp(a) from LDL. As a result, information on Lp(a) concentration and a ratio of Lp(a)-protein to Lp(a)-C was obtained. Cholesterol in the lipoproteins was determined after scanning an agarose gel enzymatically stained with cholesterol dehydrogenase and nitroblue tetrazolium chloride dye [101]. The sensitivity of this assay allows for the detection of Lp(a)-c concentrations as low as 30 mg/dL, lower Lp(a)-c concentrations could not be reliably quantified [101].

Helena Laboratories (Beaumont, TX) manufactures a cholesterol agarose gel (REP Vis) that consists of agarose in a sodium barbital buffer with EDTA, guanidine hydrochloride, and magnesium chloride. Before or after ultracentrifugation, lipoproteins are separated in this gel by electrophoresis and the bands stained with enzymatic reagent containing cholesterol esterase, cholesterol dehydrogenase, and diaphorase. LDL, Lp(a), and HDL cholesterol are determined by densitometry [102]. Still, Lp(a) mass measurement is more sensitive than the electrophoresis/cholesterol reported by Baudhuin, however this Lp(a)-c assay was able to detect levels as low as 2mg/dl Lp(a)-c [102].

12. Ultracentrifugation as an Analytical Tool for Separation of Lipoproteins

The operating principle of analytical ultracentrifugation is the movement of particles in a centrifugal field due to differences in density. The particles being separated can be solids or liquids dispersed in a liquid. Ultracentrifugation is a non-intrusive, rapid separation. It has the limitations that low concentrations of solute are needed for the appropriate separation and there is an uncertainty in density of particles if adsorbed materials such as surfactants are present. The present section is an overview of different ultracentrifugation techniques illustrating the principles for lipoproteins profiling.

A. Sequential Flotation and Rate Zone Centrifugation

This approach has been the standard method for the isolation of lipoproteins and specifically of Lp(a). This method is labor intensive and it can take many hours to achieve a complete lipoproteins separation. Sequential flotation was introduced by Havel in 1955 [103]. Briefly, the density of a serum sample is adjusted with a salt (e.g. KBr) and placed underneath a less dense solution. During centrifugation, the buoyant lipoproteins float leaving the denser particles at the bottom of the tube. The top fraction is removed and the bottom fraction is re-adjusted to a higher density and centrifuged again. This steps are repeated until the lipoproteins are completely separated. In sequential flotation, the lipoproteins are separated into density ranges predetermined by the densities chosen for the top and bottom solution.

Rate zone ultracentrifugation was introduced by Brakke in the 1950s [104]. In this technique a density gradient is formed prior to centrifugation by layering the sample on the top or of the gradient. A differential density will be created between

the solution and the layered sample, which will settle along the gradient until it reaches its equilibrium point. In a rate separation, particles float or sediment at different velocities depending mainly on their buoyant densities. The formation of this type of gradient is easy and fast, and it is especially useful when a wide gradient is needed. Some disadvantages of this type of gradient include poor resolution and reproducibility and gradient instability.

B. Equilibrium Density Centrifugation

The second way for a density gradient formation the density gradient is established during centrifugation. A small amount of sample is mixed with a salt solution, and then centrifuged to form a salt density gradient from low density at the top of the centrifuge tube to high density at the bottom of the tube. Depending on the size of the particle, the sedimentation of floating force will drive it to form a narrow band at its isopycnic density. Density gradients formed by this way are rather shallow, considerably increasing the resolution of the separation, particles are concentrated while being separated and the separation is predictable. Once equilibrium has been reached the concentration profile not longer changes with centrifugation time.

C. Advantage of BiEDTA Salts as Density Gradient Forming Solutes

Bismuth-EDTA salts (BiEDTA), are very soluble salts which is optimum for equilibrium density ultracentrifugation. Traditionally sucrose solutions are used for the separation of lipoproteins by rate zonal centrifugation. As mentioned previously, rate zonal centrifugation has the disadvantage of having poor resolution, poor reproducibility if solvent is changed and the gradient formed is unstable. Density separations of serum lipoproteins have been achieved and reported using a variety of BiEDTA density gradients [105, 106].

The facility of obtaining an equilibrium density sedimentation with BiEDTA salts is one of the major advantages of using this solute for the density separation of lipoproteins [107]. The separation is predictable, knowing the gradient to be formed and the density of the particles being separated. Since the sample is homogeneously mixed with the solvent before the spin, the laborious and delicate layering steps are eliminated. The particles separated are concentrated at their isopycnic densities, and the separation takes place in less time compared with other density gradient equilibrium separations. The density range and gradient shape can be influenced in a predictable manner by changing the BiEDTA salt concentration and centrifugation conditions [105]. In this specific case, the molecular weight of the ligand can be changed by complexing BiEDTA with different ions such as sodium (NaBiEDTA), bismuth (CsBiEDTA) or cadmium (CdBiEDTA), thus changing the density gradient formed.

D. NBD-Ceramide Stain for Lipoprotein Visualization

NBD C₆-Ceramide (7-nitro-2,1,3-benz-oxadiazol-4-yl) is a widely used probe for the fluorescent staining of lipids in the study of membrane lipids. It has an absorption spectra and fluorescence excitation spectra having a maxima around 340 and 460 nm in ethanolic solution, with a molar absorptivity of 20,000-25,000 M⁻¹•cm⁻¹ at 460 nm. NBD-labeled lipids are very stable and have suitable fluorescence properties like: good spectral overlap with other fluorophores like rhodamine allowing efficient energy transfer, minimal interference with other biological fluorophores, self quenching at high concentrations and environmental sensitivity [108]. However, NBD fluoresces very poorly in water solutions and suffers from photobleaching, limiting its fluorescence lifetime. According to studies performed in cellular membranes, the NBD group was found at the polar region of the membrane regardless of whether it is located on

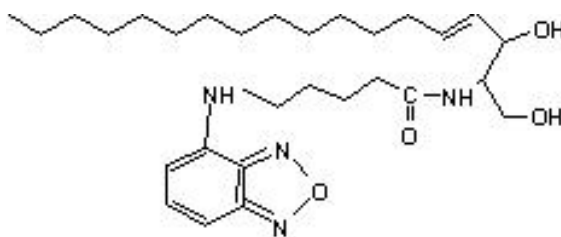


Fig. 11. NBD-C₆-Ceramide (7-nitro-2,1,3-benz-oxadiazol-4-yl)

the head or tail of the phospholipid molecule. Therefore when coupled to lipoproteins NBD may be inserted deep in the lipids core resulting in the observed effect of NBD on lipoproteins physical and chemical properties [109]. The quantitative incorporation of NBD to lipoproteins is being studied [107].

13. Capillary Electrophoresis and Lipoproteins

Lipoprotein particles are heterogeneous with respect to size, hydrated density and composition. Hydrated density remains the most common form of lipoprotein classification. Lipoproteins are viewed as particles which are heterogeneous with respect to their physical properties but homogeneous with respect to apolipoprotein composition [110]. The application of diverse separation techniques for the separation of lipoproteins based on this heterogeneity is much needed.

Capillary electrophoresis (CE) has recently emerged as a powerful separation technique for biomolecules. Capillary electrophoresis separates components of a sample on the basis of their charge and size. Capillary electrophoresis has the advantage of obtaining a separation with high efficiency in a very short time. The main advantage of CE over traditional gel electrophoresis methods is that it eliminates labor-intensive steps such as gel preparation and staining along with ease of data analysis. Another advantage is that in capillary electrophoresis high resolution separations are achieved

on small sample volumes (0.1 to 10 nL). Quantitation of the analytes separated by capillary electrophoresis is possible by analyzing CE peak intensity and correlating it with sample concentrations.

Apolipoproteins have a high affinity for detergents. Therefore, the electrophoretic behavior of apolipoproteins can be influenced by using detergents as buffer modifiers. Anionic detergents interacting with lipoproteins cause a significant increase in particle charge, thus an increase in electrophoretic mobility [111]. Tadey and Purdy separated the main apolipoproteins from HDL and LDL in a single run in less than 12 minutes [112]. The separation of these apolipoproteins was performed in a bare silica and polyacrylamide-coated capillaries. The separation of these apolipoproteins was possible using high-pH buffers and by the addition of SDS to the separation buffer. The effect of SDS on apolipoprotein CE is two-fold. First, it competes effectively with protein-protein interaction and eliminates protein aggregation. Second, it binds to lipoproteins, altering their size, shape and net charge resulting in a modification of their electrophoretic mobilities [112]. This interaction was attributed to the presence of non-interacting detergent binding sites on the apolipoprotein. VLDL apolipoproteins were separated again with the use of detergents [113]. It was concluded that the presence of detergents in the sample buffer significantly improved the separation.

Following with the surfactants idea to increase separation efficiency, Hu and co-workers developed a method for the separation of Lp(a) and reduced Lp(a) by CE [114]. In this study a high-pH borate buffer (pH=10) was mixed with SDS and acetonitrile (ACN) to improve separation efficiency. The SDS used consisted of 70% sodium dodecyl sulfate and 30% sodium tetradecyl sulfate, which increased its hydrophobicity and interaction with lipoproteins. Lp(a) separated by CE under this conditions had significantly different electrophoretic mobility than LDL and HDL.

CHAPTER II

EXPERIMENTAL

This chapter focuses on the description of the experimental settings for all the procedures developed or applied for the research reported in this dissertation. Detailed protocols for all experimental sections can be found in Chapter V of this dissertation.

1. Chemicals and Materials

N-acetyl-D-glucosamine, phosphate buffered saline (PBS) tablets, agarose coupled wheat germ agglutinin (Catalog No. L-1882), phosphorylase *b*, cross-linked molecular weight markers (Catalog No. 9012-69-5), and 2-mercaptoethanol were purchased from Sigma (St. Louis, MO). Ethylenediaminetetraacetic acid monosodium bismuth salt (NaBiEDTA) was from TCI America (Portland, OR). The cesium bismuth EDTA (CsBiEDTA) was synthesized in our laboratory as reported previously [105]. NBD C₆-ceramide (NBD) was from Molecular Probes (Eugene, OR). L-Proline was purchased from Dickinson & Co. (Franklin Lakes, NJ). The 3-8% NuPAGE Tris-acetate SDS-PAGE minigels, Novex Tris-acetate SDS running buffer (20x), NUPAGE sample buffer (4x), nitrocellulose membranes (0.2- μ m pore size), WesternBreeze Chromogenic Western Blot Detection Kit (Catalog Nos. WB7103, WB7105, WB7107), and NuPAGE transfer buffer (20x) were purchased from Invitrogen (Carlsbad, CA). Polyclonal anti-apoB antibody was purchased from Chemicon International (Catalog No. AB742, Temecula, CA). Sucrose was purchased from EM Science (Catalog No. SX1075-1, Gibbstown, NJ).

2. Blood Draw and Serum Collection

Blood from normolipidemic subjects was drawn into a Vacutainer-brand series collection tubes following a 12-h fast (7 mL, sterile interior, with STT gel and clot activator; Beckton Dickinson Vacutainer Systems, Franklin Lakes, NJ). Serum was separated from red blood cells by centrifugation at 3200 rpm for 10 min at 4°C. The supernatant (serum) was aspirated from red blood cells, separated into 200 μL aliquots, and used immediately or stored at -86°C until used.

3. Determination of Lp(a) Concentration

Lp(a) concentration in serum and Lp(a) concentration in recovered Lp(a) samples were determined using DiaSorin's Lp(a) kit (DiaSorin) adapted for analysis in a micro plate reader [115]. This assay for Lp(a) is designed for the quantitative determination of Lp(a) levels in human plasma by immunoprecipitin analysis. For this assay, 5 μL of serum sample or recovered Lp(a) was placed in a well of a micro plate and mixed with 300 μL of DiaSorin's Lp(a) diluent solution. Background absorbance of the diluted samples was read at 340 nm (absorbance A). A 50- μL volume of DiaSorin's Lp(a) antibody reagent, diluted 2:3 in phosphate buffer, was added to each plate well containing diluted sample and mixed thoroughly. Samples were incubated for 10 minutes at 37°C and individual absorbances at 340 nm read (absorbance B). A calibration curve was obtained following the same procedure but using DiaSorin's Lp(a) calibration set in lieu of serum and subtracting the background (absorbance A) from the immunoprecipitated samples (absorbance B). Lp(a) concentration was calculated according to the linear regression obtained from the Lp(a) calibration curve.

All absorbance measurements were made in a μQuant spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT) and 96 well microtitre plates were used.

4. Lp(a) Isolation from Serum by Lectin Affinity

Lp(a) was removed from serum by carbohydrate affinity according to the method developed by Seman et al. with some modifications [87], [115]. Briefly, 50 μL of serum, 100 μL of PBS-200 mM proline (PBS-p), and 50 μL of agarose-WGA (equal to 0.336 mg WGA/mL gel packaged) were mixed together and vortexed briefly. The homogeneous mixture was incubated for 30 minutes at room temperature under continued mixing in a M-60 orbital shaker (Labnet International Inc., Edison, NJ). After incubation, the WGA-Lp(a) complex was sedimented by a slow centrifugation (5 min at 6000 rpm).

A control sample (Lp(a) serum) was prepared by following the Lp(a) extraction protocol (above) in the absence of WGA.

After Lp(a) was extracted from serum, the remaining serum (Lp(a)-depleted serum) was separated from the WGA-Lp(a) complex for further analysis. Lp(a) and Lp(a)-depleted serum samples were ultrafiltrated to achieve similar sample composition. Briefly, the samples were transferred into the top chamber of a 10 000 molecular weight cutoff filter (Microcon YM-10, Millipore, Bedford, MA), and ultrafiltrated two times for 8 minutes at 10,000 rpm, replacing the volume displaced with density gradient solution. Samples in the top chamber (Lp(a) and Lp(a)-depleted serum) were recovered by inverting the filter into a new reservoir and centrifuging for 10 minutes at 10,000 rpm.

5. Separation of Lipoprotein Particles by Single Spin Ultracentrifugation

A. NaBiEDTA Density Gradient

Lipoproteins were separated according to their hydrated density as described previously [105, 106]. Lp(a) and Lp(a)-depleted serum samples were individually mixed

with 10 μL of 2 mg/mL NBD and enough NaBiEDTA density gradient solution to obtain a final sample volume of 1200 μL . The samples were allowed to stand for 30 minutes at 5°C in order to equilibrate NBD C₆-ceramide uptake by lipoproteins. A 1000- μL volume of each sample was transferred to an ultracentrifuge tube (1.5-mL, thick-walled, polycarbonate, Beckman-Coulter, Palo Alto, CA). Ultracentrifugation was carried out in a Beckman Optima TLX tabletop ultracentrifuge equipped with a 30° fixed angle TLA 129.2 rotor. A NaBiEDTA density gradient was formed by ultracentrifugation for 6 hours, at 120,000 rpm and 5°C [105, 106].

B. Sucrose Density Gradient

Lipoprotein separation in a sucrose gradient was employed for preparative purposes. A serum sample or recovered Lp(a) sample was stained with 10 μL NBD (2mg/mL) and let stand for 30 minutes at room temperature in order to equilibrate NBD uptake by lipoproteins. After staining, the sample was diluted with deionized water to a total volume of 400 μL , and layered above 800 μL of a 20 (w/v) of a sucrose solution. The lipoprotein fractions were separated according to their density by a 4-h ultracentrifuge spin at 120,000 rpm and 20° with an acceleration and deceleration step of 5 minutes each [2].

C. Digital Analysis

After ultracentrifugation, the tubes were carefully layered with 150 μL of deionized water. Tubes were imaged and analyzed as reported previously [105]. A digital Optronics Microfire Camera (Goleta, CA), and a Dolan-Jenner (Lawrence, MA) MH-100 metal halide continuous light source were used for imaging. Light from the source was filtered orthogonally using Schott Glass (Elmsford, NY) filters: BG12 for NBD excitation (466 nm) and OG515 for emission (536 nm). For analysis, the images

were converted to an 1800 x 1200 matrix using Origin 7.0 (Microcal Software Inc., Northampton, MA). The intensity values of the 10 center columns of the tube were averaged, and plotted against a tube coordinate scale and density gradient.

6. Differential Density Lipoprotein Profiling

Differential density lipoprotein profiling (DDLp) was used to determine the mean density of Lp(a) in a serum sample [115]. Lp(a)'s mean density was obtained after differentially comparing the Lp(a) serum profile and the Lp(a)-depleted serum profile. Briefly, DDLp samples consisted of a serum sample treated with WGA (Lp(a)-depleted serum) as described in this Experimental Section and a second serum sample that was not treated with WGA (control sample). The samples were prepared in duplicate. Both samples were stained with NBD, mixed with a solution of 10% NaBiEDTA and ultracentrifuged for 6 hours at 5°C and 120,000 rpm. After centrifugation, tubes were layered with 150 μ L of distilled water and imaged according to the specifications mentioned previously. To obtain a Lp(a) DDLp, the mean Lp(a)-depleted serum profile was subtracted from the mean Lp(a) serum profile and the difference graphed as a differential density Lp(a) profile (DDLp).

7. Lp(a) Recovery from Lp(a)-WGA Complex

Lp(a) was recovered from the WGA-Lp(a) complex by incubation of the complex in PBS-200 mM *N*-acetyl-D-glucosamine (PBS-g), following the protocol established by Seman et al. with some modifications [87, 115]. For this purpose, a 150 μ L aliquot of serum was mixed with 1.00 mg WGA/mL gel packaged (150 μ L Sigma WGA agarose-bound lectin) and 100 μ L PBS-p. The sample was incubated at room temperature for 30 minutes under constant mixing in a M-60 orbital shaker (Labnet International

Inc., Edison, NJ). After incubation, the sample was centrifuged for 5 minutes at 6,000 rpm to sediment the WGA-Lp(a) complex formed. The supernatant (Lp(a)-depleted serum) was removed from the sample and the precipitated WGA-Lp(a) complexed transferred to a 45 μm filter (Ultrafree-MC 0.45 μm centrifugal filter units, Millipore, Bedford, MA).

Transfer of the WGA-Lp(a) complex to the top chamber of the filter was possible by adding 150 μL of PBS-p to the tube containing the sedimented WGA-Lp(a) complex. Then, the sample was well mixed and transferred to the top chamber of a 45- μm filter. The sample tube was rinsed with 150 μL of PBS-p to completely transfer all WGA-Lp(a) complex left after the first transfer. The sample was rinsed twice for 90 seconds at 6000 rpm with 150 μL PBS-p to drain any unbound lipoproteins or serum proteins from the complex. Then, 300 μL of PBS-g were added to the top chamber, and the filter contents were briefly mixed by vortexing. The homogeneous samples were incubated for 30 minutes at room temperature and continued mixing using a M-60 orbital shaker (Labnet International Inc., Edison, NJ). After incubation, the sample was spun for 90 seconds at 6000 rpm, and the filtered solution containing recovered Lp(a) in PBS-g collected. PBS-g was removed from the sample by ultrafiltration for 3 minutes in a 100,000 molecular weight cutoff filter (Microcon YM-10, Millipore, Bedford, MA) at 6,000 rpm until the original volume was replaced 2.5 times by the density gradient solution (NaBiEDTA or sucrose).

A. Recovered Lp(a) Ultracentrifugation

Recovered Lp(a) in the density gradient solution was mixed with 10 μL NBD (2 mg/mL) and let stand at room temperature for 30 minutes for sucrose gradient or at 5°C for NaBiEDTA gradient. Enough volume of density gradient medium was added to obtain a total final sample volume of 1200 μL . Ultracentrifugation and

imaging of these samples were performed following the specifications described in the ultracentrifugation section (above).

8. Lipoprotein Fraction Collection after Ultracentrifugation

After ultracentrifugation and imaging, the tubes containing Lp(a) were slowly frozen in liquid nitrogen, without disturbing of the gradient [116]. Lipoprotein fractions were collected by cutting the tube at the appropriate positions with a high speed thin blade (0.254 mm wide) scroll saw (Model 1672, 16-inches, 2-speed, Dremel, Racine, WI). Cut positions were determined from the profile obtained from the digital imaging and analysis.

9. Lp(a) Characterization by Capillary Electrophoresis

Lp(a) fractions were collected after removing Lp(a) from serum by WGA, recovering Lp(a) from the WGA-Lp(a) complex, and purifying Lp(a) by ultracentrifugation. Two Lp(a) fractions collected in this way were mixed together to increase Lp(a) concentration in the sample. The sample volume was adjusted to 400 μL with deionized water and transferred to the top chamber of a 100 000 molecular weight cutoff filter (Millipore).

Lp(a) fractions were prepared for capillary electrophoresis (CE) by removing the density gradient medium by ultrafiltration. The volume displaced by filtration was replaced with 7 mM sodium borate buffer. The samples were ultrafiltrated 5 times for 3 minutes at 6000 rpm or until the initial sample volume was displaced 3 times by the sodium borate buffer.

Capillary electrophoresis experiments were performed using a Beckman P/ACE Model 5510 instrument, equipped with a photodiode array detector. The capillary

unit was prepared using a Beckman capillary cartridge assembly (Beckman Instruments, Fullerton, CA) and untreated fused silica capillaries with inner diameter (i.d.) of 75 μm and an outer diameter (o.d.) of 365 μm (Polymicro Technologies, Phoenix, AZ), following Beckman instructions. Migration times and corrected peak areas were automatically obtained using the data analysis feature of the Beckman P/ACE Station Migration software. NaOH was used for capillary conditioning. The background electrolyte consisted of 13 mM sodium borate (Fisher Scientific), 3.5 mM SDS (70% SDS (C_{12} -chain), 25% sodium myristil sulfate (C_{14} -chain), and 5% sodium cetyl sulfate (C_{16} -chain), Sigma Chemical Co., St. Louis MO), and 20% (v/v) acetonitrile (99.8% purity, EM Industries, Inc., Gibbstown, NJ), pH 9.25 [117]. A 0.001% dimethyl sulfoxide (DMSO) solution was used as electrophoretic marker (EOF). The time program used for these analyses is presented in Table 6. Absorbance at 214 nm was obtained for the analysis of the proteins separation by capillary electrophoresis.

Electropherogram data generated by the Beckman P/ACE Station Migration software was exported as an ASCII file and imported into Origin 7.0 software. The electropherogram was reproduced using Origin 7.0 graphing, baseline and peak tools and converted into a Joint Photographic Experts Group (.jpeg) file.

10. Lp(a) Characterization by Transmission Electron Microscopy

For Lp(a) characterization by transmission electron microscopy (TEM), Lp(a) was removed from serum sample by WGA. Lp(a) was recovered from the WGA-Lp(a) complex and ultracentrifuged in a 10% NaBiEDTA density gradient as specified in the Experimental Section. The UC Lp(a) fraction was collected and submitted for analysis to the Texas A&M Microscopy and Imaging Center.

Lp(a) was prepared using a modification of the standard droplet technique as de-

Table 6. Capillary electrophoresis time program

Step	Time	Function	Duration
1		Buffer Rinse	2.00 min
2		Inject EOF	1.00 sec
3		Inject Sample	4.00 sec
4		Inject Buffer	2.00 sec
5	0.00	Separate-Voltage 17.5 KV	20.00 min
6	0.00	Auto Zero	
7	20.00	Stop Data	
8	20.00	Rinse-0.1N NaOH	1.00 min
9	21.00	Rinse-H ₂ O	1.00 min
10	22.00	End	

scribed by Harris and Agutter [118, 119]. Three microliters of Lp(a) sample were directly applied to a freshly glow-discharged carbon-coated copper grid (G400), washed with distilled water, and negatively stained with an aqueous solution of uranyl acetate (1% w/v, pH 4.25). Images were recorded at a calibrated magnification of 40, 800x in a JEOL 1200 EX transmission electron microscope operated at an acceleration voltage of 100 kV. The instrument specifications were as follows: 0.45 nm-resolution, double condenser projection lens, 60-120 kV, bright/dark field imaging, electron diffraction, eucentric goniometer (+/- 60). Data was collected using a Gatan 673 video camera.

11. Apo(a) Gel Electrophoresis and Immunoblotting

Samples were prepared for electrophoresis by mixing 15 μL of sample with 3 μL of sample buffer and 2 μL of 10% 2-mercaptoethanol [115]. Samples were reduced by incubation for 7 minutes in boiling water. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on a 3-8% Tris-acetate gradient slab minigel using a XCell SureLock Mini-Cell (Invitrogen, Carlsbad, CA). The gel was rinsed and positioned in the cell and the inner chamber filled with running buffer. Fifteen μL of reduced sample were loaded to each well in a gel. The outer chamber was then filled with running buffer and the electrophoresis cell locked. Electrophoresis took place for 1.25 hours at 150 V constant and 3 W. The power supply was EPS 3500 XL (Catalogue number 19-3500-01, Pharmacia Biotech, Piscataway, NJ). After electrophoresis, the gel was stained with BioSafe Coomassie Blue (BioRad, Hercules, CA).

A. Western Blot

When necessary, samples were electroblotted onto nitrocellulose membranes using the XCell II blot module (Invitrogen, Carlsbad, CA). The instructions provided by the manufacturer were followed for electroblotting. Electroblotting took place for 1.5 hours at 30 V constant.

B. WesternBreeze Chromogenic Immunodetection Protocol

Blotted proteins were revealed using Invitrogen's WesternBreeze anti-goat Chromogenic Western Blot immunodetection Kit and goat anti-apo(a) (DiaSorin, Stillwater, MN) or goat polyclonal anti-apoB (Chemicon Int., Temecula, CA) as primary antibody. Invitrogen's Chromogenic Western Blot kit consisted of a blocker/diluent (Part A) which is concentrated buffered saline solution containing detergent; Part

B is blocker/diluent with concentrated Hammersten casein solution; a concentrated buffer saline solution with detergent as antibody wash and a ready-to-use solution of BCIP/NBT substrate for alkaline phosphatase as the chromogenic substrate. The secondary antibody was a ready-to-use solution of alkaline phosphatase conjugated, affinity purified anti-rabbit IgG. The solutions were prepared following Invitrogen's indications. Briefly, a blocking solution was prepared by mixing 4 mL of blocker/diluent (Part A) and 2 mL of blocker/diluent (Part B) with 14 mL of deionized water. The primary antibody was prepared by a 1:1000 dilution with deionized water. Ten ml of the antibody wash solution was diluted with 150 mL of deionized water.

Invitrogen's instructions for immunodetection were followed [120]. In summary, the blotted membrane was placed in the plastic dish provided in the kit and it was rinsed twice with deionized water for 5 minutes. The water was decanted after each rinse. Ten mL of blocking solution were added to the membrane and incubated for 30 minutes on a rotary shaker set at 1 revolution/second. The blocking solution was decanted and the membrane rinsed twice with water for 5 minutes. Then, the membrane was incubated with 10 mL of the primary antibody solution for 1 hour and rinsed three times for 5 minutes with 20 mL of antibody wash solution. The wash solution was decanted and 10 mL of secondary antibody added to the membrane. This solution was incubated with the membrane for 30 minutes after which the membrane was washed three times with 10 ml of antibody wash solution. The next step was to rinse the membrane twice for two minutes with 20 mL of deionized water. Protein bands were developed by incubating the membrane in 5 mL of chromogenic substrate for a variable amount of time ranging from 10 to 60 minutes. Incubation was stopped when purple bands were visible. The membrane was rinsed twice with 20 mL of water for two minutes. Finally, the membrane was placed on a filter paper and let dry to open air.

C. Imaging of Gels and Membranes

Stained gels and revealed membranes were digitalized by scanning, and stored as uncompressed Tagged Image File Format (TIF) files. When necessary, gels were analyzed using Kodak Digital Science Image Analysis software (part of the Kodak EDAS 120 system) and Origin 7.0 software (Microcal Software Inc., Northampton, MA).

12. Reduced Lp(a) Characterization by Capillary Electrophoresis

Lp(a) samples obtained after ultracentrifugation were placed in the top chamber of a 100 000 molecular weight cutoff filter (Millipore) and sucrose density gradient removed by ultrafiltration. Seven ultrafiltration cycles were carried out for 3 minutes each at 6000 rpm, replacing the volume filtered with CE background buffer.

Lp(a) samples were reduced with phosphines. An Lp(a) sample was mixed with 0.8 μ mole per mL (100 μ L) of Tris(2-carboxyethyl)phosphine hydrochloride, (TCEP) disulfide reducing gel (effective TCEP concentration of at least 8 mM, Catalog no. 77712, Pierce Biotechnology Inc., Rockford, IL). The sample was incubated under constant shaking for 7 minutes at 85°C and 10 minutes at room temperature. Reduced Lp(a) sample was recovered by filtration in a 45 μ m filter (Ultrafree-MC 0.45 μ m centrifugal filter units, Millipore, Bedford, MA) for 45 seconds, at 7000 rpm. Agarose-bounded TCEP was left on the filter top, and reduced Lp(a) solution is recovered in the filtrate.

Capillary electrophoresis experiments were performed using a Beckman P/ACE Model 5510 instrument equipped with a photodiode array detector. The capillary unit was prepared using a Beckman capillary cartridge assembly (Beckman Instruments, Fullerton, CA) and untreated fused silica capillaries with inner diameter (i.d.)

of 75 μm and an outer diameter (o.d.) of 365 μm (Polymicro Technologies, Phoenix, AZ), following Beckman instructions. Migration times and corrected peak areas were automatically obtained using the data analysis feature of the Beckman P/ACE Station Migration software. NaOH was used for capillary conditioning. The background electrolyte consisted of 13 mM sodium borate (Fisher Scientific), 3.5 mM SDS (70% purity, Sigma Chemical Co., St. Louis MO), and 20% (v/v) acetonitrile (99.8% purity, EM Industries, Inc., Gibbstown, NJ), pH 9.25 [117]. A 0.001% dimethyl sulfoxide (DMSO) solution was used as electrophoretic marker (EOF). Absorbance at 214 nm was obtained for the analysis of the proteins separation by capillary electrophoresis.

Electropherogram data generated by the Beckman P/ACE Station Migration software was exported as an ASCII file and imported into Origin 7.0 software. The electropherogram was reproduced using Origin 7.0 graphing, baseline and peak tools and converted into a Joint Photographic Experts Group (.jpeg) file.

A time program for the separation of reduced Lp(a) by electrophoresis can be found in Table 6.

CHAPTER III

RESULTS AND DISCUSSION

1. Determination of Lp(a) Concentration by Immunoassay

The most common method for the determination of a protein concentration is by immunoassays. Antibodies that are specific to many proteins have been developed and widely used for the calculation of the concentration of such proteins in a sample. The region of the protein (antigen) that binds to the antibody constitutes an area and number of residues similar to those in the antibody [84]. The binding of the protein to the antibody depends on factors such as thermodynamics and spatial orientation of both the protein and antibody.

The antibody specificity for a protein is compromised when multiple isoforms of that protein are present in the sample. Such is the case of Lp(a). Up to 36 different isoforms have been characterized for Lp(a). This protein heterogeneity sets up a challenging task for development of an antibody that is isoform independent, and that is suitable for Lp(a) quantitation [27]. An evaluation of several commercially available kits for Lp(a) quantitation concluded that DiaSorin's SPQ Lp(a) kit demonstrated to be the most reliable assay and the least isoform dependent [121]. For this reason, we adopted DiaSorin's assay for the purpose of Lp(a) concentration determinations.

A protocol for the calculation of Lp(a) concentration was developed by adapting a commercially available kit for Lp(a) (DiaSorin SPQ) for analysis in a microplate reader [115]. A linear calibration curve was obtained using the calibrator reagents provided in the kit and Lp(a) concentrations in mg/dL determined from this calibration curve. DiaSorin's diluent reagent was successfully substituted by a phosphate buffer in order to make the assay more compatible with other applications used in

our laboratory, such as capillary electrophoresis. Fig. 12 shows a typical calibration curve obtained for the Lp(a) assay using phosphate buffer as diluent.

The adaptation of the assay involved two main variables: sample volume in the micro plate and incubation time. While optimizing the assay, it was noted that incubation time was crucial for this assay to give reliable results. First, the reagents have to be well mixed in the well of the microplate by pipetting the contents in and out several times. This mixing has to be performed being very careful not to form any bubbles in the well that may interfere with absorbance readings. And second, incubation of the sample with the antibody (anti-Lp(a)) has to be carefully timed. DiaSorin's instructions are to incubate the sample for 5 minutes before reading the second absorbance. Several experiments showed that 5 minutes of incubation at 37°C is not enough to give reproducible results when phosphate buffer is used instead of DiaSorin's diluent reagent. A 10 minutes incubation showed to be the maximum time allowed for the immunocomplex to form resulting in good absorbance readings between samples.

Sample volume was also determinant on getting a good absorbance reading and as consequence, a robust assay. The antibody/antigen ratio was maintained at an optimum, assuring that the immunoprecipitation reaction was the same for all samples while the final volume was changed. It was noted that the absorbance readings by the instrument were increased with increasing sample volume in the well. This increasing trend in absorbance with increasing diluent volume may be explained by changes in immunoprecipitate concentration at different volumes. The particles are dispersed differently in the different sample volumes and therefore, the light is scattered in a different way in each sample. In conclusion, for Lp(a) concentration assays the total sample volume in the microwell plate should be kept constant at 300 μL and the plate incubated for 10 minutes at 37°C. Absorbance readings should be taken immediately

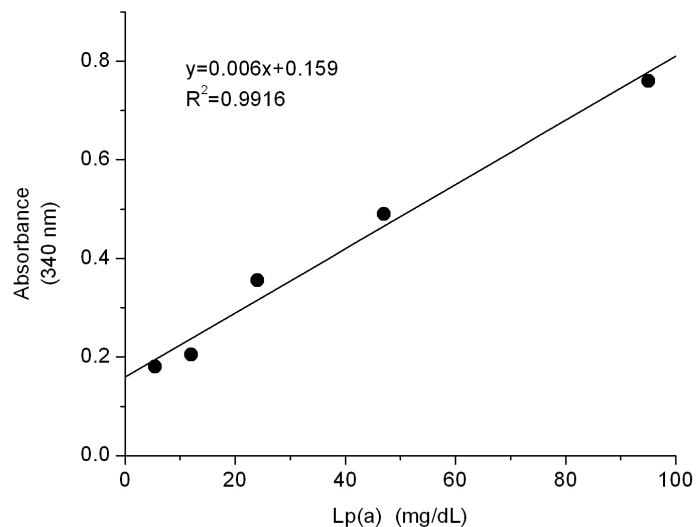


Fig. 12. DiaSorin Lp(a) immunoprecipitation calibration curve

after taking the plate out from the incubation chamber.

2. Determination of Lp(a) Concentration by Fluorometry

Fluorometry was explored as an alternative technique to determine Lp(a) concentration in serum. This technique was selected because Lp(a) can be isolated from serum by affinity chromatography with WGA and then stained with NBD C₆-ceramide. In this way, NBD fluorescence was used to determine Lp(a) concentration. Fluorometry also has the advantage of being isoform independent, since the fluorescence of NBD is dependent on the lipid core of the lipoprotein and not on the apolipoprotein identity or size.

DiaSorin Lp(a) standards were used to build a fluorometry calibration curve (Fig 13). A linear calibration curve was obtained by setting the spectrofluorometer (SLM Aminco Spectrofluorometer, Model 8100, Series 2, Spex, Jabin Yvon, Edison,

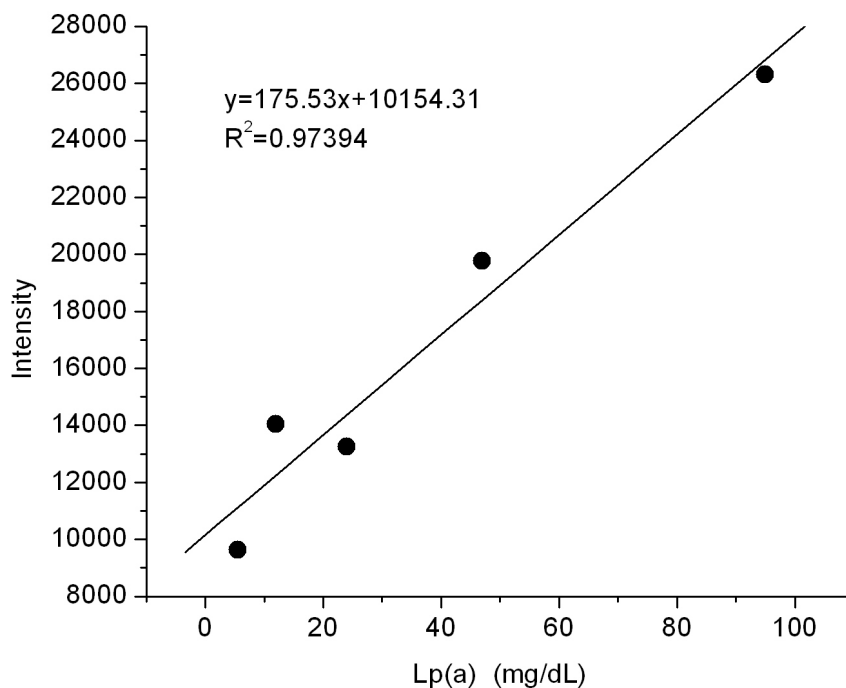


Fig. 13. DiaSorin Lp(a) fluorescent emission calibration curve. Samples were excited at 466 nm, emission intensity was recorded at 536 nm

NJ) with an excitation wavelength at 466 nm, and 426-446 emission scan. Lp(a) was stained with NBD after being isolated from serum with WGA. Lp(a) concentration in those samples was determined by fluorometry using the calibration curve constructed from Lp(a) standards.

This fluorometry approach to determine Lp(a) concentration is very straight forward, takes advantage of lipoprotein staining with NBD, and is isoform independent. Eventually, the results presented here could be linked with peak intensities in a lipoprotein density profile.

3. Lp(a) Isolation from Serum by Immunoaffinity

Lp(a) immunoaffinity was used as a first approach for the isolation of Lp(a) from serum. The antibody used for this purpose had to be isoform independent. For this purpose, DiaSorin's anti-Lp(a) antibody was used. This antibody was reported to be the most insensitive to apo(a) isoform size [27], making it a good candidate for the specific immunoprecipitation of Lp(a) in a serum sample. It is important to note that this antibody was manufactured for the immuno-determination of Lp(a) in serum based on a calibration curve created from serum standards provided in the kit and not for Lp(a) separations.

The principle of this immunoaffinity approach was to incubate a diluted serum sample with the antibody to form an immunoprecipitate. This immunoprecipitate would be used to further characterize Lp(a) after separating it from the antibody. One disadvantage of this approach is the high cost of the antibody. This defeats the purpose of the present research which is the development of low cost, user friendly diagnostic techniques for cardiovascular risk assessment. The size of the immunoprecipitate formed is very small, making it very difficult to handle it for further characterization with other analytical techniques such as capillary electrophoresis and mass spectrometry. It is possible that coupling this antibody to agarose beads may result in a bigger immunoprecipitate. A major disadvantage of using antibodies for the isolation of Lp(a) from serum is that these antibodies require the use of buffers to keep them stable. The presence of buffers in the sample to be ultracentrifuged interferes negatively with the density gradient formation.

The high specificity of antibodies for a determined protein makes Lp(a) immunoaffinity isolations from serum a very promising technique. However, there is the need for the development of apo(a) size-independent antibodies. A similar approach

to immunoaffinity was developed to overcome the multiple disadvantages of the immunoaffinity approach. This new approach is based on carbohydrate affinity and will be discussed in the following section.

4. Lp(a) Isolation from Serum by Carbohydrate Affinity

Lp(a) is highly glycosylated with 30% of apo(a)'s mass being composed of carbohydrates with a high content of sialic acid [15]. Combining the high sialic content of Lp(a) compared to other lipoproteins in serum with the specificity of the wheat germ agglutinin lectin (WGA), for this sugar makes it possible to isolate Lp(a) based on carbohydrate affinity. The large number of WGA binding sites per Lp(a) molecule allows for Lp(a) to compete strongly with other serum glycoproteins that are present in serum in a much greater molar concentrations than Lp(a) [87].

Taking advantage of the high glycosylation in Lp(a) compared to other lipoproteins a protocol was developed to selectively extract Lp(a) from a serum sample [115]. Fig. 14 demonstrates the effectiveness of the methodology developed. Panel A in Fig. 14 is a serum lipoprotein density profile of a sample known to contain high levels of Lp(a) (determined by immunoassay). Panel B in Fig. 14 is a serum lipoprotein density profile of the same sample as in Panel A after being treated with WGA.

Lp(a) was recovered from the WGA-Lp(a) complex and ultracentrifuged under the same conditions as the samples in Panels A and B of Fig. 14. The Lp(a) density profile is portrayed in Fig. 15. In this figure, the Lp(a) sample was profiled along side with the lipoprotein density profile from where Lp(a) was removed (parent density profile), which is the same as in Panel B of Fig. 14. The specificity of WGA for Lp(a) is demonstrated by a complete removal of the Lp(a) peak from the sample treated with WGA (Fig. 14 and 15). Furthermore, Lp(a) recovered from the WGA-Lp(a)

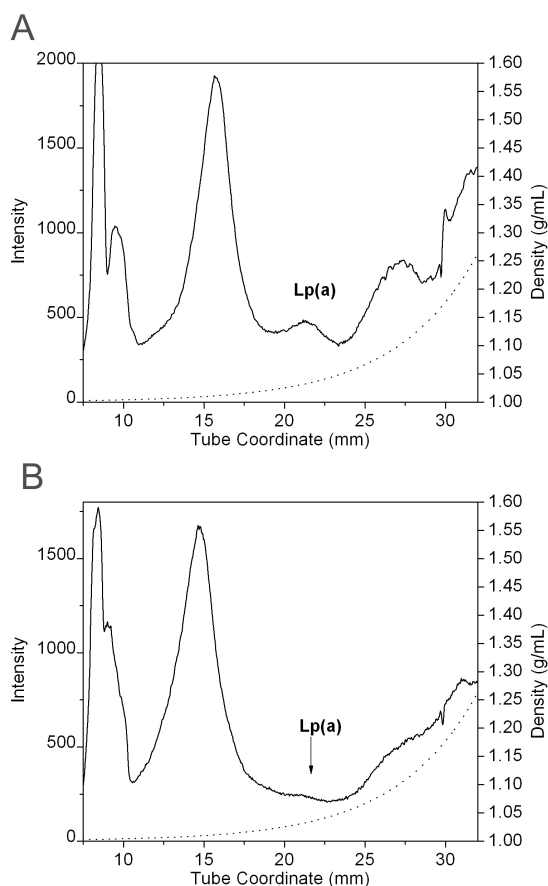


Fig. 14. Lp(a) extraction from serum by carbohydrate affinity. A) Serum lipoprotein density profile; B) Serum lipoprotein density profile after treatment with WGA

complex can be related to its parent lipoprotein density profile by being located at the same position as the original Lp(a) peak (Fig. 14 and 15).

In the research reported here, an analytical method was developed for the extraction of Lp(a) from serum by carbohydrate affinity. This method is an important and novel tool in the characterization of Lp(a). The following section focuses on the discussion of the advantages of using carbohydrate affinity for the study of Lp(a).

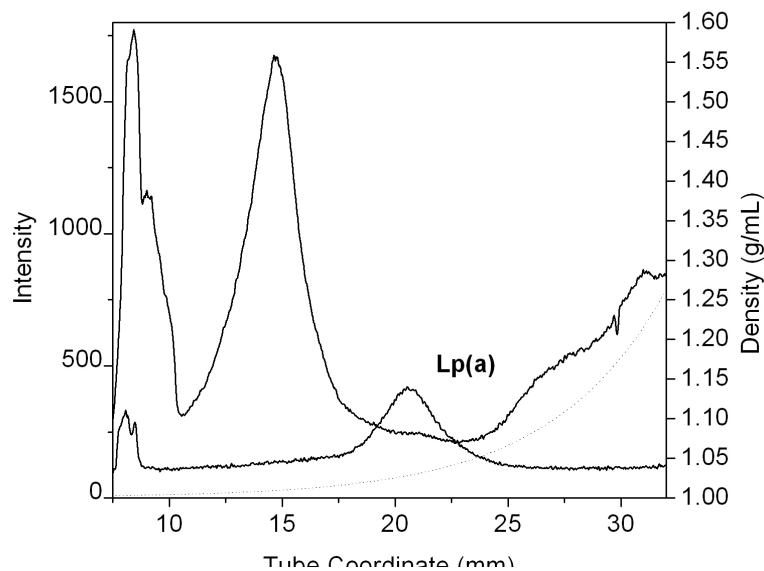


Fig. 15. Lp(a) extraction from serum by carbohydrate affinity. Comparison of a serum lipoprotein density profile from panel B in Fig. 14 with its Lp(a) density profile

5. Lp(a) Density Determination by Differential Density Lipoprotein Profiling (DDLPP)

Differential Density Lipoprotein Profiling (DDLPP) was developed to determine the density of a specific feature in the density profile by differentially comparing a serum profile with a profile from where a component (LDL, HDL, Lp(a), etc) was specifically removed [115]. In a DDLPP the component being studied is removed while all of the other components in the lipoprotein profile are unaltered in density and peak intensity. DDLPP methodology is important because it gives relevant information such as lipoprotein density and isoform characteristics that are significant for the assessment of lipoproteins as markers for cardiovascular disease.

This section elaborates on the results obtained by DDLPP for the study and characterization of Lp(a). There are three steps for DDLPP: Preparation of a control sample (Lp(a) serum), removal of Lp(a) from serum (Lp(a)-depleted serum), and

differential density lipoprotein profiling. These three steps are discussed in detail in this section.

A. Serum Lipoprotein Density Profile (Lp(a) Serum)

Fig. 16A is an example of the mean lipoproteins density profile obtained for an Lp(a) serum (control sample). Serum used in this figure contain elevated Lp(a). Lp(a) levels for this serum were 111 mg/dL according to DiaSorin's Lp(a) kit. The profile in Fig. 16A presents a characteristic lipoprotein density distribution for lipoproteins obtained by ultracentrifugation using 10% NaBiEDTA as density gradient. Each of the peaks in Fig. 16A corresponds to one lipoprotein class: VLDL, LDL, and HDL. The peak in this figure between the LDL-HDL density region was identified as Lp(a). The artifact in the HDL peak is a result of an optical effect due to the ultracentrifuge tube shape and should not be mistaken as an HDL inherent characteristic [115].

The Lp(a) serum profile was run in duplicate and averaged to achieve better statistics and facilitate the comparison between Lp(a) and Lp(a)-depleted serum profiles. Using this protocol to study several Lp(a)-containing samples, it was observed that the Lp(a) mean density varies, sometimes being closer to the LDL density and sometimes being closer to the HDL density. This wide range of Lp(a) densities is attributable in part to the existence of different Lp(a) isoforms within samples [34]. Lp(a) density heterogeneity can also be attributable to metabolic changes such as triglyceride levels in serum and LDL levels [30]. This metabolic Lp(a) density heterogeneity is best observed when the same subject is studied over a long period of time. Lp(a) density results presented herein correspond to the same draw of serum sample and are not significantly affected by LDL serum levels or variations in triglyceride levels.

B. Lp(a)-Depleted Density Profile

Lp(a) was specifically removed from a second aliquot of the same serum sample from where the Lp(a) serum profile was obtained (Fig 16A) [115]. Lp(a) was removed from serum by carbohydrate affinity using WGA, following the procedures described in the Experimental Section. Fig. 16B shows the mean lipoprotein density profile for the Lp(a)-depleted serum sample. Serum samples were diluted in a saline buffer containing proline (PBS-p) to remove any endogenous bound Lp(a) or apo(a) [122]. It has been demonstrated that there is a noncovalent interaction of Lp(a) with triglyceride rich lipoproteins (TRL). The noncovalent forces between Lp(a) and TRL are greatly and preferentially influenced by proline [122]. The dissociation of Lp(a) from TRL is directly dependent on proline concentrations over the range of 1-100 mM. Proline demonstrated selectivity as an Lp(a) dissociative agent, suggesting some degree of steric specificity of binding. This idea is compatible with the model of a specific krigle-binding pocket of apo(a) interacting with a proline moiety of apoB in TRL. Proline also inhibits the binding of apo(a) to apoB-100 *in vitro* [123].

Compared to what is observed in Fig. 16A (Lp(a) serum), in Fig. 16B the peak assigned to Lp(a) is missing from the lipoprotein density profile. All other lipoproteins in serum remained intact with the same density profile after the incubation with WGA, leaving the lipoprotein profile essentially unaffected. Only the Lp(a) density region was affected where the Lp(a) peak is missing in the Lp(a)-depleted lipoprotein density profile.

C. Differential Density Lipoprotein Profile (DDLDP)

Fig. 16C shows an overlay of the lipoprotein profiles from Fig. 16A and B. In this figure, it is evident that only the Lp(a) was removed when the sample was treated

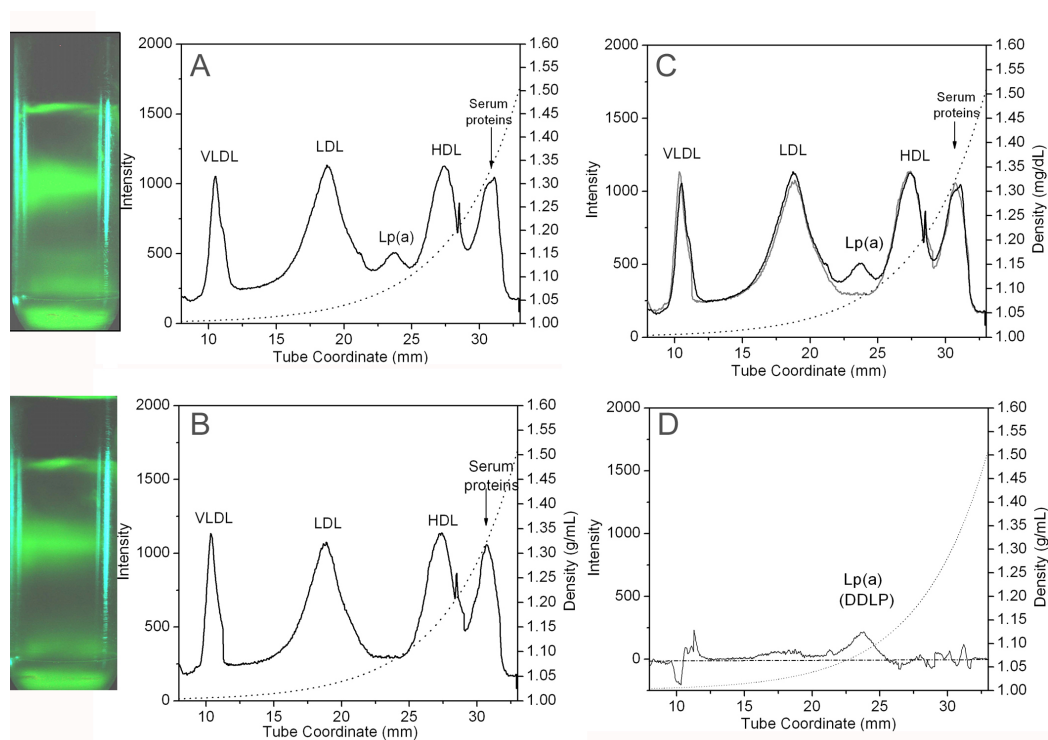


Fig. 16. Differential Density Lipoprotein Profiling (DDL): A) Lipoprotein density profile; B) Lp(a)-depleted density profile; C) Overlay of A and B; D) Lp(a) DDL

with WGA (bottom profile in Fig. 16C). Both density and peak intensity of all other lipoproteins remain unaltered after treatment of serum with WGA [115].

A differential density lipoprotein profile was obtained by subtracting the mean Lp(a)-depleted serum profile (Fig 16B) from the mean Lp(a) serum profile (Fig 16A). These profiles were obtained after staining the same amount of serum (Lp(a) and Lp(a)-depleted) with the same amount of NBD. In both cases, the stain is at saturation level to guarantee that VLDL, LDL, and HDL take up the same amount of NBD independently of the presence or absence of Lp(a). Having NBD at saturation levels makes it possible for an accurate comparison between density profiles.

The DDLP is shown in Fig. 16D. This profile corresponds to the differential density profile for Lp(a). The major peak observed in this profile represents the density region from where Lp(a) was removed. Other artifacts in the profile in Fig. 16D resulted from the slight shift in the density gradient between samples and should not be mistaken as Lp(a) features.

With an Lp(a) DDLP (Fig 16D), and knowing the density gradient formed by NaBiEDTA, the mean density of Lp(a) removed from serum can be calculated. For the sample profiled in Fig. 16 the mean Lp(a) density was 1.086 g/mL.

D. DDLP of Non-Lp(a) Containing Serum

The efficiency of WGA in the removal of Lp(a) was demonstrated by assaying serum samples known not to contain Lp(a). The subjects assayed were normolipidemic subjects with no detectable Lp(a) (measured with DiaSorin's Lp(a) kit). The Lp(a) and Lp(a)-depleted serum lipoprotein profile for these samples are presented in Fig. 17A and B. The VLDL, LDL, and HDL peaks in Fig. 17A remained unchanged after the serum was incubated with WGA. This result confirms the high specificity of WGA for Lp(a) [115].

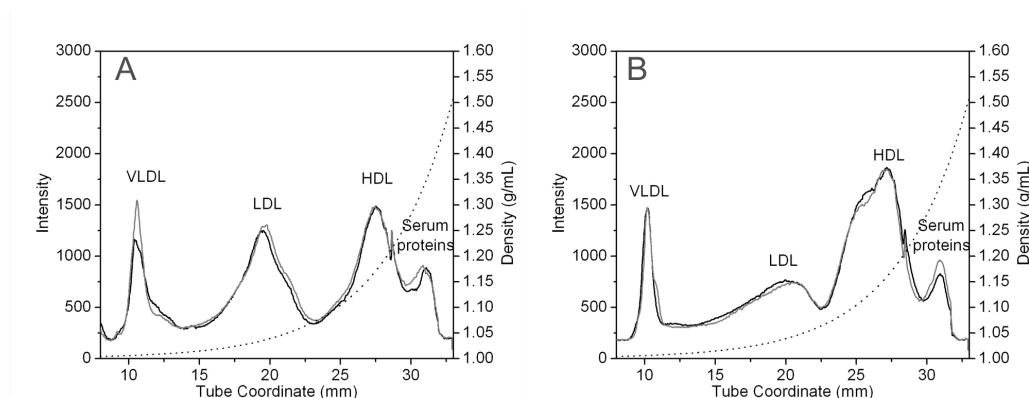


Fig. 17. Differential Density Lipoprotein Profiling on normolipidemic subjects with no detectable Lp(a) (by immunoassay)

A more complex case is presented in Fig. 17B. This sample contains a feature or shoulder in the low density of HDL region, corresponding to HDL₁. It was known from the immunoassay that this sample did not contain any detectable amount of Lp(a). However, when the serum was incubated with WGA, there was a difference detected in the lipoprotein density profile in the HDL density region. Most likely, in the absence of Lp(a), WGA complexes with other glycosylated lipoproteins. In this case, the feature removed from the HDL peak suggests that a glycosylated HDL subclass might be present in this sample (see Fig. 17B). These results are consistent with the suggestion by Seman et al. concerning a competitive binding of WGA to Lp(a) in the presence of other lipoproteins [87]. These findings make it possible for the WGA technology to be implemented in other lipoprotein subclasses for characterization of sialylated lipoprotein and its study as cardiovascular risk markers [115].

6. Lp(a) Recovery and Density Determination

Lp(a) extracted from serum was recovered from the WGA-Lp(a) complex formed by incubation of the sample in PBS-g (PBS-200 mM *N*-acetyl-D-glucosamine). The high concentration of *N*-acetyl-D-glucosamine in this buffer interacts with WGA, releasing

the intact Lp(a) back into solution. Ultrafiltration was used to exchange the PBS buffer with the density gradient solution (NaBiEDTA or sucrose) used for serum lipoproteins separation. As mentioned earlier, removing the PBS buffer and having the Lp(a) sample diluted in a density gradient solution, the mean density obtained for the sample is not affected by the introduction of a third component to the density gradient. The density gradient formed is then similar to the density gradient used for the serum lipoprotein separation. The top profile in Fig. 18 shows a typical lipoprotein density profile for a sample containing Lp(a) [115].

The density of recovered Lp(a) is correlated with Lp(a)'s inherent density when both density gradients are identical [115]. Fig. 18 demonstrates the feasibility of this comparison. An aliquot of the sample in the top profile was treated with WGA following the procedures explained in the Experimental Section. Lp(a) was recovered from the WGA-Lp(a) complex, mixed with 10% NaBiEDTA, and stained with NBD. A density gradient was generated by ultracentrifugation for 6 hours at 120,000 rpm at 5°C.

The lipoprotein density profile for this sample is represented by the bottom profile in Fig. 18. A single peak at the reported density range for Lp(a) was obtained [28]. More importantly, both Lp(a) peaks (lipoprotein density profile on the top and recovered Lp(a) density profile on the bottom, Fig. 18) are at the same density. This shows how Lp(a) is recovered intact from serum after treatment with WGA. Lp(a) isolated and recovered by the method presented herein retained its inherent density and immunoreactive properties. This result confirms the fact that Lp(a) is effectively removed from serum by WGA and that its density is accurately calculated from the Lp(a) density profile.

The Lp(a) mean density for this particular sample is 1.086 g/mL (Fig 18). This density is the same as the density obtained for the same sample by DDLP (Fig 16D).

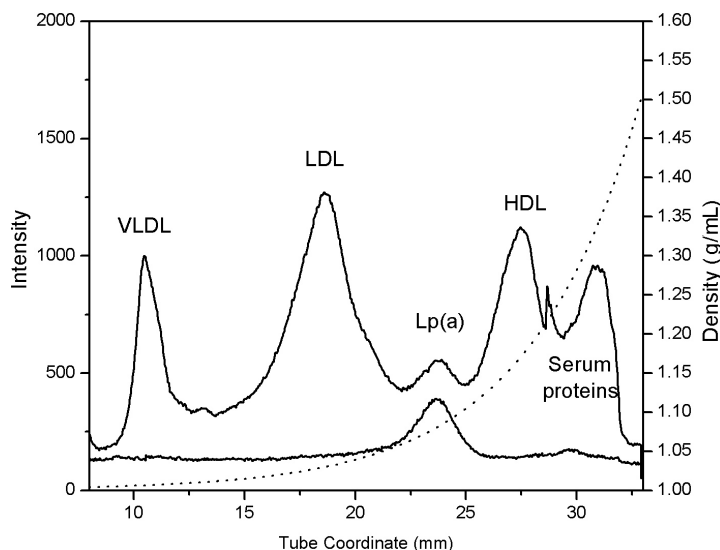


Fig. 18. Lp(a) density profile in 10% NaBiEDTA. Lp(a) recovered from serum after being extracted by WGA (bottom profile)

The profile presented in Fig. 19 was obtained by taking a picture of the same sample from the bottom profile in Fig. 18. In order to get a more detailed profile of the sample, the exposure time while taking the picture of the tube was increased by a factor of 3, from 15 ms to 50 ms. As a result, the Lp(a) peak intensity was increased and more details in the profile are detected. One particular detail of the profile observed when the exposure time of the imaging camera was increased is the presence of other proteins at high density. These proteins presumably represent glycosylated serum proteins that were extracted by WGA along with Lp(a), but were successfully separated from Lp(a) by ultracentrifugation.

The presence of Lp(a) in the recovered fraction was confirmed by Western blot. An aliquot of serum known to contain Lp(a) was spun in the ultracentrifuge and lipoprotein fractions were collected by the freeze and cut method described in the Experimental Section. Fig. 20 shows the tube positions from where VLDL, LDL, Lp(a), HDL, and serum proteins fractions were collected. These fractions were ana-

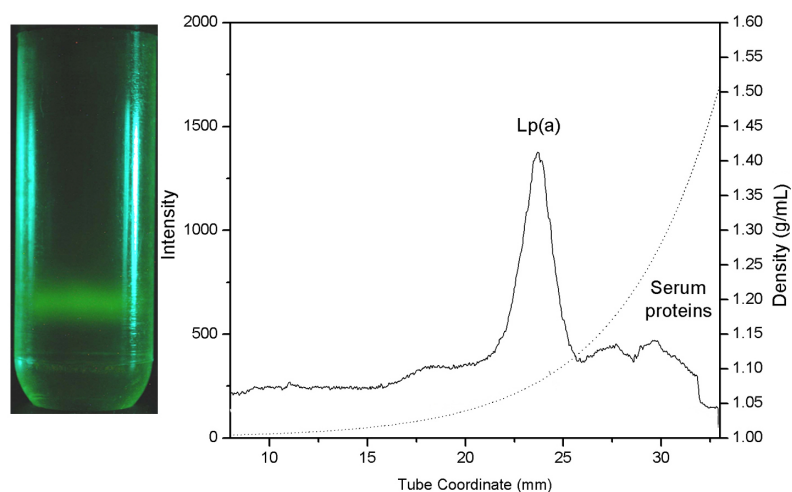


Fig. 19. Lp(a) density profile in 10% NaBiEDTA. Detailed Lp(a) density profile obtained by increasing the imaging camera exposure time by a factor of 3, from 15 ms to 50 ms

lyzed by SDS-PAGE immediately followed by Western blot. All proteins transferred to the nitrocellulose membrane were assayed against anti-Lp(a) following the protocol elaborated in the Experimental Section.

Western blot results are presented in Fig. 20 only for those fraction testing positive for Lp(a). As it can be seen in this figure, after assaying all lipoprotein fractions, Lp(a) was mainly found in the Lp(a) density region and a small amount of it in the serum proteins region. It has previously been reported that Lp(a) exists in serum as free apo(a) in less than 3% of total apo(a). Evidence of the presence of non-covalently linked apo(a) to VLDL has also been reported [25], in which case apo(a) may have been detached from VLDL in the ultracentrifugation step, and separated into the serum protein region. The amount of Lp(a) found in this high density region is minimum compared to the amount of apo(a) present in the Lp(a) density region. It is for this reason that free apo(a) in the serum protein region was considered insignificant for the purposes of this research.

The presence of Lp(a) in the main peak of a recovered Lp(a) density profile

was also confirmed by Western blot. For this purpose, Lp(a) was extracted from serum by WGA and recovered from the WGA-Lp(a) complex after incubation with PBS-g. Lp(a) obtained in this way was stained and ultracentrifuged as explained previously. After ultracentrifugation the tube was imaged and slowly frozen. An Lp(a) fraction was collected by cutting the tube at the density region corresponding to the main peak. Fig. 21 shows the profile obtained where Lp(a) is the main feature. This fraction was analyzed by SDS-PAGE and immunoblotted against anti-Lp(a). Western blot results are shown also in Fig. 21, confirming the presence of pure Lp(a) in this fraction.

Analyzing Lp(a) by Western blot also demonstrated that Lp(a) is recovered intact after extraction with WGA, retaining its inherent properties. Lp(a) was only found at the reported density for Lp(a) (Fig 20 and Fig 21), meaning that the integrity of Lp(a) was not disturbed by the multiple steps required for the extraction/recovery of Lp(a) from serum. More importantly it also demonstrates that Lp(a) obtained by this method preserves its immunoreactivity, confirming once more that the integrity of Lp(a) has not been altered by the experiment.

A. Importance of the Integrity of Density Gradient Solution of Lp(a) Differential Density Analysis

The density gradient formed by different solutes is very sensitive to mixture composition. Ideally a density gradient forming solution should only contain the density gradient forming solute and sample. However, the sample may contain other components resulting from sample preparation steps. These components, when present in the density gradient solution, would slightly change the density gradient formed. This effect was minimized in the DDLP protocol by keeping the composition of both samples (Lp(a) and Lp(a)-depleted) serum almost identical and replacing the PBS

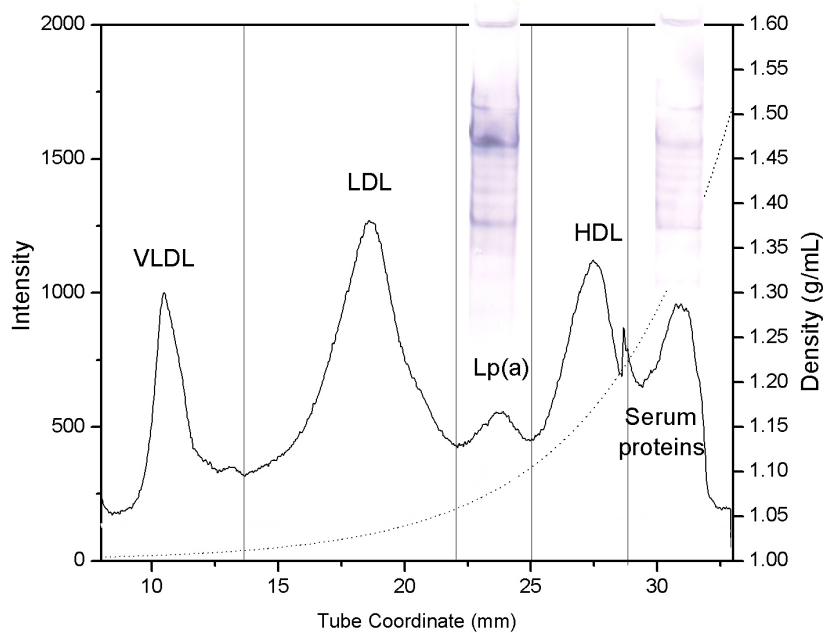


Fig. 20. Identification of density fractions containing Lp(a). Western blot results are shown for those fractions that were positive for Lp(a)

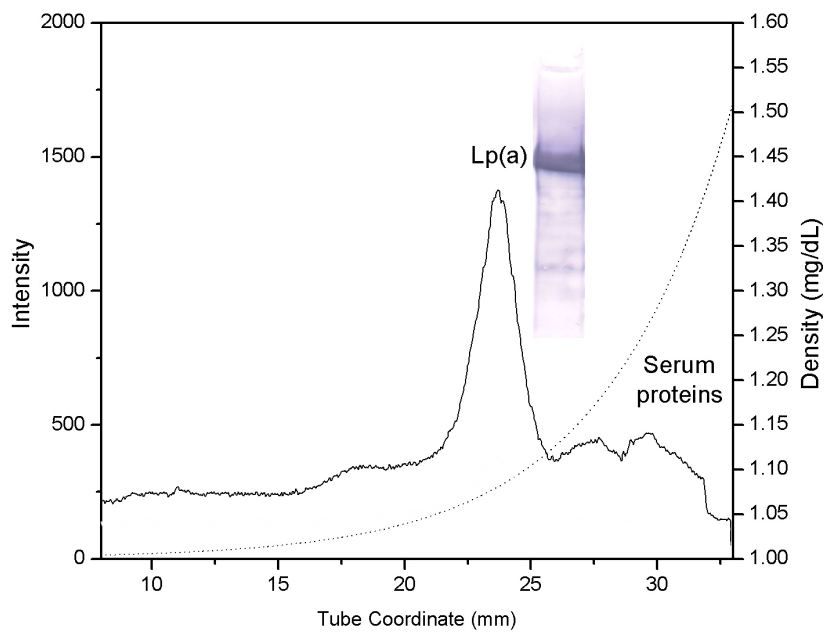


Fig. 21. Recovered Lp(a) density profile. Presence of Lp(a) was confirmed by Western blot

buffer in the sample with density gradient solution prior to ultracentrifugation.

Fig. 22 demonstrates the effect that a third component has in the density profile. Here, the same amount of serum was diluted with three different solutions: a)water, b)PBS-p, and c)PBS-g. Lipoproteins peak position in each sample are described in Table 7.

Table 7. Comparison of lipoproteins peak position when the density gradient is affected by a third component in the mixture (From Fig 22)

Lipoprotein	H ₂ O	PBS-p	PBS-g
LDL (mm)	17.5	14.5	15.1
Lp(a) (mm)	24.0	21.5	22.5
HDL (mm)	27.2	26.8	27

Density gradient formation has been described elsewhere [105, 106]. For the purpose of this research a normal density gradient is that formed when the sample is diluted with a small amount of water (Fig 22A). After comparing the lipoprotein profiles obtained for sample diluted in water, PBS-p and PBS-g the most drastic disturbance in the density gradient was observed when the sample was mixed with PBS-g prior to ultracentrifugation (Fig 22C). In this case, the lipoprotein density distribution is shifted considerably to a higher density. This shift does not by itself mean that the density of the lipoproteins is higher in PBS-g. It only means that the density gradient formed in the presence of a third component is different than when it is not present.

This effect turns out to be relevant when the density of a recovered Lp(a) sample is to be compared with that of Lp(a) in serum. After being recovered from the WGA-Lp(a) complex, Lp(a) is diluted in a PBS-g solution. This solution has to be removed from the sample in order to achieve accurate density measurements from the recovered

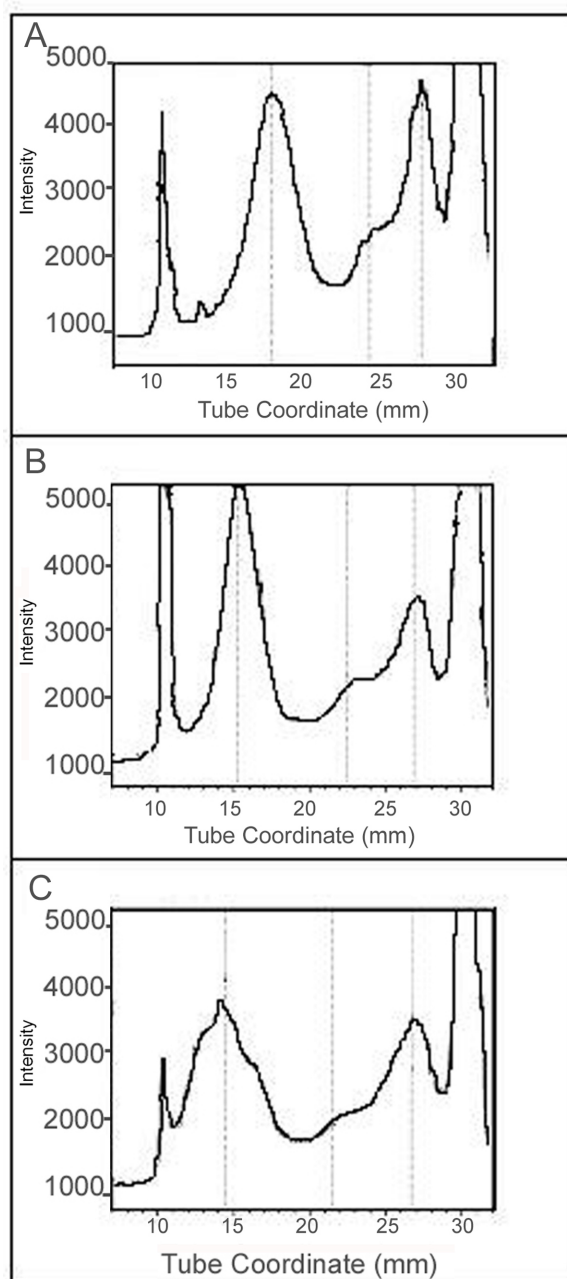


Fig. 22. Third component influence in density gradient formation. NaBiEDTA density gradient formed with serum sample diluted in: A) water; B) PBS-p; C) PBS-g

Lp(a) density profile. Dialysis or ultrafiltration can be used for this purpose. Only after removing the third component from the sample mixture, densities of different samples and lipoproteins can be accurately compared.

7. Lp(a) Purification by Ultracentrifugation

Ultracentrifugation of the recovered Lp(a) sample served a dual purpose. First, it is an efficient way to determine Lp(a) density and is a novel way to determine the presence of Lp(a) isoforms masked under other lipoprotein peaks in the serum lipoprotein profiles [115].

Second, Lp(a) is further purified by ultracentrifugation. This result was demonstrated in the Coomassie Blue-stained gel in Fig. 23. High molecular weight markers (phosphorylase *b*, cross-linked) were used to determine the approximated molecular weight of the proteins in the SDS-PAGE gel (lane 1, Fig 23). Lane 2 in this figure contains all proteins extracted from serum by WGA. Apo(a) is present at an approximate molecular weight of 330,000 Da.

Other serum proteins that contain sialic acid and could be present in this non-purified Lp(a) sample were identified by their molecular weight as: α -2-macroglobulin (170 kDa), haptoglobin (approximately 40 kDa), ceruloplasmin (130 kDa), hemopexin (51 kDa) and α -2-acid glycoprotein (55-81 kDa). Any of these proteins are expected to be present when Lp(a) is extracted from a serum sample by WGA. With the SDS-gel gradient used for these experiment (3-8 % SDS-PAGE), and the molecular weight markers used (from 97 kDa to 400 kDa) only 2-Macroglobulin and ceruloplasmin are expected to be detected with a migration of more than 34 mm down the gel.

Lane 3 in Fig. 23 consists of Lp(a) extracted from serum by WGA and separated from other serum proteins by ultracentrifugation. The Lp(a) fraction purified by this

ultracentrifugation method recovered by the freeze-cut technique, resulted in a single band with an approximate molecular weight of 330,000 Da.

8. Study of the Effectivity of Lp(a) Removal from Serum by Carbohydrate Affinity

The effect of the amount of serum on the recovery of Lp(a) using was also studied. For these experiments, the volume of WGA was kept constant while the amount of serum incubated with WGA was varied. Increasing the amount of serum used increased the amount of Lp(a) recovered. This effect was studied by observing the intensity in fluorescence of the recovered Lp(a) samples. For all experiments, 50 μL of WGA (equivalent to 0.336 mg WGA/mL gel) were used to incubate 0, 10, 20 and 50 μL of a Lp(a)-containing serum. Fluorescence at 536 nm of the recovered Lp(a) samples was measured after staining with 10 μL of NBD. The SLM Aminco Spectrofluorometer (Model 8100, Series 2, Spex, Jabin Yvon, Edison, NJ) was used to obtain fluorescence spectra. A calibration curve was generated and is presented in Fig. 24. A linear relationship in fluorescence was observed by increasing the amount of serum treated with a constant amount of WGA. This results confirm the hypothesis that the amount of Lp(a) recovered after WGA extraction is quantitative when the amount of Lp(a) in a sample is compared to its fluorescence intensity.

The effectiveness of WGA in removing Lp(a) was evidenced by this qualitative fluorescence study. When attached to lipoproteins, NBD fluoresces with a maximum excitation of 466 nm. The quantitative properties of the NBD-lipoprotein system were also evident in this experiment. The intensity detected by the fluorometer increased as the amount of serum in the sample was increased (or amount of Lp(a) recovered was increased). This experiment demonstrates the versatility of using fluorescent tags

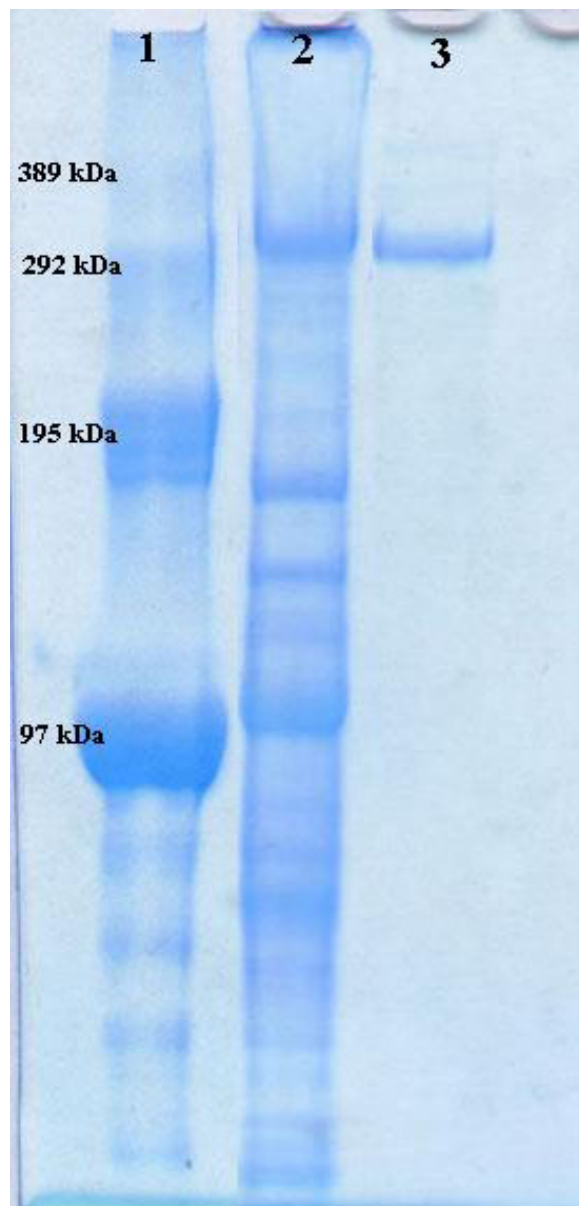


Fig. 23. Coomassie Blue-stained SDS-PAGE gel demonstrating Lp(a) purification by UC. Lane 1: Molecular weight markers; Lane 2: Proteins extracted from serum by WGA; Lane 3: Lp(a) extracted from serum by WGA and purified by UC

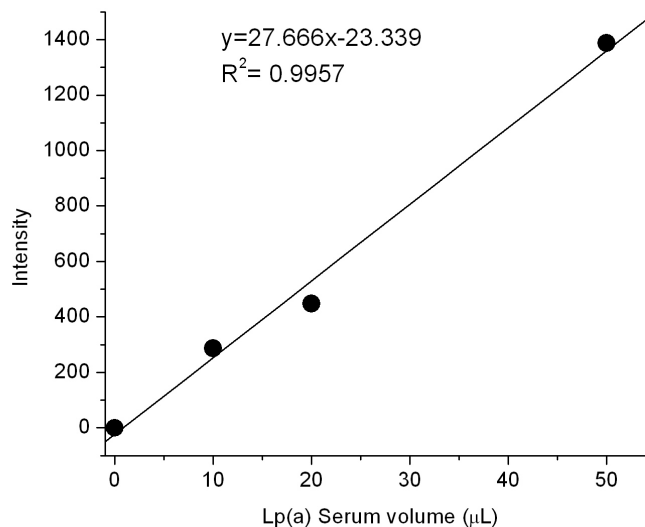


Fig. 24. Fluorescence calibration curve of recovered Lp(a) stained with NBD (Emission intensity at 536 nm)

in the quantitative study of lipoproteins. This approach could be a complement of immunoassay measurement by obtaining lipoprotein(a) concentrations by fluorescence intensity.

Efficiency of recovered Lp(a) is also possible by analysis of the density profile. Fig. 25 shows a series of density profiles obtained from the same samples used in the fluorescence study described before. Lp(a)'s mean density in each profile in Fig. 25 is identified by a vertical line. The mean density of the Lp(a) peak is equivalent in all samples. As expected, more Lp(a) is recovered as the amount of serum is increased, resulting in an increase in Lp(a) peak intensity. As mentioned earlier, this approach demonstrates the feasibility of using the imaging technology with fluorescent tags to obtain quantitative data from the lipoprotein density profiles. From this results, it seems that a 1:1 ratio of serum-to-WGA is ideal for the extraction of Lp(a) from serum. Results presented in a later section on the effect of WGA in extraction of Lp(a) confirmed this conclusion (see Section 9.

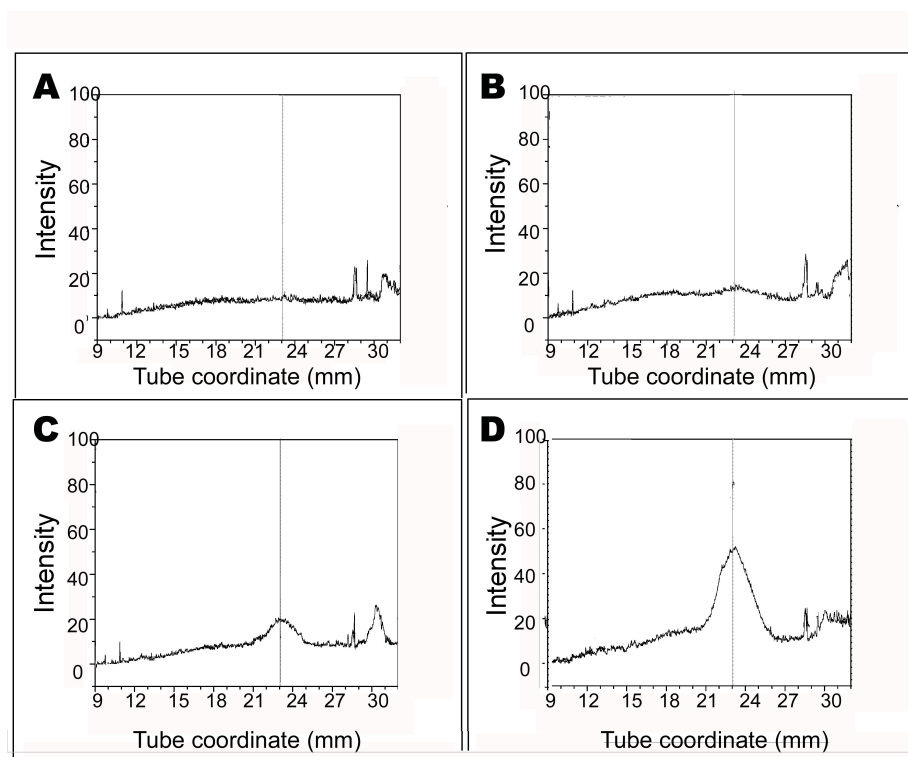


Fig. 25. Recovered Lp(a) density profile from A) 0 μL serum; B) 10 μL serum; C) 20 μL serum; D) 50 μL serum. All samples were incubated with 0.336 mg WGA/mL gel (50 μL Sigma WGA)

9. Optimization of Lp(a) Isolation from Serum by WGA

Lectin affinity is sometimes compared to antibody affinity [88]. Because of this similarity, it was expected that the Lp(a)-WGA interaction will follow a Heidelberger and Kendall type curve (Fig. 7). Therefore, the amount of WGA used was optimized in order to achieve the most effective Lp(a) isolation without isolating other lipoproteins along with Lp(a). For this purpose, a study was performed where the same amount of Lp(a)-containing serum was treated with varying amounts of WGA.

For a 150 μL serum sample, the amount of WGA needed to obtain maximum removal of Lp(a) from serum without any other lipoprotein being removed simultaneously was determined to be 0.672 mg WGA/mL gel (or 100 μL Sigma agarose-WGA sample). This was determined after incubating 150 μL of serum with various amounts of WGA ranging from 0.168 mg WGA/mL gel to 2.352 mg WGA/mL gel (equivalent to 25 to 350 μL Sigma agarose-WGA sample). Lp(a) was recovered from these samples by the methods described in the Experimental Section, stained with 10 μL NBD, and ultracentrifuged in a 20% sucrose gradient. A sucrose gradient was chosen for preparative purposes for two main reasons. First, it has been reported that sucrose helps by protecting lipoproteins' biological properties during storage [124]. Second, salt gradients such as NaBiEDTA interfere with other analytical analyses like capillary electrophoresis. This makes it necessary to remove the density gradient solution from the sample prior to analysis. Sucrose does not interfere with secondary analyses.

Lp(a) peak areas were calculated for each sample and graphed against WGA volume used (Fig 26). Lp(a) removal seems to approach a maximum at around 1.344 mg WGA/mL gel (200 μL of WGA) and then starts declining with increasing amounts of WGA used. If Fig. 26 is compared with a Heidelberger-Kendall curve (Fig. 7), then a maximum amount of 0.672 to 1.008 mg WGA/mL gel should be

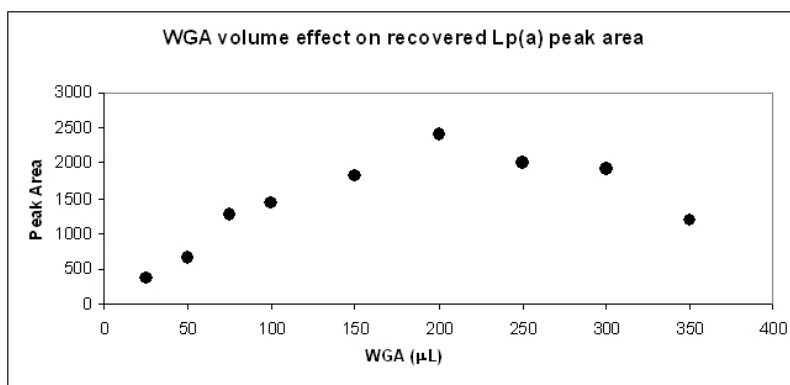


Fig. 26. Optimization of WGA needed to maximize Lp(a) extraction from 150 μL of serum

used to remove Lp(a) from 150 μL of serum and be in the region of the curve where maximum extraction is achieved.

Fig. 27 is a graphic example on the results obtained for the study where Lp(a) was recovered after incubating the same amount of serum with varying amounts of WGA. In this figure, the Lp(a) peak intensity is increased as the amount of WGA used was increased from Panel A down to Panel D. This particular sample contains two Lp(a) isoforms that are clearly distinguished even at low WGA concentrations. As expected, the two isoforms are better resolved as the amount of WGA used was increased (profiles C and D in Fig 27).

At higher WGA concentrations other lipoprotein species start to be removed from serum along side with Lp(a). Fig. 27D shows the extreme case where 150 μL of serum were incubated with 2.352 mg WGA/mL gel. In this case, Lp(a) was recovered with high degree of efficiency, however other lipoproteins were also removed from serum by WGA. These other species were found at a density similar to LDL and are suspected to be glycosylated LDL particles. This phenomenon is explained by the competitive nature of Lp(a) extraction with WGA [87]. When an excess WGA is present in the sample Lp(a) is effectively removed from serum but there are extra

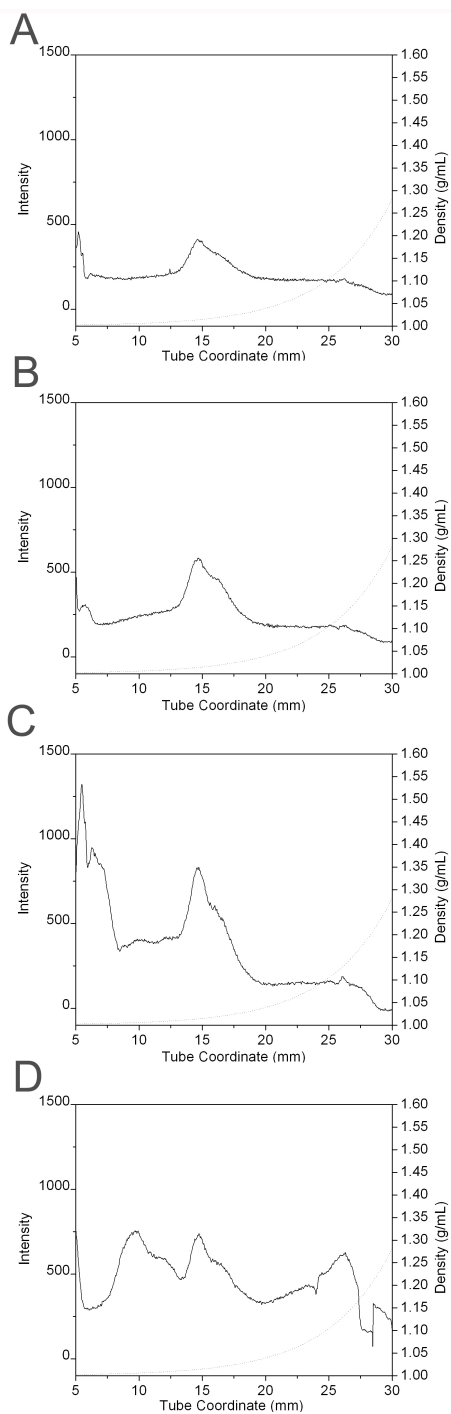


Fig. 27. Recovered Lp(a) density profiles for 150 μ L of serum incubated with different WGA volumes: A) 0.504 mg WGA/mL gel; B) 0.672 mg WGA/mL gel; C) 1.68 mg WGA/mL gel; D) 2.352 mg WGA/mL gel

WGA binding sites available to bind to other lipoproteins containing sialic acid. In the serum sample presented in Fig. 27C and D, some LDL particles presented this glycosylation property. It is possible that different serum samples may contain other lipoproteins such as VLDL or HDL that are glycosylated and will be removed from serum after incubating it with an excess of WGA. Evidence of glycosylation in other apolipoproteins was presented in Table 4.

10. Lp(a) Percent Recovery

For quantitative purposes, Lp(a) recovery was followed using DiaSorin's Lp(a) kit and a percent recovery in Lp(a) concentration established. Total Lp(a) concentration in a serum sample was calculated using DiaSorin Lp(a) kit and compared to Lp(a) concentration in a recovered Lp(a) sample (from the WGA-Lp(a) complex). This approach resulted in a Lp(a) percent recovery efficiency of around 82% (in Lp(a) concentration). Lp(a) was undetectable in the Lp(a)-depleted serum sample, meaning that Lp(a) was completely removed from serum but a percentage of it was lost during the sample handling for Lp(a) recovery from the WGA-Lp(a) complex.

The efficiency of the recovery of Lp(a) from WGA after being extracted from serum was determined by analyzing the peak areas for Lp(a) peaks in a density profile. The samples compared for this experiment consisted of a) an Lp(a) containing serum sample (Fig. 28A), and b) Lp(a) recovered from the serum sample (Fig. 28B). This Lp(a) sample was from the sample in Fig. 28A. Both samples were stained with NBD and ultracentrifuged in a NaBiEDTA density gradient. Lp(a) baselines were corrected to facilitate density and area comparisons. Both peaks, Lp(a) in lipoproteins density profile and Lp(a) peak in recovered sample, were treated in the same way. The peak minima were obtained, and this number subtracted to normalize

the baseline. The peaks were re-graphed independently and the area under the curve obtained using Origin 7.0 software.

These results as well as normalized Lp(a) peaks obtained are shown in Fig. 28. Peak areas were analyzed in order to get a qualitative idea on Lp(a) recovery. The mean densities for both Lp(a) peaks differ slightly. The mean density of recovered Lp(a) (1.08421 g/mL) is slightly lower than that of Lp(a) in a serum sample (1.09048). This shift in density is observed because in the absence of other lipoproteins the Lp(a) band is sharper and thus, resulting in a narrower, more focused peak. This peak area comparison is only possible when Lp(a) peak is resolved in the lipoprotein density profile.

Peak areas for this sample were quite similar for both Lp(a) peaks: recovered Lp(a) had an area under the curve of 437 (Fig 28 peak A) while serum Lp(a) has 453 (Fig 28 peak B). The percent recovery of Lp(a) following the WGA-Lp(a) protocol is of 96% (in peak area) of total serum Lp(a). This comparison indicates that Lp(a) is recovered from serum by WGA with a high yield.

The Lp(a) peak area results qualitatively demonstrate that Lp(a) is effectively recovered from serum after treatment with WGA.

11. Lp(a) Size Characterization by Transmission Electron Microscopy

Traditionally, Lp(a) has been characterized by its density and apo(a) molecular weight. It has been widely demonstrated that Lp(a) heterogeneity is mainly due to apo(a)'s variable size. Apo(a) has been reported to have a molecular weight range of 300 to 800 kDa. This size variability added to metabolic changes in Lp(a)'s lipid core may result in an Lp(a) size heterogeneity. Lp(a) metabolism is still not well understood, however it is known that Lp(a) dramatically resembles LDL in that it

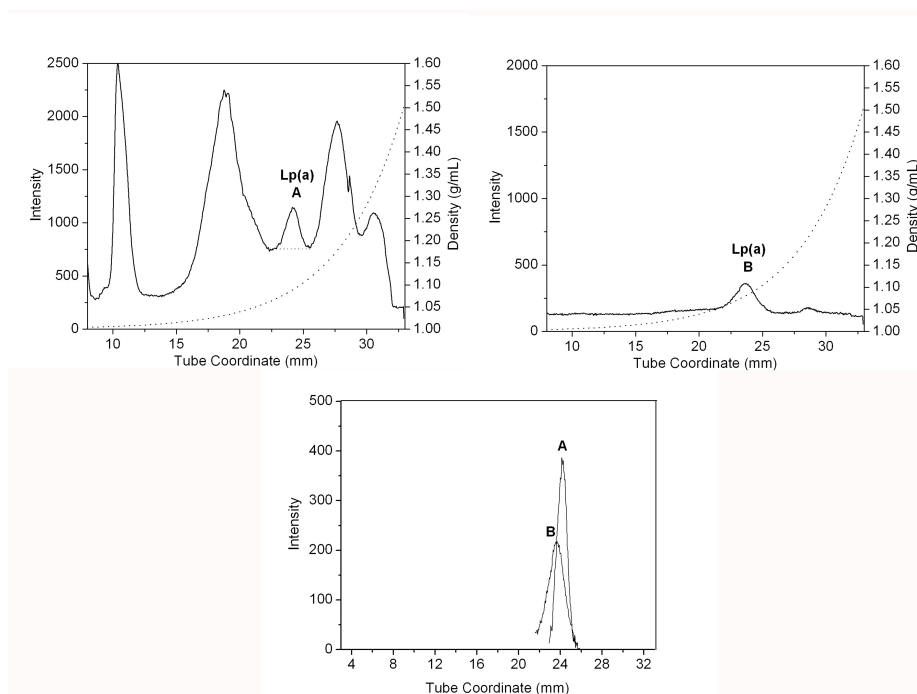


Fig. 28. Lp(a) peak area comparison. A) Lp(a) peak from a serum sample in a lipoprotein density profile; B) Lp(a) peak after extraction from serum and recovery from WGA. Both profiles were run under identical conditions (10% NaBiEDTA, 10 μ L NBD). Baselines were corrected for comparison purposes

contains an apoB-100 apoprotein attached to a lipid core containing cholesterol, phospholipids, cholesterol esters and triglycerides. LDL average particle size is reported to be 18 to 25 nm while Lp(a) particle size has been reported to be between 25 and 35 nm [4]. It is possible then to characterize Lp(a) by its particle size and in this way differentiate it from other lipoproteins and possibly between Lp(a) isoforms.

As a first approach for Lp(a) size characterization Transmission Electron Microscopy (TEM) was used. A pure sample of Lp(a) was produced following the procedures described in the Experimental Section. This sample was analyzed using TEM instrument available at the Texas A&M Microscopy and Imaging Center.

An example of the results obtained by TEM is presented in Fig. 29. In this example, the purity of the Lp(a) sample after purification of ultracentrifugation is confirmed by observing only one type of particle in the sample. If the sample should contain different Lp(a) isoforms and their size varies considerably, then it could be possible to differentiate them by TEM. The size calculated for this Lp(a) sample was approximately 35 nm in diameter, which falls into the reported particle size range for Lp(a).

Lp(a) used in Fig. 29 was recovered from a 10% NaBiEDTA density gradient. The integrity of the particle demonstrated by the round shape of every particle confirms that the salt gradient is not perturbing the lipoproteins. These type of salt gradients do not compromise the structural characteristics of lipoproteins. This demonstrates the versatility of TEM to be applied as a secondary method for the determination of lipoprotein particle size.

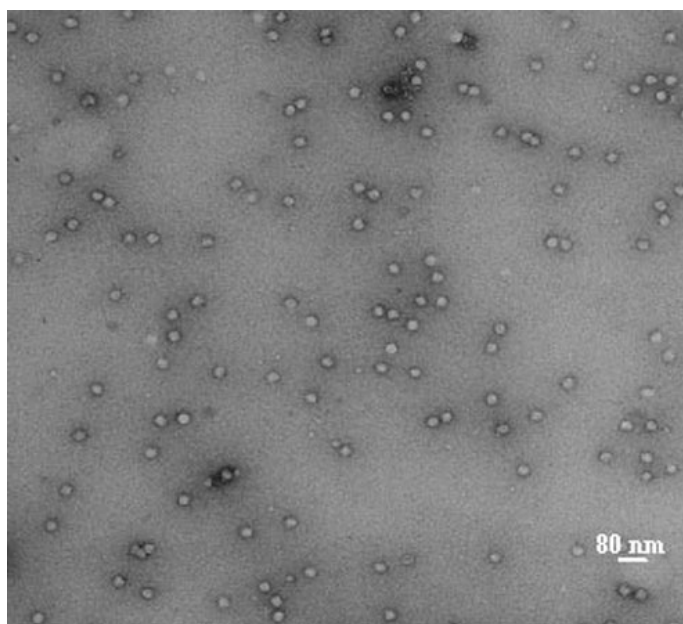


Fig. 29. Lp(a) particle size obtained by Transmission Electron Microscopy. Lp(a) size in this sample is approximately 35 nm

12. Apolipoprotein(a) Characterization

Apolipoprotein(a) is the main source of size heterogeneity in Lp(a). Apo(a) molecular mass varies between 300 and 800 kDa. It is not clear what is the main effect of these size heterogeneity in CDH. It has been demonstrated that the smaller apo(a) isoforms are the more atherogenic and its size is genetically determined. An single individual may exhibit none, one or two different apo(a) isoforms [27].

The characterization of apo(a) was possible after obtaining a pure sample of Lp(a) by the carbohydrate affinity technology described herein. Lp(a) was obtained after incubation of serum with WGA and then recovered from the WGA-Lp(a) complex and purified by ultracentrifugation. This pure sample of Lp(a) was incubated in a reducing buffer containing 2-mercaptoethanol to break the disulfide bond between apo(a) and apoB-100.

Apo(a) molecular weight was determine by SDS-PAGE. This is the traditional

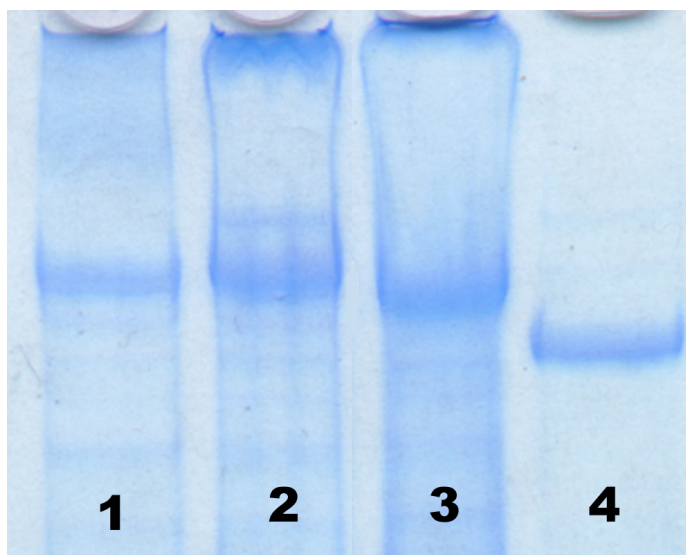


Fig. 30. Apo(a) molecular weight isoforms characterization by SDS-PAGE. Lanes 1 to 4 represent apo(a) isoforms obtained from four different Lp(a) samples

way for the elucidation of apo(a) molecular weight isoforms. In this research, this approach was followed for an initial screening for Lp(a) heterogeneity, and complemented with other analytical techniques such as ultracentrifugation and capillary electrophoresis.

This technique was demonstrated to be effective in the separation of different apo(a) isoforms. Fig. 30 shows the results obtained for a series of Lp(a) samples separated in an SDS-PAGE gel. Each lane in a gel was loaded with a different reduced Lp(a) sample. After electrophoresis, the gel was stained with Coomassie Blue. Different apo(a) isoforms were obtained for each sample as shown in Fig. 30. Of special attention is lane 2 where two apo(a) isoforms were observed with significantly different molecular weight. This result represents a case of a heterozygote subject.

The identity of apo(a) in these samples was confirmed by Western blot. The samples as loaded into the gel contained both apo(a) and apoB-100, both resulting from the reductive cleavage of the disulfide bonds in Lp(a). In order to discriminate apo(a) protein bands from apoB, a gel was transferred to a nitrocellulose membrane

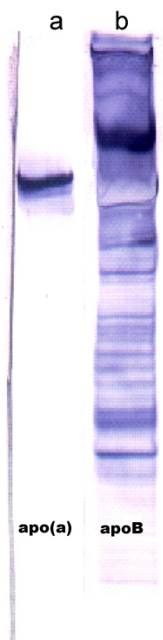


Fig. 31. Western blot of a reduced Lp(a) sample assayed against apo(a) and apoB and tested against apo(a) and apoB. Fig. 31 shows an example of how apo(a) (in lane a) in these samples is significantly lower in molecular weight when compared to apoB (in lane b). Only one result is presented here, however similar results were obtained for all Lp(a) samples assayed by this method. The apo(a) band immunodetected in lane a of Fig. 31 corresponds to the apo(a) band in lane 3 of Fig. 30.

Many apoB-containing proteins were identified in Lane b in Fig. 31. It has been reported that apoB is present in humans as many classes, such as apoB-100, apoB-48, etc. The primary antibody used to detect apoB in the western blot was not specific for apoB-100 and as a consequence will bind to any other apoB protein resulting from either apoB-100 fragmentation or metabolism.

A. Reduced Lp(a) Stability Study

The stability of the Lp(a) sample prepared over days was studied. For this purpose, Lp(a) was extracted from serum during five consecutive days. Each day, the recovered Lp(a) fraction was reduced with 2-mercaptoethanol, dried under vacuum and stored at -20°C . On the fifth day a fresh apo(a) sample was obtained and dried. Then all the five samples were reconstituted with $12.5\ \mu\text{L}$ dH₂O.

Evidence of a better structural stability was observed when Lp(a) is stored in its reduced form (as apo(a)) and stored at -20°C . For each sample (over the 4 days storage), two distinct bands were observed (Fig. 32), both corresponding to the molecular weight range for apo(a) in this sample. The evidence of two bands instead of just one may be due to the second reduction when the samples were reconstituted in dH₂O, and sample buffer and incubated for 5 minutes at 100°C prior to loading the samples into the gel. The reducing buffer contains substances such as glycerol, SDS and 2-mercaptoethanol. For this experiment $1\ \mu\text{L}$ of Invitrogen's antioxidant reagent was added after reduction and prior to drying. The addition of the antioxidant may have helped in preserving the protein during storage. It is recommended that this antioxidant be added to all samples to be stored for more than 24 hours. According to the results obtained in this experiment apo(a) can be stored for a period of 4 days at -20°C without disturbing its main characteristics.

13. Lp(a) Characterization by Capillary Electrophoresis

Capillary electrophoresis was selected for the characterization of Lp(a) and reduced Lp(a). Capillary electrophoresis has the advantage of obtaining a separation with high efficiency in a very short period of time. The main advantage of capillary electrophoresis over traditional electrophoretic methods is that it eliminates labor-intensive steps.

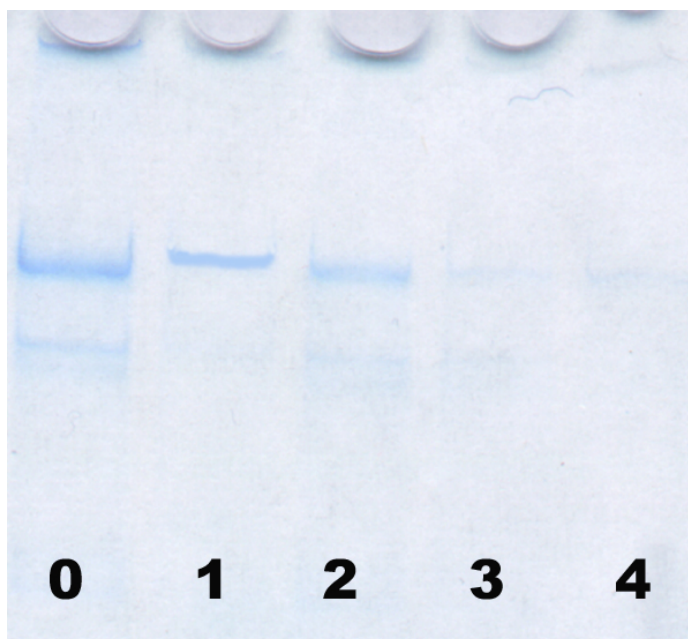


Fig. 32. Apo(a) stability study. Each lane number corresponds to the number of days the reduced Lp(a) sample was stored at -20°C . Lp(a) were reduced with 2-ME and dried under vacuum before storage

The availability of software specially design to analyze data generated by the CE provides with an unmeasurable advantage for the analysis of proteins with CE.

A Lp(a) sample was obtained from a serum sample known to contain high levels of Lp(a) (according to immunoassay results). A pure sample of Lp(a) was generated by the carbohydrate affinity separation technique described previously. An unstained Lp(a) fraction was recovered by the freeze-cut technique described earlier. This sample was ultrafiltrated, as described in the Experimental Section, to remove all density gradient solution from the sample. The volume of density gradient solution ultrafiltrated was replaced with CE running buffer.

The removal of density gradient solution from the sample becomes extremely important when salt gradients are used. These solutions absorb strongly at the wavelengths used for lipoprotein analysis (190 and 214 nm). When the density gradient solution is not removed from the sample, it is detected by the CE as a very strong

and broad peak that interferes and most of the times masks the lipoprotein peak. Removal of the density gradient solution by a cycle of ultrafiltrations proved to be an effective way to preserve the integrity of lipoproteins while exchanging the solution they are contained in.

A representative electropherogram of an intact Lp(a) sample is presented in Fig. 33. The main peak in this figure represents an Lp(a) peak and has a mobility of $-17 \times 10^{-5} \text{ cm}^2/\text{Vs}$. As specified in the Experimental Section, samples prepared for CE were in a borate buffer solution. This buffer may cause some degree of delipidation of the lipoproteins. Evidence of this partial delipidation of lipoproteins when in presence of borate buffer is represented by the peak in Fig. 33 with mobility higher than $-25 \times 10^{-5} \text{ cm}^2/\text{Vs}$. This effect, while not significant for an Lp(a) electropherogram, is also induced by SDS present in the background electrolyte which may contribute to the high mobility of lipids in the assay of samples of Lp(a) reduced after capillary electrophoresis.

An apoB standard sample (Cat. No. A5353, Sigma Aldrich, St. Louis, MO) was analyzed by CE to identify its electrophoretic mobility. Fig. 34 shows the electropherogram obtained from this sample under the same electrophoretic conditions as Lp(a). From these experiments, it was determined that apoB had an electrophoretic mobility of $-20 \times 10^{-5} \text{ cm}^2/\text{Vs}$. This mobility is significantly higher than that of Lp(a). This difference in electrophoretic mobilities will become extremely important when a reduced Lp(a) sample is analyzed by CE (next section). Capillary electrophoresis is a versatile, user-friendly technique that is useful in the characterization of Lp(a) isoforms according to its electrophoretic mobility.

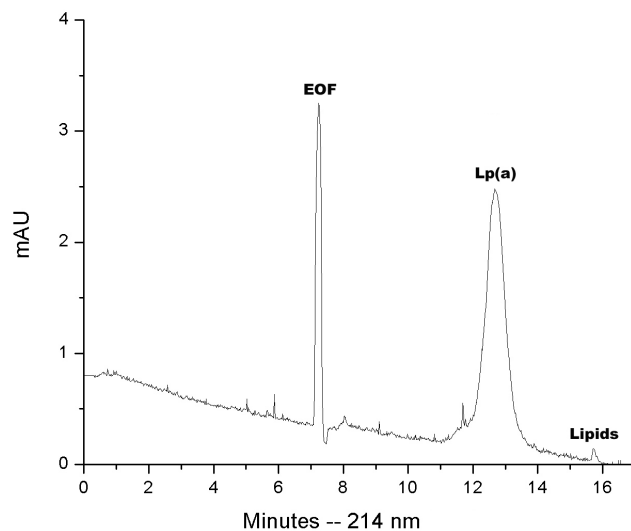


Fig. 33. Electropherogram of Lp(a). Background electrolyte consisted of 13 mM sodium borate, 3.5 mM SDS , and 20% (v/v) acetonitrile, pH 9.25. Under these conditions this Lp(a) sample has an effective mobility of $-17 \times 10^{-5} \text{ cm}^2/\text{Vs}$

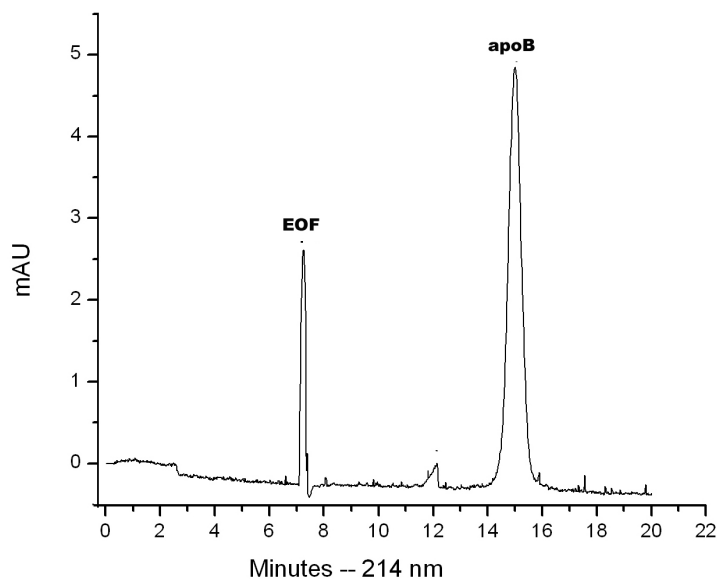


Fig. 34. Electropherogram of apoB. Background electrolyte consisted of 13 mM sodium borate, 3.5 mM SDS , and 20% (v/v) acetonitrile, pH 9.25. Under these conditions apoB has an effective mobility of $-20 \times 10^{-5} \text{ cm}^2/\text{Vs}$

14. Reduced Lp(a) Characterization by Capillary Electrophoresis

Capillary electrophoresis was also used for the characterization of Lp(a) after reduction of the disulfide bonds. The traditional reducing agents used for reduction of disulfide bonds are 2-mercaptoethanol and dithiothreitol (DTT). The use of these reducing agents in the study of Lp(a) by capillary electrophoresis had two major drawbacks. First, presence of the reducing agents in the sample being analyzed by CE resulted in peak with strong intensity that interfered with the analysis of the reduced proteins. Second, in order to overcome the first drawback the reducing agent has to be removed from the sample before capillary electrophoresis. It was noted that some of the sample was lost while removing the reducing agent from it. This resulted in electropherograms with very low peak intensity making it very difficult for the analysis of the reduced proteins.

A different approach for the reduction of disulfide bonds in proteins was followed. Trialkylphosphines (TCEP) were used as reducing agents. In aqueous solutions, TCEP stoichiometrically and irreversibly reduces disulfides. TCEP has been shown to be significantly more stable than DTT at pH values below 8.0 [125]. TCEP has been commercially available since 1992. Pierce Biotechnology manufactures a TCEP disulfide reducing gel that consists of the phosphine covalently linked to 4% crosslinked beaded agarose. This product was used in this research for the reduction of Lp(a) prior to capillary electrophoresis. The bulky size of the agarose bead made it simpler to remove the reducing agent from the sample after reduction by a simple filtration. This successfully minimizes the reducing agent interference with the analysis.

A typical electropherogram obtained for a reduced Lp(a) sample is presented in Fig. 35. Evidence of incomplete reduction was detected by the presence of a

peak with electromobility of $-21 \times 10^{-5} \text{ cm}^2/\text{Vs}$ which corresponds to Lp(a) for this sample (see Fig. 33). When Lp(a) is reduced, the bond between apoB and apo(a) is cleaved leaving apo(a) and Lp(-a) as products. Lp(-a) would consist of an Lp(a) molecule from where apo(a) has been removed. This Lp(-a) molecule was found to have an electrophoretic mobility of $-26 \times 10^{-5} \text{ cm}^2/\text{Vs}$. However, when this Lp(-a) molecule is partially delipidated, the products of the reduction would be apo(a), Lp(-a) and apoB. Evidence of this partial delipidation was observed also in the sample in Fig. reffig:CEapo(a). The peak labeled as apoB had a similar electromobility as a standard apoB (Fig. 34). After analyzing this results two peaks were left to identify. As mentioned earlier, lipids would have a mobility higher than any other proteins in the sample. Such is the case of the peak observed at last in Fig. 35, which had a mobility of $-28 \times 10^{-5} \text{ cm}^2/\text{Vs}$. Finally, apo(a) was identified as a peak between the EOF and apoB with mobility of $-18 \times 10^{-5} \text{ cm}^2/\text{Vs}$. This findings are in complete accordance with those reported by Hu and coworkers for capillary electrophoresis of apo(a) [114]. Capillary electrophoresis proved to be an effective technique for the characterization of reduced Lp(a) and apo(a) isoform phenotyping.

The identity of apo(a) and Lp(a) peaks in the electropherogram was determined by incubating the reduced Lp(a) sample from Fig. 35 with WGA. Reduction of the lipoprotein with phosphines does not affect the sialylation in the apolipoprotein. Lp(a), reduced Lp(a) and apo(a) bind to WGA in a similar manner. In this way, these particles will form a complex with WGA, leaving all other non-apo(a) proteins in solution. This WGA complex was removed from the sample by ultrafiltration and the filtrate analyzed by CE. Fig. 36 shows the results from this experiment where only one peak was detected with mobility of $-19.9 \times 10^{-5} \text{ cm}^2/\text{V}$. This mobility corresponds to the mobility of apoB as presented in Fig. 34. When the electropherograms from Figs. 35 and 36 were compared it was noted that the apo(a) and Lp(a)-labeled peaks

were absent after the incubation with WGA and only the apoB peak remained in the electropherogram. This experiment allowed for the differential identification of apo(a) and reduced Lp(a) by capillary electrophoresis.

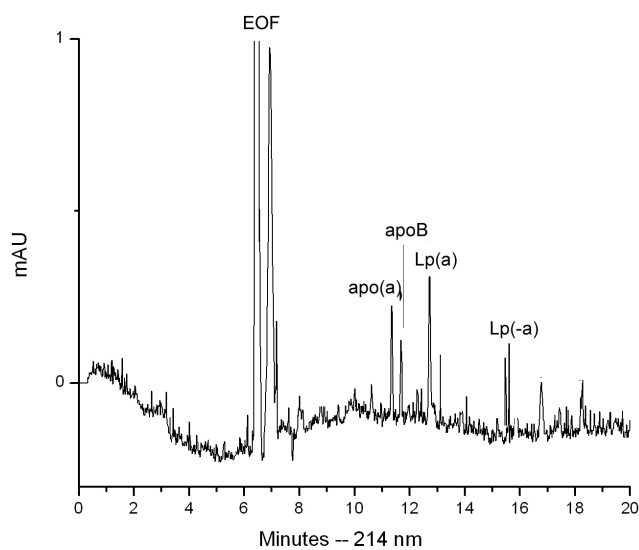


Fig. 35. Electropherogram of reduced Lp(a). Background electrolyte consisted of 13 mM sodium borate, 3.5 mM SDS, and 20% (v/v) acetonitrile, pH 9.25. Under these conditions, apoB has an effective mobility of $-19.9 \times 10^{-5} \text{ cm}^2/\text{Vs}$; apo(a) of $-18 \times 10^{-5} \text{ cm}^2/\text{Vs}$; Lp(a) of $-21 \times 10^{-5} \text{ cm}^2/\text{Vs}$ and Lp(-a) of $-26 \times 10^{-5} \text{ cm}^2/\text{Vs}$

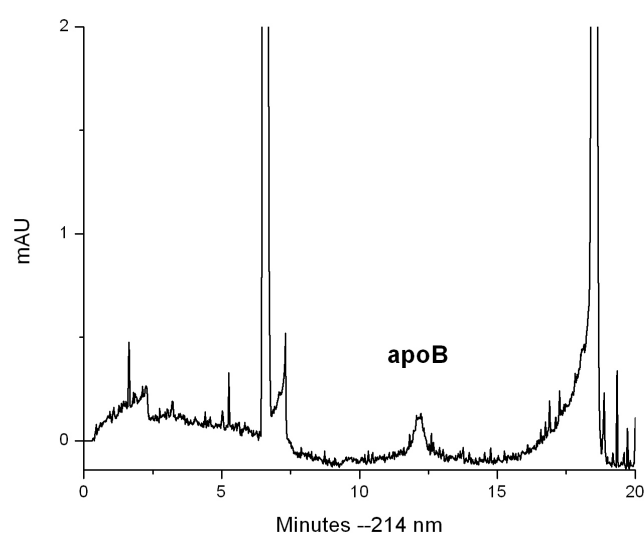


Fig. 36. Electropherogram of reduced Lp(a) after incubation with WGA. Background electrolyte consisted of 13 mM sodium borate, 3.5 mM SDS , and 20% (v/v) acetonitrile, pH 9.25. The only peak detectable after treatment of a reduced Lp(a) sample with WGA corresponds to that of apoB with an effective mobility of $-19.9 \times 10^{-5} \text{ cm}^2/\text{Vs}$

CHAPTER IV

APPLICATION: LIPOPROTEIN(a) CHARACTERIZATION IN CASE STUDIES

The purpose of this chapter is to demonstrate the applicability of the methods developed during the course of this research. A series of samples sourced from a variety of subjects were subjected to Differential Density Profiling for Lp(a) and to the characterization of Lp(a) after recovery from the WGA-Lp(a) protocol. The protocols used in these samples were the same for all of them and were as detailed in the Experimental Section.

The main goal of this research was to develop a rapid, easy-to-use method for the characterization of Lp(a) by density and other inherent properties such as molecular weight, size and electromobility. The consortium of samples presented in this chapter demonstrate the capability of the methodology developed for the characterization of Lp(a) in different samples. The samples assayed for this purpose correspond to randomly selected normolipidemic subjects as well as Lp(a) standard samples sourced from Northwestern Lipid Research Laboratory.

Each of the Lp(a)-containing samples were assayed for:

- Differential Density Lipoprotein Profiling (DDLPP) for Lp(a).
- Density determination of recovered Lp(a).
- Identification of apo(a) isoform by SDS-PAGE and Western blot with immunodetection against Lp(a).
- Characterization of Lp(a) by Capillary Electrophoresis.

1. Lp(a) Characterization Subject A.

Suspected Lp(a) from Broad Separation between HDL and LDL in the Lipoprotein Density Profile

Sample A was collected from a normolipidemic male subject in his mid 40s. The lipoprotein density profile of this subject revealed a shoulder in the LDL region and a bump in the density region between LDL and HDL (Fig. 37A). Serum sample for this subject was treated with WGA following the DDLP protocol. The results obtained are shown in Fig. 37. Lp(a) was revealed in the density region where the bump was observed while leaving the LDL shoulder intact (Fig. 37B and C). The DDLP for this sample resulted in two peaks, the first one being Lp(a) and the second one serum proteins removed by WGA (see Panel D in Fig. 37). Lp(a) density for this sample was obtained from the recovered Lp(a) profile in 10% NaBiEDTA (Fig. 38), and was calculated as 1.086 g/mL.

The Lp(a) fraction was collected and analyzed by SDS-PAGE and Western blot. Lane *a* in both figures on page 114) represents the apo(a) isoform contained in Lp(a) from subject A. This subject only showed one apo(a) isoform according to western blot results.

Fig. 39 shows the electropherogram obtained for the Lp(a) fraction of subject A. Here, Lp(a) is present as a single sharp peak with an electromobility equal to $-22 \times 10^{-5} \text{ cm}^2/\text{Vs}$.

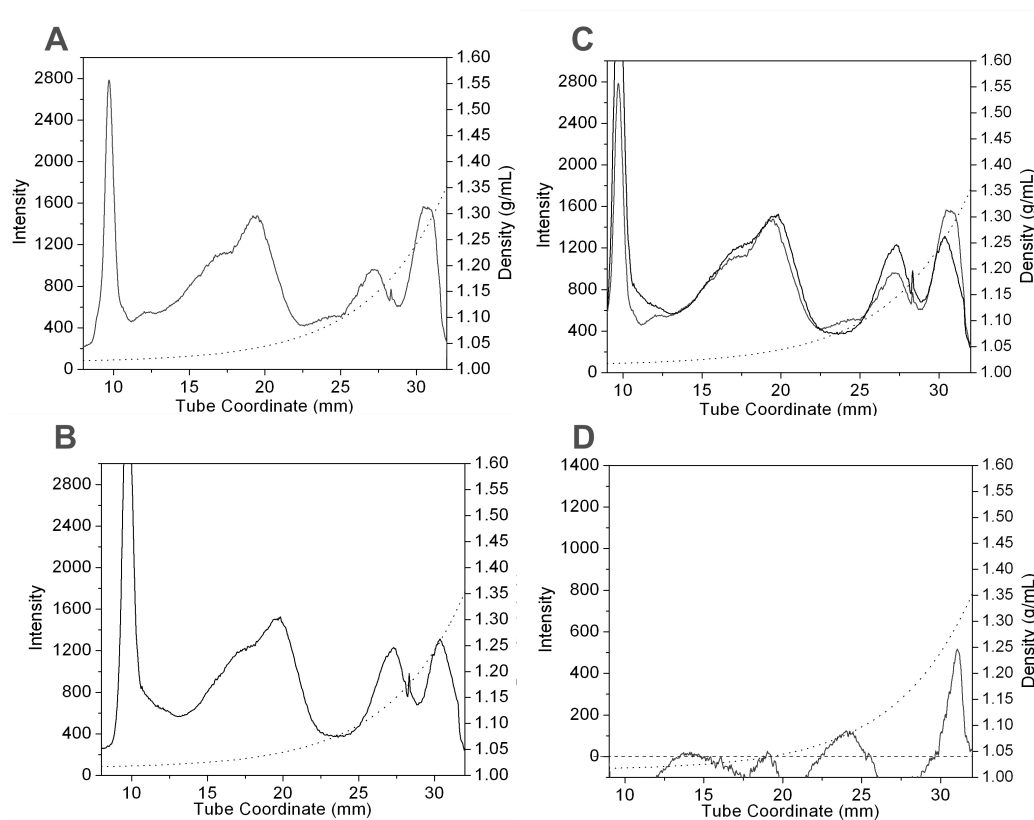


Fig. 37. Differential Density Lipoprotein Profiling (DDL) for subject A: A) Lipoprotein density profile; B) Lp(a)-depleted density profile; C) Overlay of A and B; D) Lp(a) DDLP

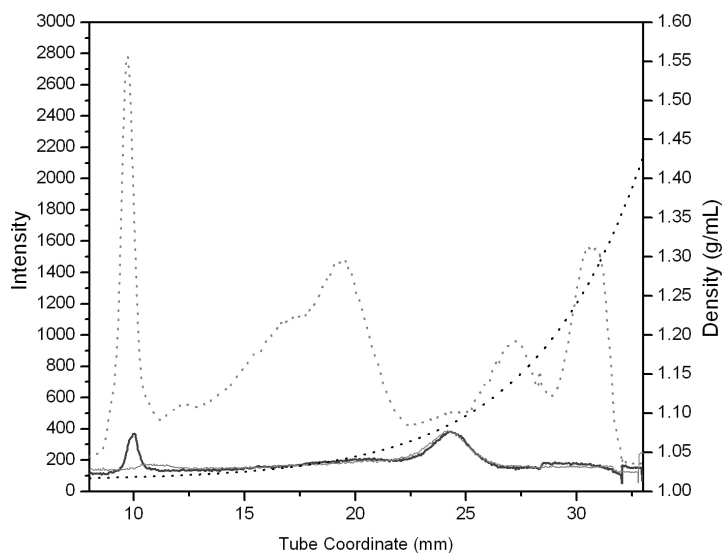


Fig. 38. Subject A, Lp(a) density profile in 10% NaBiEDTA. Lp(a) density is 1.086 g/mL

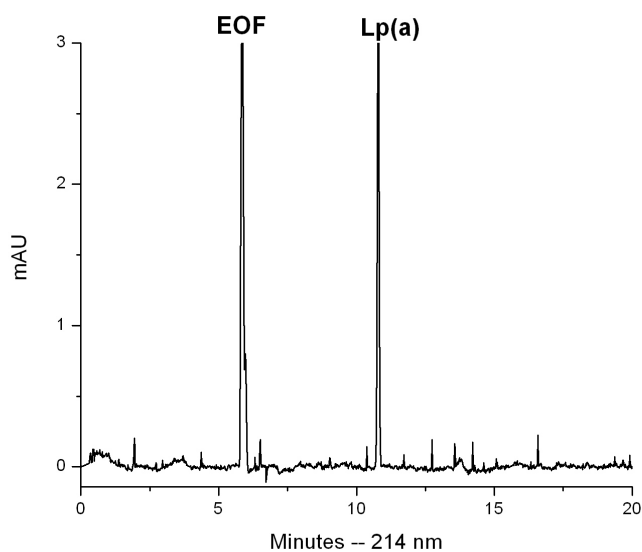


Fig. 39. Electropherogram of Lp(a) from subject A. Lp(a) mobility of $-22 \times 10^{-5} \text{ cm}^2/\text{Vs}$. Background electrolyte consisted of 13 mM sodium borate, 3.5 mM SDS, and 20% (v/v) acetonitrile, pH 9.25

2. Lp(a) Characterization Subject B.

Lp(a) Peak Resolved in the Lipoprotein Density Profile

Sample B was collected from a normolipidemic male subject in his early 30s. The lipoprotein density profile of this subject contains a peak close to the buoyant density of HDL that was suspected to be Lp(a) (Fig. 40A). Serum sample for this subject was treated with WGA following the DDLP protocol. The results obtained are shown in Fig. 40. Lp(a) was revealed in the density region where the Lp(a) peak was observed (Panel C in Fig. 40). Lp(a) density for this sample was obtained from the recovered Lp(a) profile in 10% NaBiEDTA (Fig. 41), and was calculated as 1.088 g/mL.

The Lp(a) fraction was collected and analyzed by SDS-PAGE and Western blot. Lane *b* in both figures on page 114, represents the apo(a) isoform contained in Lp(a) from subject B. This subject only showed one apo(a) isoform according to western blot results.

Fig. 45 shows the electropherogram obtained for the Lp(a) fraction of subject A. Here, Lp(a) is present as a single peak with an electromobility equal to -19.7×10^{-5} cm²/Vs. Following the Lp(a) peak, there is a second a peak with higher mobility which was identified as lipids from a partial delipidation of the lipoprotein.

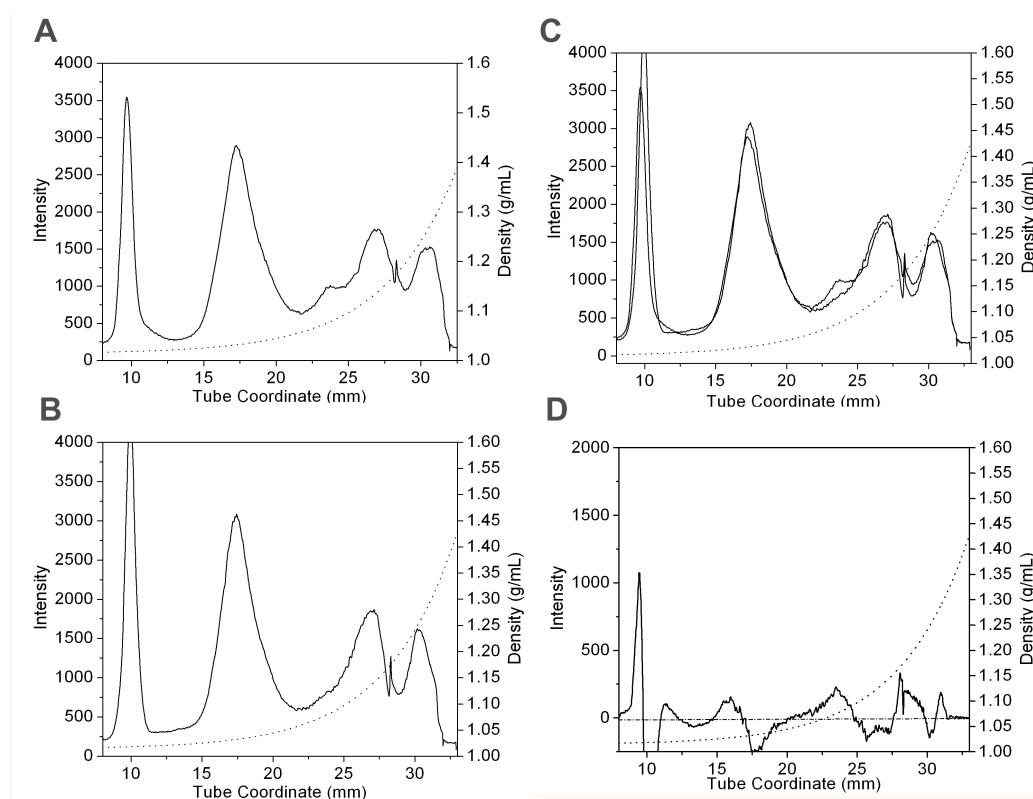


Fig. 40. Differential Density Lipoprotein Profiling (DDL) for subject B: A) Lipoprotein density profile; B) Lp(a)-depleted density profile; C) Overlay of A and B; D) Lp(a) DDLP

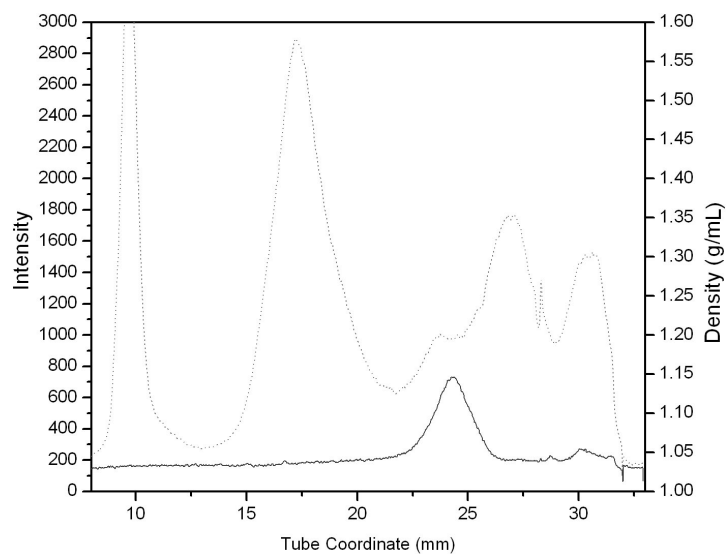


Fig. 41. Subject B, Lp(a) density profile in 10% NaBiEDTA. Lp(a) density is 1.088 g/mL

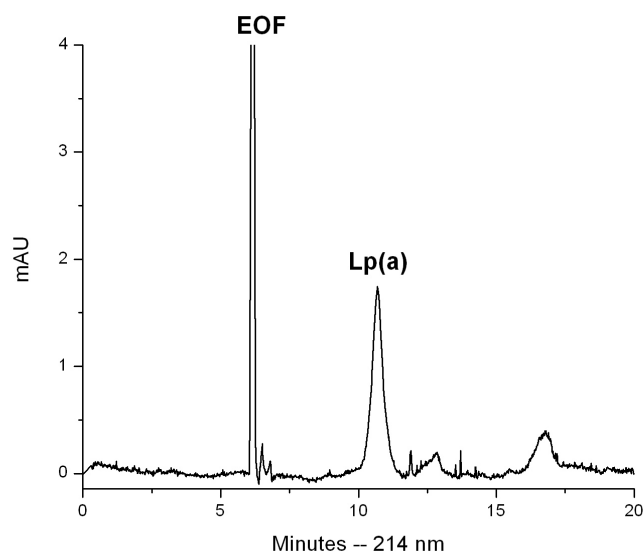


Fig. 42. Electropherogram of Lp(a) from subject B. Lp(a) mobility of $-19 \times 10^{-5} \text{ cm}^2/\text{Vs}$. Background electrolyte consisted of 13 mM sodium borate, 3.5 mM SDS, and 20% (v/v) acetonitrile, pH 9.25

3. Lp(a) Characterization Subject C.

Sample Containing Two Apo(a) Isoforms

Sample C was collected from a normolipidemic male subject in his early 40s. The lipoprotein density profile of this subject is very peculiar. LDL is clearly resolved in a doublet and there is the presence of the Lp(a) peak close to the buoyant HDL density (Fig. 43A).

Serum sample for this subject was treated with WGA following the DDLP protocol. The results obtained are shown in Fig. 43. As it can be seen in Fig. 43B, the LDL doublet was not modified after treatment of the serum with WGA. Therefore, the presence of Lp(a) in the LDL region was ruled out.

Lp(a) for this sample was found to be present in the Lp(a) peak close to HDL. The DDLP for Lp(a) shown in Fig. 43 D shows a very well defined differential Lp(a) peak. Lp(a) density for this sample was obtained from the recovered Lp(a) profile in 10% NaBiEDTA (Fig. 44), and was calculated as 1.087 g/mL.

The Lp(a) fraction was collected and analyzed by SDS-PAGE and Western blot. Lane *c* in both figures on page 114, represents the apo(a) isoform contained in Lp(a) from subject C. These results showed a unique Lp(a) feature not noticed with DDLP. Two apo(a) isoforms were present in this sample, and their presence was confirmed by western blot (see Lane *c* in the bottom figure on page 114). No doublet in the Lp(a) peak was observed in the Lp(a) density profile (Fig. 44), or in the lipoprotein density profile in Fig. 43A.

Capillary electrophoresis turned out to be a powerful analytical technique in the study of Lp(a) isoforms. Even when the Western blot confirmed the presence of two apo(a) isoforms, their separation was not possible by ultracentrifugation. Fig. 42 shows the electropherogram obtained for the Lp(a) fraction of subject C.

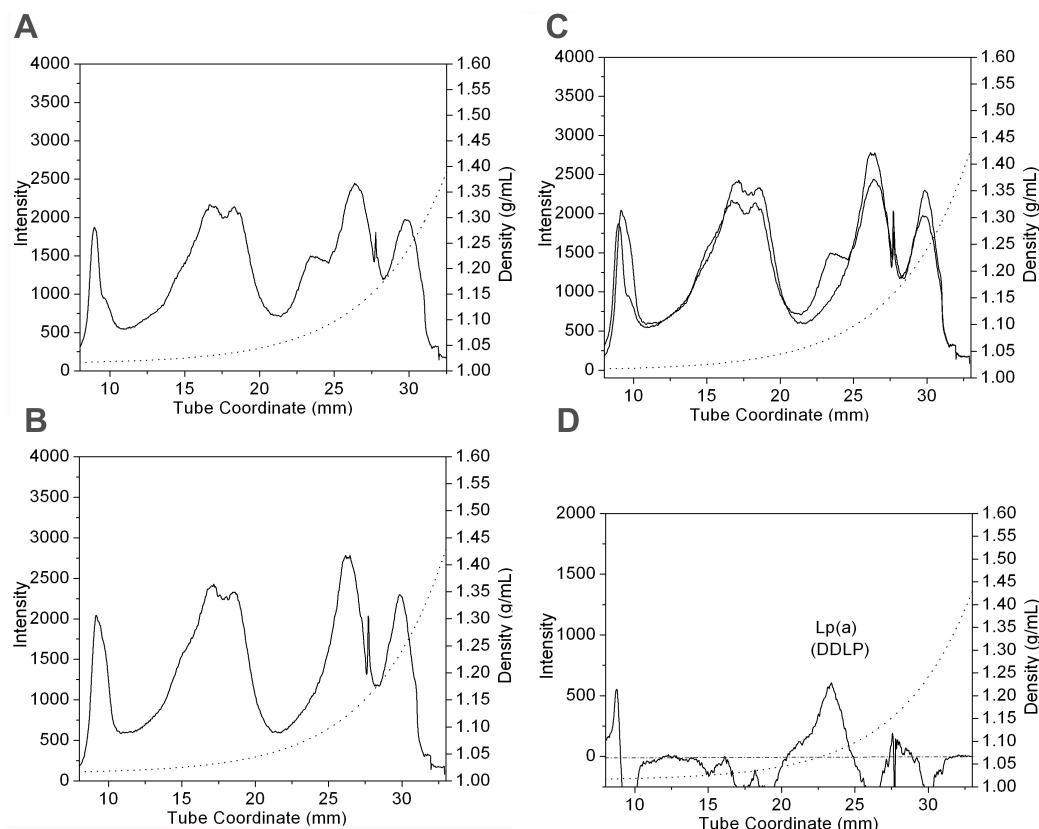


Fig. 43. Differential Density Lipoprotein Profiling (DDL) for subject C: A) Lipoprotein density profile; B) Lp(a)-depleted density profile; C) Overlay of A and B; D) Lp(a) DDL

Here, two main peaks were observed with mobilities of a) $-19 \times 10^{-5} \text{ cm}^2/\text{Vs}$ and b) $-23 \times 10^{-5} \text{ cm}^2/\text{Vs}$, each corresponding to a Lp(a) isoform. Partial delipidation was also observed, resulting in a third peak with the highest mobility ($-28 \times 10^{-5} \text{ cm}^2/\text{Vs}$).

The results obtained from a serum sample of subject C seem to indicate that apo(a) isoformism is not responsible for Lp(a) density heterogeneity. However, more results are needed in order to confirm this hypothesis.

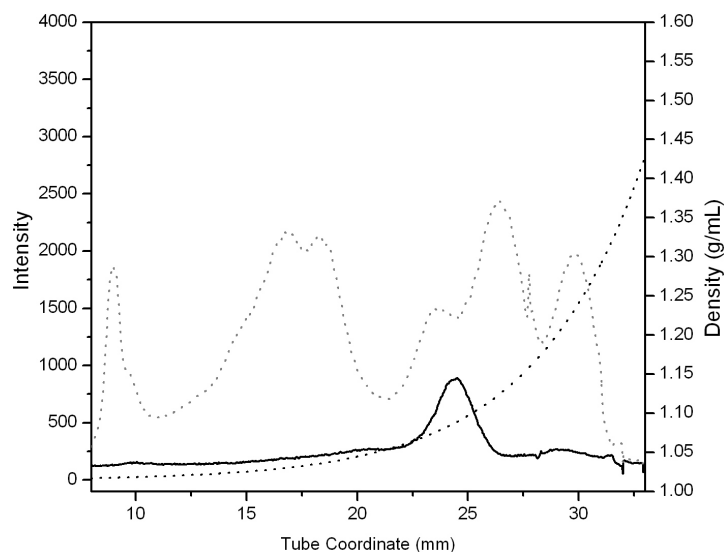


Fig. 44. Subject C, Lp(a) density profile in 10% NaBiEDTA. Lp(a) density is 1.087 g/mL

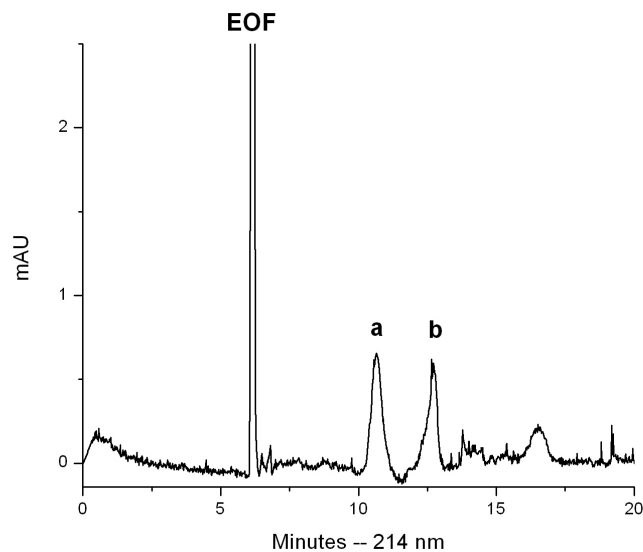


Fig. 45. Electropherogram of Lp(a) from subject C. Two Lp(a) isoforms were resolved with mobility of a)- $19 \times 10^{-5} \text{ cm}^2/\text{Vs}$, and b)- $23 \times 10^{-5} \text{ cm}^2/\text{Vs}$. Background electrolyte consisted of 13 mM sodium borate, 3.5 mM SDS, and 20% (v/v) acetonitrile, pH 9.25

4. Lp(a) Characterization Subject D.

No Evidence of Lp(a) in the Lipoprotein Density Profile

Sample D was collected from a normolipidemic female subject in her early 20s. One characteristic of the lipoprotein density profile of this sample is a shoulder in the HDL region and no evidence of Lp(a) (Fig. 46A). Even though Lp(a) was not resolved in the lipoprotein density profile, it was detected in this sample by immunoassay.

A serum sample of subject D was treated with WGA and Lp(a) recovered from it. The results for Lp(a) DDLP are presented in Fig. 46. No drastic difference was observed after the sample was treated with WGA (Panel B in Fig. 46). But when the Lp(a) serum profile and the Lp(a)-depleted serum profile are directly compared (Panel C in Fig. 46), it is now evident that a feature of the lipoprotein density profile was changed. When these two samples were subtracted from each other the Lp(a) DDLP presented in Panel D of Fig. 46 was obtained. Here, a Lp(a) differential density profile was uncovered after removing all LDL and HDL interferences from the sample.

Lp(a) was recovered from the WGA-Lp(a) complex and separated in a 10% NaBiEDTA density gradient. Fig. 47 shows the Lp(a) density profile for this sample. Interestingly, Lp(a) was detected as being buried in the LDL and HDL peaks as a doublet at a density of 1.069 g/mL.

The Lp(a) fraction containing the doublet was collected and analyzed by SDS-PAGE and Western blot. Lane *d* both figures on page 114, represent the apo(a) isoform contained in Lp(a) from subject D. The existence of two Lp(a) isoforms was not detected by western blot when assayed against Lp(a). It is possible that this subject serum contains two distinct Lp(a) particles with different density but carrying the same apo(a) isoform.

Fig. 48 shows the electropherogram obtained for the Lp(a) fraction of subject D.

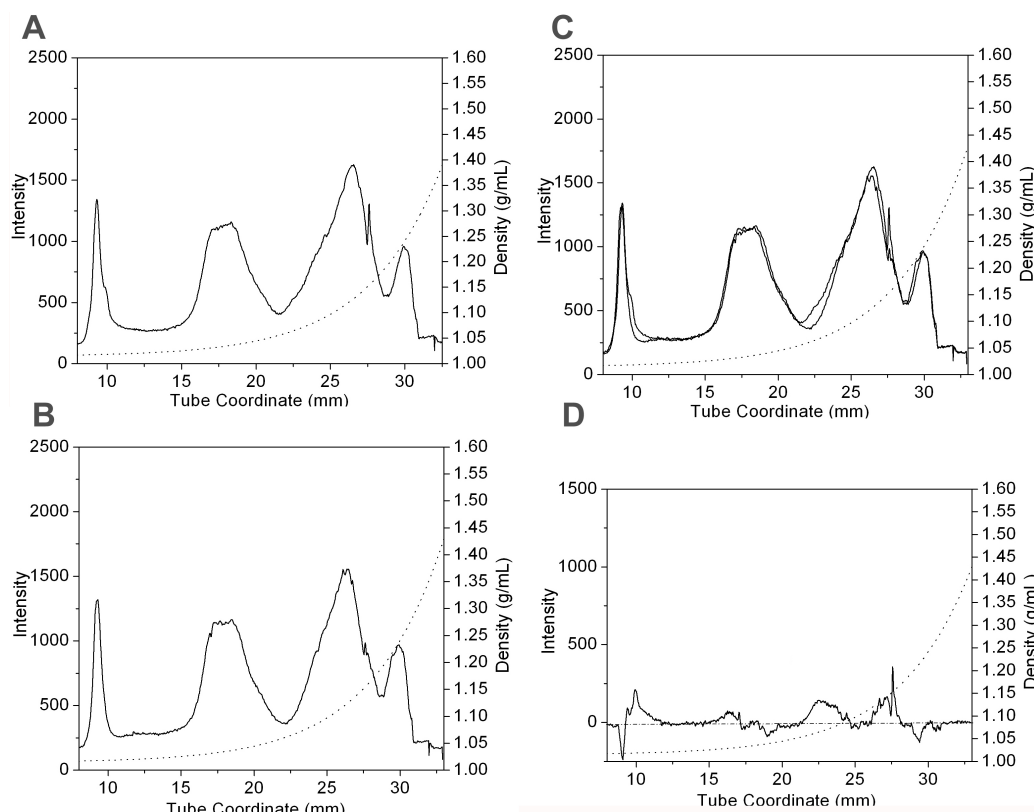


Fig. 46. Differential Density Lipoprotein Profiling (DDLDP) for subject D: A) Lipoprotein density profile; B) Lp(a)-depleted density profile; C) Overlay of A and B; D) Lp(a) DDLP

Two distinct features were observed in this electropherogram. First, there is a sharp peak with mobility of $-22 \times 10^{-5} \text{ cm}^2/\text{V}$, followed by a smaller and broader peak with mobility of $-28 \times 10^{-5} \text{ cm}^2/\text{Vs}$.

The results obtained from this subject reinforces the conclusion that apo(a) isoformism is not reflected in Lp(a) density heterogeneity. Subject D does not have two distinct apo(a) isoforms but in contrast it contains two Lp(a) isoforms that were resolved by density and electromobility in capillary electrophoresis. These results were opposite to those obtained with subject C, where only one Lp(a) isoform was resolved with DDLP, but two apo(a) isoforms resolved with SDS-PAGE and CE.

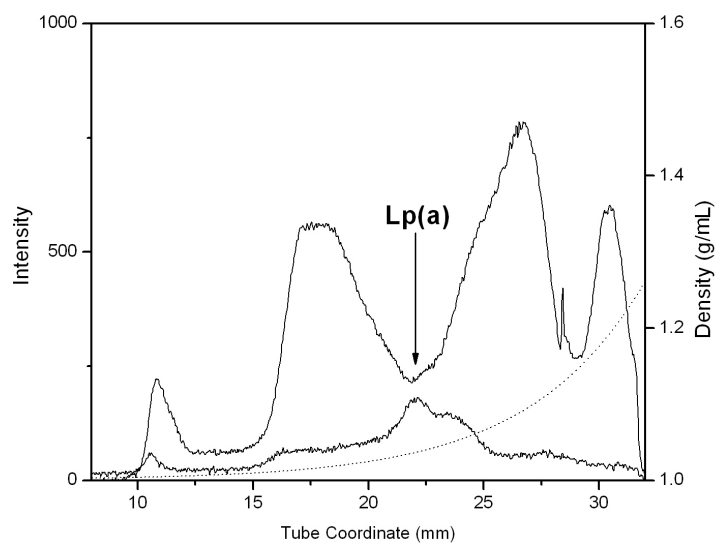


Fig. 47. Subject D, Lp(a) density profile in 10% NaBiEDTA. Lp(a) density is 1.069 g/mL

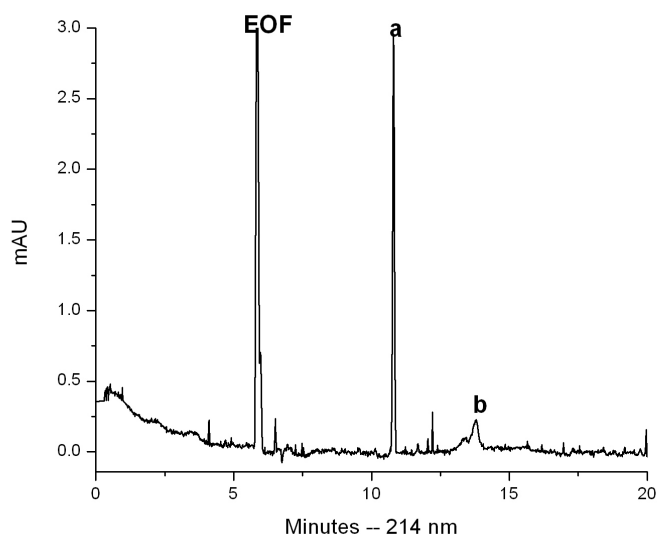


Fig. 48. Electropherogram of Lp(a) from subject D. Lp(a) mobility of a)- 22×10^{-5} cm^2/Vs and b)- 28×10^{-5} cm^2/Vs . Background electrolyte consisted of 13 mM sodium borate, 3.5 mM SDS, and 20% (v/v) acetonitrile, pH 9.25

5. Lp(a) Characterization Sample E.

Lp(a) Serum from Northwestern Lipid Research Lab

Sample E was sourced from Northwestern Lipid Research laboratory (NWLR). Apo(a) in this sample has been characterized as having 18 KIV repeats, and is present in a concentration of 74 nmol/L Lp(a). This sample was treated in the same way as all the previous subjects. Its lipoprotein density profile reveals a shoulder in the HDL peak (Fig. 49A). This peak was differentially identified as Lp(a) as shown in Fig. 49D.

Lp(a) density for this sample was calculated as 1.143 g/mL. The density profile for Lp(a) recovered from the WGA-Lp(a) complex for this sample is presented in Fig. 50. Since this sample had already been characterized by NWLR, it was known to contain a single Lp(a) isoform. This was confirmed by a single peak obtained by ultracentrifugation and a single band in the SDS-PAGE/Western Blot analysis. Lane e in both figures on page 114, represent the apo(a) isoform contained in Lp(a) of this standard sample.

The Lp(a) fraction from this sample was also analyzed by capillary electrophoresis. The results for this analysis are shown in Fig. 51. A single, sharp peak was obtained for this Lp(a) sample. The electromobility detected for this Lp(a) was of $-22 \times 10^{-5} \text{ cm}^2/\text{Vs}$.

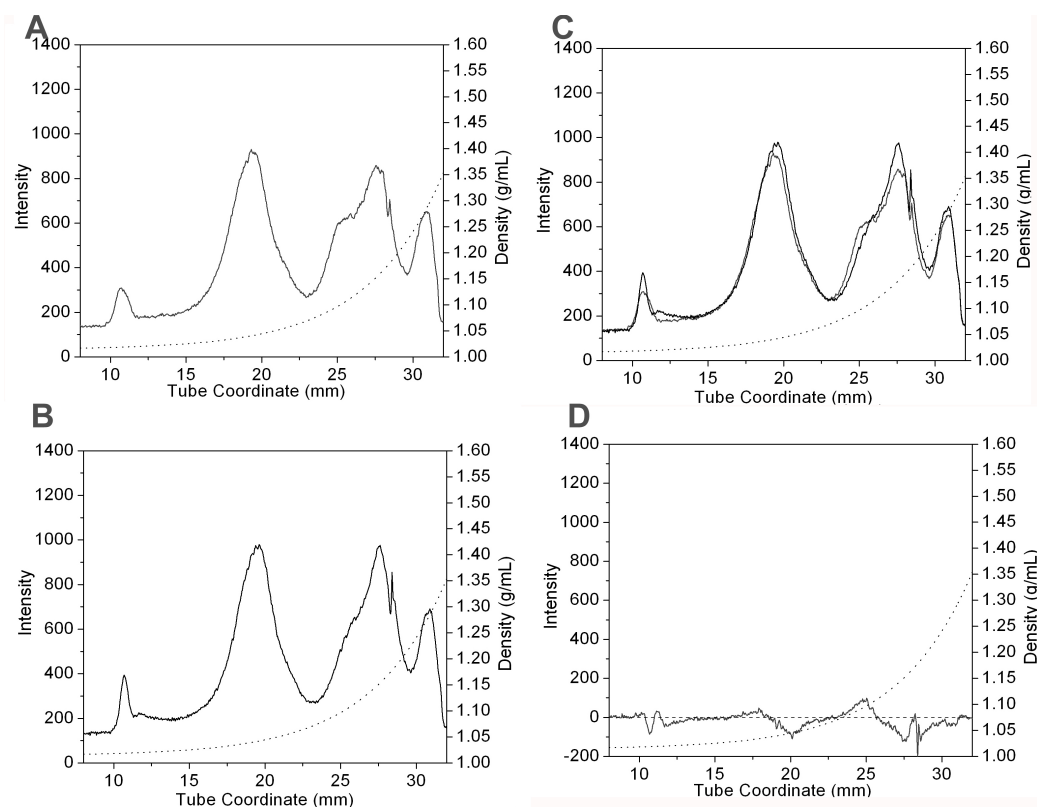


Fig. 49. Differential Density Lipoprotein Profiling (DDL) for sample E: A) Lipoprotein density profile; B) Lp(a)-depleted density profile; C) Overlay of A and B; D) Lp(a) DDLP

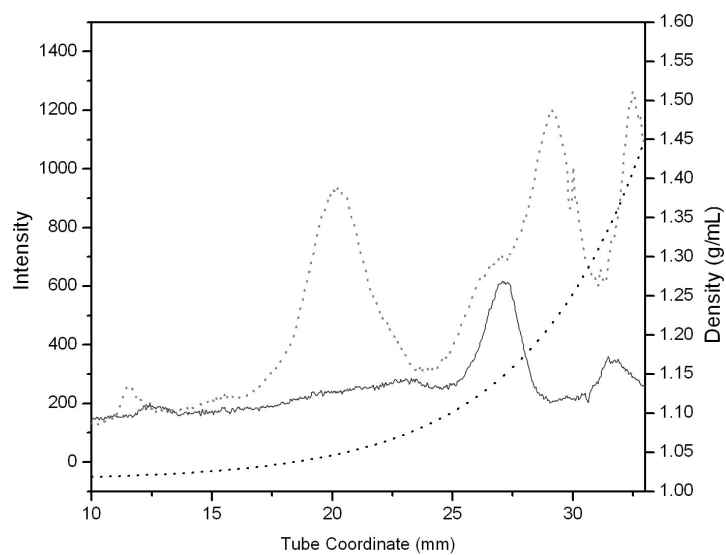


Fig. 50. Sample E, Lp(a) density profile in 10% NaBiEDTA. Lp(a) density is 1.143 g/mL

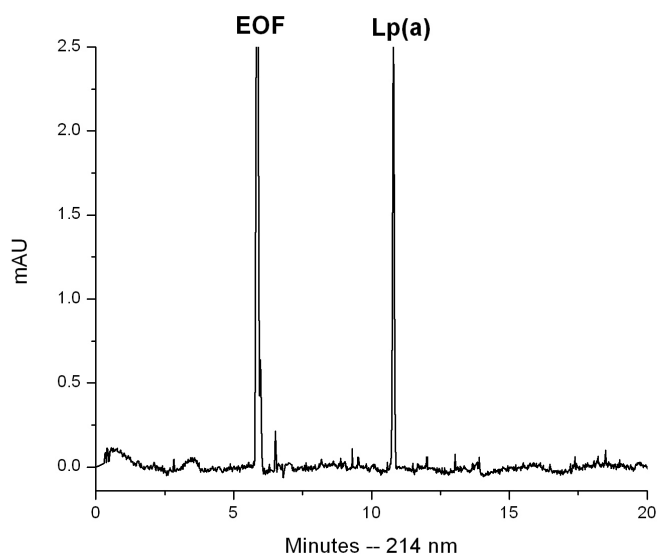


Fig. 51. Electropherogram for Lp(a) from sample E. Lp(a) mobility of $-22 \times 10^{-5} \text{ cm}^2/\text{Vs}$. Background electrolyte consisted of 13 mM sodium borate, 3.5 mM SDS, and 20% (v/v) acetonitrile, pH 9.25

6. Lp(a) Characterization Sample F.

Lp(a) Serum from Northwestern Lipid Research Lab

Sample F was also sourced from Northwestern Lipid Research laboratory (NWLR). Apo(a) in this sample has been characterized as having 14 KIV repeats, and is present in a concentration of 114.1 nmol/L Lp(a). A first glance at this sample's lipoprotein density profile does not reveal the presence of Lp(a). However it presents a very broad and dense HDL peak and almost no LDL (Fig. 52A). This is an example of serum samples where Lp(a) is masked by the over abundant LDL or HDL peaks. It appears that Lp(a) was not resolved under the conditions established for DDLP. The only peak detected in the differential profile (Fig. 52D) is well in the HDL region.

It was only after Lp(a) being recovered from the WGA-Lp(a) complex, and analyzed by itself that it could be easily determined. Lp(a) was buried in the buoyant density range of Lp(a), and it appears to feature double isoformism in the Lp(a) density profile presented in Fig. 53. The density for this Lp(a) in a 10% NaBiEDTA density gradient was calculated to be 1.012 g/mL.

Apo(a) in this Lp(a) sample contained fewer KIV repeats than sample E and it was expected to show higher mobility when the two samples were compared in SDS-PAGE. Lane *f* in the top figure on page 114 corroborates this. When compared to apo(a) from sample E, which contains more KIV repeats, apo(a) from sample F traveled longer in the gel.

From the information provided by NWLR this sample only contained one apo(a) isoform. This was in accordance with the western blot results where only one apo(a) isoform was observed (Lane *f* in the bottom figure on page 114. However, the Lp(a) density profile suggests the presence of two Lp(a) isoforms.

Capillary electrophoresis was used once more to identify Lp(a) isoforms. Fig. 54

is the electropherogram obtained for the Lp(a) fraction of sample F. In here, two distinct, well resolved peaks are observed. One having an electrophoretic mobility of $-24 \times 10^{-5} \text{ cm}^2/\text{Vs}$, followed by a second Lp(a) peak with mobility of $-27 \times 10^{-5} \text{ cm}^2/\text{Vs}$. A smaller peak at higher mobility ($-30 \times 10^{-5} \text{ cm}^2/\text{Vs}$) suggests the presence of lipids resulting from partial delipidation of the lipoprotein.

In conclusion, the case studies presented in this chapter demonstrated that Lp(a) can be characterized by using a combination of analytical techniques. It is possible to detect Lp(a) isoformism by ultracentrifugation and capillary electrophoresis of the intact lipoprotein. Lp(a) density heterogeneity is not related to apo(a) size, but to density differences in the lipoprotein particle. Apo(a) isoformism is easily detected by a combination of SDS-PAGE and western blot analyses.

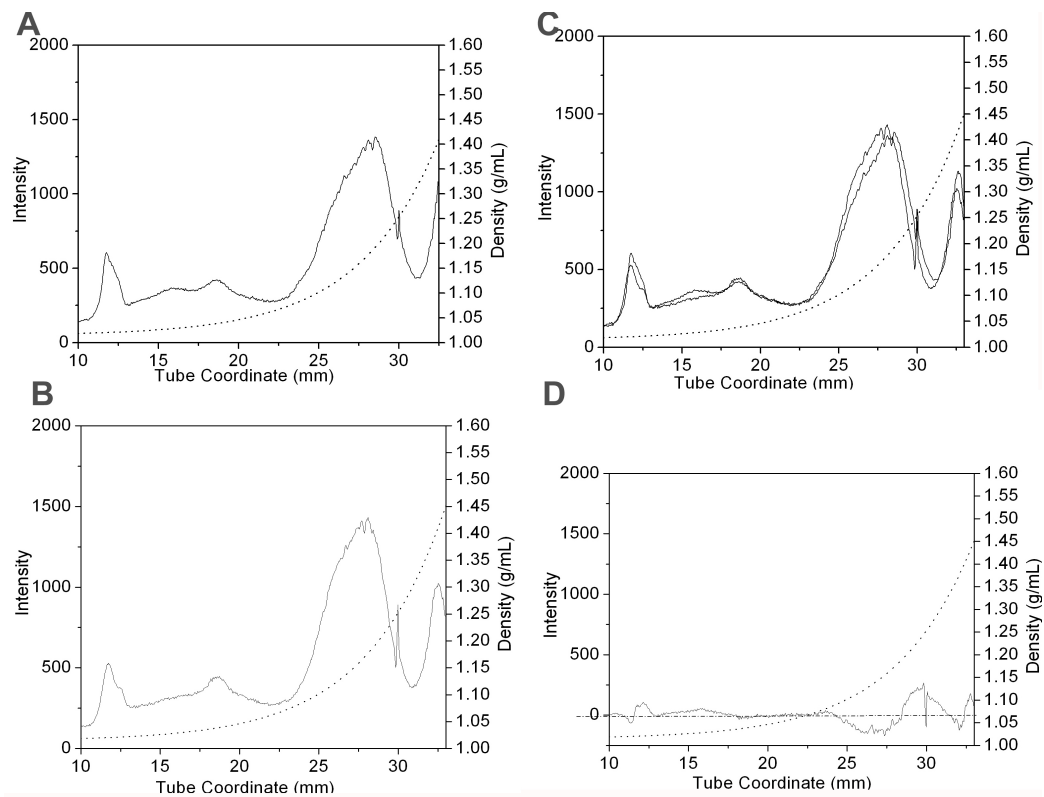


Fig. 52. Differential Density Lipoprotein Profiling (DDLP) for sample F: A) Lipoprotein density profile; B) Lp(a)-depleted density profile; C) Overlay of A and B; D) Lp(a) DDLP

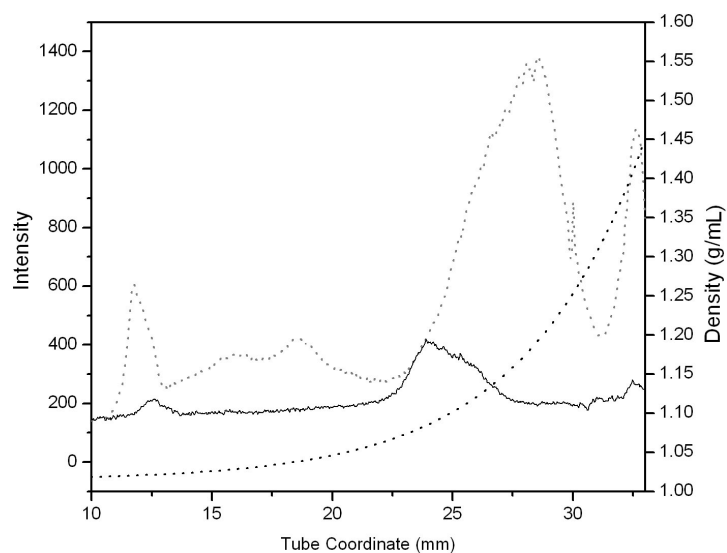


Fig. 53. Sample F, Lp(a) density profile in 10% NaBiEDTA. Lp(a) density is 1.012 g/mL

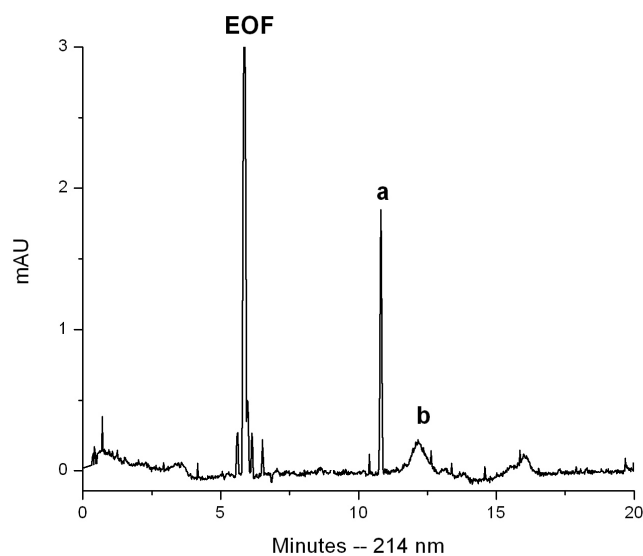


Fig. 54. Electropherogram for Lp(a) from sample F. Lp(a) mobility of a)- $24 \times 10^{-5} \text{ cm}^2/\text{Vs}$, and b)- $27 \times 10^{-5} \text{ cm}^2/\text{Vs}$. Background electrolyte consisted of 13 mM sodium borate, 3.5 mM SDS, and 20% (v/v) acetonitrile, pH 9.25

7. SDS-PAGE/Western Blot Analysis of Lp(a) Case Studies

All Lp(a) samples from subjects A through F were reduced with 2-mercaptoethanol and analyzed by gel electrophoresis. SDS-PAGE and Western Blot with immunodetection procedures were followed as explained in the Experimental Section. After separation by gel electrophoresis the gel was either stained with Coomassie Blue (Bio-Rad) (Fig. 55) or Western Blotted to a nitrocellulose membrane and immunodetected against apo(a) (Fig. 56).

The methodology for the extraction of Lp(a) from serum reported in this dissertation is apo(a) isoform independent. This statement was proved to be correct after the analysis of different apo(a) samples by SDS-PAGE. Apo(a) from subjects A through F showed increasing electrophoretic mobilities in the gel, apo(a) from subject F being the fastest while apo(a) from subject A being the slowest (see Fig. 55). It is well known that molecular weight of proteins determined by SDS-PAGE is not accurate and is relative to the amount of SDS present in the experiment. The purpose of the analysis of apo(a) isoforms by SDS-PAGE in this section was not the determination of the molecular weight but the characterization of Lp(a) subclasses within subjects. It was for these reasons that no apo(a) molecular weights are reported for these samples.

Fig. 56 is the results of the western blot transfer of a gel identical to that in Fig 55 where anti-Lp(a) was used as a primary antibody and immunodetected as specified in the Experimental Section. The results presented in Fig. 56 demonstrate that all the samples from subjects A through F contained different apo(a) isoforms clearly resolved by SDS-PAGE.

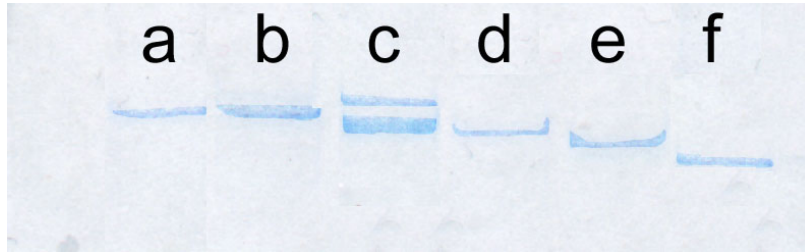


Fig. 55. Coomassie blue stained 3-8% SDS-PAGE of case study Lp(a) samples. Lanes: a)apo(a) from subject A; b)apo(a) from subject B; c)apo(a) from subject C; d)apo(a) from subject D; e)apo(a) from sample E; f)apo(a) from sample F

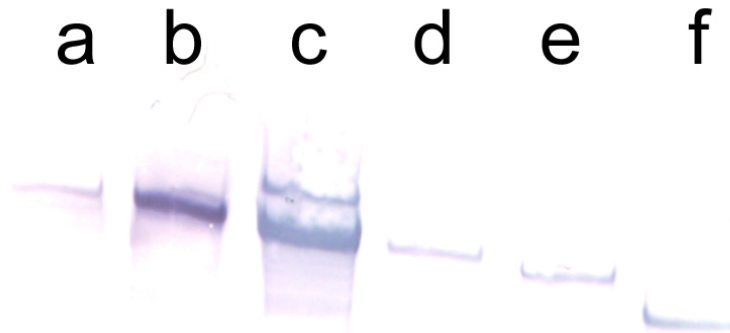


Fig. 56. Western Blot of apo(a) samples from case study subjects. Apo(a) was immunodetected after SDS-PAGE and Western blot. a)apo(a) from subject A; b)apo(a) from subject B; c)apo(a) from subject C; d)apo(a) from subject D; e)apo(a) from sample E; f)apo(a) from sample F

8. Case Studies Conclusions

Differential Density Lipoprotein Profiling (DDLDP) provides with relevant information on Lp(a) density and Lp(a) distribution in the lipoprotein density profile. More importantly, it is possible to determine Lp(a) subclasses in the density profile. Table 8 is a summary of the results obtained for each of the case studies samples. Subjects C and F contained two very distinct Lp(a) subclasses (by density ultracentrifugation) but only one apo(a) isoform. These two Lp(a) subclasses were resolved in the density profile only after recovering Lp(a) from the WGA-Lp(a) complex. These results are an insight on the specificity of WGA for Lp(a) and the power of analytical ultracentrifugation in the study of lipoprotein subclasses. This is the first time that Lp(a) subclasses have been reported.

Lp(a) from subject C had only one Lp(a) subclass by density ultracentrifugation, but two well resolved apo(a) isoforms by SDS-PAGE/Western Blot. These findings leads to the conclusion that apo(a) isoform size is not completely responsible for Lp(a) density heterogeneity. It is expected that being an LDL-like molecule Lp(a)'s lipid content will vary among individuals, leading to the existence of Lp(a) subclasses as is in the case of all other lipoproteins in serum.

Capillary Electrophoresis is a powerful technique that is capable of resolve both Lp(a) subclasses and apo(a) isoforms. For every subject with two Lp(a) subclasses determined by density ultracentrifugation two Lp(a) peaks with different effective electromobility was observed (see subjects D and F in table 8). As well as two peaks were observed for the subject whose serum Lp(a) was composed of two distinct apo(a) isoforms (subject C in table 8).

In conclusion, Lipoprotein(a) was characterized by DDLDP after its extraction from serum by WGA. Lp(a) subclasses were resolved by analytical density ultracen-

trifugation after recovery of Lp(a) from the WGA-Lp(a) complexes, and their respective electromobility determined by capillary electrophoresis. Apo(a) isoforms from each Lp(a) sample were characterized qualitatively by SDS-PAGE and later by capillary electrophoresis. The consortium of samples analyzed in this case study section represent a variety of Lp(a) subclasses with densities ranging from 1.012 to 1.143 g/mL. The use of capillary electrophoresis as a secondary characterization technique results in relevant information for the complete characterization of Lp(a) and its assessment as a cardiovascular risk marker.

Table 8. Summary of Lp(a) characterization in case studies

Subject	Density (g/mL)	Lp(a) density subclass	Lp(a) effective mobility	apo(a) isoforms
A (page 94)	1.086	ONE	$-22 \times 10^{-5} \text{ cm}^2/\text{Vs}$	ONE
B (page 97)	1.088	ONE	$-19 \times 10^{-5} \text{ cm}^2/\text{Vs}$	ONE
C (page 100)	1.087	ONE	$-19 \times 10^{-5} \text{ cm}^2/\text{Vs}$ $-23 \times 10^{-5} \text{ cm}^2/\text{Vs}$	TWO
D (page 103)	1.069	TWO	$-22 \times 10^{-5} \text{ cm}^2/\text{Vs}$ $-28 \times 10^{-5} \text{ cm}^2/\text{Vs}$	ONE
E (page 106)	1.143	ONE	$-22 \times 10^{-5} \text{ cm}^2/\text{Vs}$	ONE
F (page 109)	1.012	TWO	$-24 \times 10^{-5} \text{ cm}^2/\text{Vs}$ $-27 \times 10^{-5} \text{ cm}^2/\text{Vs}$	ONE

CHAPTER V

SUMMARY AND CONCLUSIONS

The study reported here demonstrates the analytical power of linking ultracentrifugation with affinity separations for the characterization of serum lipoproteins. The primary objective of this research was to develop a rapid method for the separation, purification, density measurement, and characterization of Lp(a) from serum using a procedure that is isoform independent. The complexity of Lp(a) presents a challenge for the determination of the risk it represents for the cardiovascular system. The objective was met by taking advantage of the high glycosylation of Lp(a) and using a novel density gradient ultracentrifugation method for lipoprotein separation.

Lp(a) was selectively removed from serum following a simple protocol using the lectin wheat germ agglutinin (WGA) that demonstrated high affinity for the carbohydrates in Lp(a). The lipoprotein density profile of a serum sample containing high levels of Lp(a) was compared with the lipoprotein density profile of the same serum sample from where Lp(a) was removed. Lp(a)'s mean density was determined from the Differential Density Lipoprotein Profile (DDLPP).

The application of the DDLPP technology for the characterization of Lp(a) in case studies demonstrated that Lp(a) in a sample can be discriminated against all other lipoproteins such as LDL and HDL using DDLPP. This is true even when Lp(a)'s presence is not evident in the serum lipoproteins density profiles. Lp(a) density isoforms were determined by ultracentrifugation in a 10% NaBiEDTA density gradient solution.

Lp(a) was quantitatively removed from serum and recovered with more than 80% recovery efficiency. Lp(a) recovered from serum by the method described in this dissertation retained its inherent hydrated density and immunoreactivity. Lp(a) was

purified by ultracentrifugation. The Lp(a) fraction was further characterized by other analytical techniques such as SDS-PAGE coupled with Western Blot and immunodetection, and by capillary electrophoresis. Apo(a) isoformism was studied using these techniques. The characterization of Lp(a) in the case studies demonstrated that capillary electrophoresis is a very powerful analytical tool for the characterization of Lp(a) isoforms by electromobility.

The procedures described herein are relevant in a clinical setting for the detection of Lp(a) isoforms based on density and electrophoretic characteristics. In conclusion, this DDLP methodology gives relevant information such as Lp(a) density and Lp(a) isoform characteristics that are important for the assessment of Lp(a) as a marker for cardiovascular disease.

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APPENDIX A

DETAILED EXPERIMENTAL PROTOCOLS

Lp(a) concentration: DiaSorin Lp(a) kit

1. Let serum, DiaSorin Lp(a) standards, DiaSorin Lp(a) controls and DiaSorin Lp(a) antibody to reach room temperature.
2. Get a clean micro plate and use the template in Fig 57 to determine where the samples will be placed.
3. Prepare enough antibody solution by diluting DiaSorin's Lp(a) in a 2:3 ratio with dH₂O. Vortex briefly. Each blank, standard, control, and sample will use 50 μ L of this antibody solution.
4. Place 5 μ L of each serum, standard, control, and blank sample in a micro well plate IMMEDIATELY after briefly vortexing each of them.
5. Carefully add 300 μ L of DiaSorin's diluent to each well in the plate. Be careful not to form any bubbles in the well.
6. Read plate's absorbance at 340 nm. This is ABSORBANCE 1.
7. Remove the plate from the UV/Vis instrument and add 50 μ L of the previously prepared antibody solution (from step 3 to each well. Carefully mix by pipetting the well contents up and down for five times without forming bubbles. *Not using reverse pipetting for this step prevents the formation of bubbles in the well.*

8. Incubate the micro well plate for 10 minutes at 37°C.
9. Read the plate absorbance after incubation at 340 nm. This is ABSORBANCE 2.
10. Correct each absorbance (ABSORBANCE 1 and 2) by subtracting the blank absorbance.
11. Calculate NET absorbance by subtracting ABSORBANCE 2 from ABSORBANCE 1.
12. Create a calibration curve for DiaSorin's Lp(a) standards. Use NET absorbance in the y-axis and Lp(a) standard concentration in the x-axis. Obtain a linear regression for this curve.
13. Use this linear regression to determine Lp(a) concentration in the serum sample.

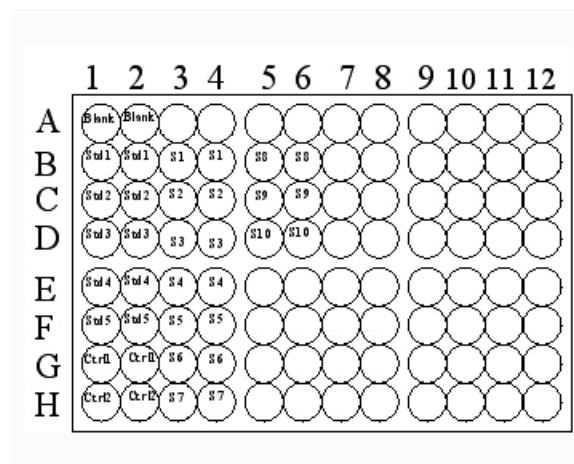


Fig. 57. Plate template for Lp(a) kit

Differential Density Lipoprotein Profiling (DDLDP)

This protocol is to be followed to obtain a Lp(a) Differential Density Lipoprotein Profile (DDLDP). Lp(a) is removed by carbohydrate affinity using the lectin WGA. The lipoprotein profile obtained from this sample (Lp(a)-depleted) is compared with a serum lipoprotein profile for the same sample (Lp(a)-control). The difference represents a Lp(a) DDLDP.

Materials PBS-p = PBS-200 mM L-Proline

PBS-g = PBS-200 mM glucosamine

Agarose-coupled WGA (Sigma, 6.72 mg Lectin/mL packed gel)

1 mL eppendorf tubes

10,000 molecular weight cut-off filter

Procedure

1. In a 1 mL eppendorf tube prepare Lp(a)-control samples and Lp(a)-depleted samples by duplicated.

Lp(a)-control	Lp(a)-depleted
50 μ L serum	50 μ L serum
50 μ L WGA (shake well before using)	
100 μ L PBS-p	150 μ L PBS-p

2. Vortex samples briefly to homogenize the solution. Incubate at room temperature for 30 minutes in the M-60 Orbital shaker set to 13,000 rpm.

3. Centrifuge ALL samples for 3 minutes at 6,000 rpm. This will sediment the WGA-Lp(a) complex. (*Lp(a) can be recovered from this complex, see the Lp(a) extraction from serum protocol*).
4. Discern the volume of supernatant present in the Lp(a)-depleted samples (approximately 240 μ L). Transfer the supernatant (Lp(a)-depleted sample) to the top chamber of a 10,000 molecular weight cut-off filter.
5. For Lp(a)-control samples, transfer the SAME volume as the Lp(a)-depleted samples (from step 4) to the top chamber of a 10,000 molecular weight cut-off filter.
6. Centrifuge ALL samples for 5 minutes at 10,000 rpm. Add 150 μ L PBS-p to the top chamber of the filter. Repeat twice.
7. Recover the samples by inverting the filter into a new bottom chamber and centrifuge for 8 minutes at 10,000 rpm.
8. Decipher the volume of sample recovered, and complete to 1200 μ L with the density gradient medium.
9. Stain for 30 minutes with 10 μ L 1 mg/mL NBD.
10. Follow protocol for Ultracentrifugation and profiling.

Profiling for DDLP

1. Profile each sample using Origin 7.0.
2. Duplicate the template spread sheet in origin (right click on the blue border, select *duplicate*).

3. Keep the following columns: Row, Tube and Density. Delete the rest.
4. Add two columns and label them *Control1* and *Control2* respectively. Add a third column and label it *ControlAvg*.
5. Copy and paste the last column of the profile from each Lp(a)-control sample into the columns just created in the new spread sheet (*Control1* and *Control2*).
6. Average these two columns in the *ControlAvg* column. (Right click on the column title, select *Set Column Values*, and type in: $((col(Control1)+col(Control2))/2)$. Click OK.
7. Repeat the same procedure (steps 2 to 6) for the Lp(a)-depleted samples *WGA1*, *WGA2* and *WGAAvg*.
8. Duplicate a spread sheet, keep the Row, Tube and Density columns. Add three new columns *ControlAvg*, *WGAAvg* and *DDLp*.
9. Copy and paste the *ControlAvg* and *WGAAvg* columns into the newly created spread sheet.
10. Set the *DDLp* column to subtract the *WGAAvg* column from the *ControlAvg* column.(Right click on the column title, select *Set Column Values*, and type in: $((col(ControlAvg)-col(WGAAvg)))$).
11. Create a graph that contains the Lp(a)-control average profile, Lp(a)-depleted average profile and Lp(a) DDLp.

Lp(a) Extraction from Serum by Lectin Affinity

This protocol is to be followed for the preparation of Lp(a) samples to be used in secondary characterization studies such as Capillary Electrophoresis and Mass Spectrometry.

1. In a 1 mL eppendorf tube dilute 150 μL of serum with 150 μL PBS-200 mM L-Proline
2. Add 100 μL WGA (or the equivalent to 672 mg lectin/gel) and vortex briefly to homogenize the sample.
3. Incubate at room temperature under constant shaking. Set the M-60 orbital shaker at 13,000 rpm and 30 minutes.
4. Centrifuge the samples for 3 minutes at 6,000 rpm to sediment the WGA-Lp(a) complex.
5. Carefully remove the supernatant from the sample (approximately 260 μL of supernatant will be present).
6. Add 150 μL PBS-200 mM L-proline to the tube containing the WGA-Lp(a) complex. Mix the contents by pipetting them up and down (use a BLUE pipette tip for this).
7. When a homogeneous sample is obtained, transfer the sample to a 45 μm filter using the same blue pipette tip.
8. Add another 150 μL aliquot of PBS-200 mM L-proline to the eppendorf tube. Mix well and transfer the rest of the WGA-Lp(a) complex to the 45 μL filter. By now, the top chamber of the filter should contain 300 μL of PBS-200 mM L-proline and most of the WGA-Lp(a) complex.

9. Centrifuge for 30 seconds at 6,000 rpm. Discard bottom chamber.
10. Add 150 μL PBS-200m mM L-proline to the top chamber. Centrifuge for 30 seconds at 6,000 rpm. Repeat twice.
11. Discard bottom chamber and replace it with a clean 1 mL eppendorf tube. Add 300 μL PBS-200 mM glucosamine to the top chamber. Cap the filter (use parafilm for this if the original cap was removed).
12. Vortex briefly to homogenize the sample. Incubate at room temperature under constant shaking. Set the M-60 orbital shaker at 13,000 rpm and 30 minutes.
13. Centrifuge for 30 seconds at 6,000 rpm. Lp(a) in PBS-200 mM glucosamine is recovered in the bottom chamber (filtrate).

Lp(a) Purification by Ultracentrifugation

Lp(a) that was recovered from the WGA-Lp(a) complex is purified by ultracentrifugation in a density gradient. PBS-200 mM glucosamine has to be removed from the Lp(a) sample by ultracentrifugation and replaced with the density gradient solution.

1. Transfer 300 μL of the Lp(a) solution recovered from WGA to a 100,000 molecular weight cut-off filter.
2. Centrifuge for 3 minutes at 6,000 rpm. Discard filtrate (bottom chamber).
3. Add 400 μL of density gradient. Repeat until initial sample volume has been replaced 2.5 times.
4. Concentrate sample by centrifuging for 7 minutes at 6,000 rpm.
5. Recover Lp(a) sample by inverting the top chamber of the filter into a clean bottom chamber and centrifuging for 8 minutes at 10,000 rpm.

6. Complete the sample volume to 1200 μL with density gradient medium.
7. Add 10 μL of 1 mg/mL NBD and vortex.
8. Stain for 30 minutes at 5°C for NaBiEDTA or room temperature for sucrose gradients.
9. Aliquot 1000 μL of stained sample to a ultracentrifuge tube. Follow UC protocol established for the density gradient medium used.
10. After ultracentrifugation, layer each tube with 150 μL of dH_2O . Proceed to tube imaging.

Imaging of UC tubes for Lp(a)

1. Use the Optronics Microfire Camera (System II) using the BG12 filter for NBD excitation and OG515 for emission.
2. Increase the exposure time from 15.8 ms to 50 ms. This will increase the Lp(a) peak fluorescent intensity. Keep the exposure time at 15.8 if sample is to be compared with a lipoprotein density profile.
3. All other settings are to be kept the same. Target intensity of 30%, Gain of 1.000, MONO and 12 Bit Snap adjustment.
4. Take the picture and obtain a profile it using Origin 7.0

Differential Density Lipoprotein Profiling Flow Diagram

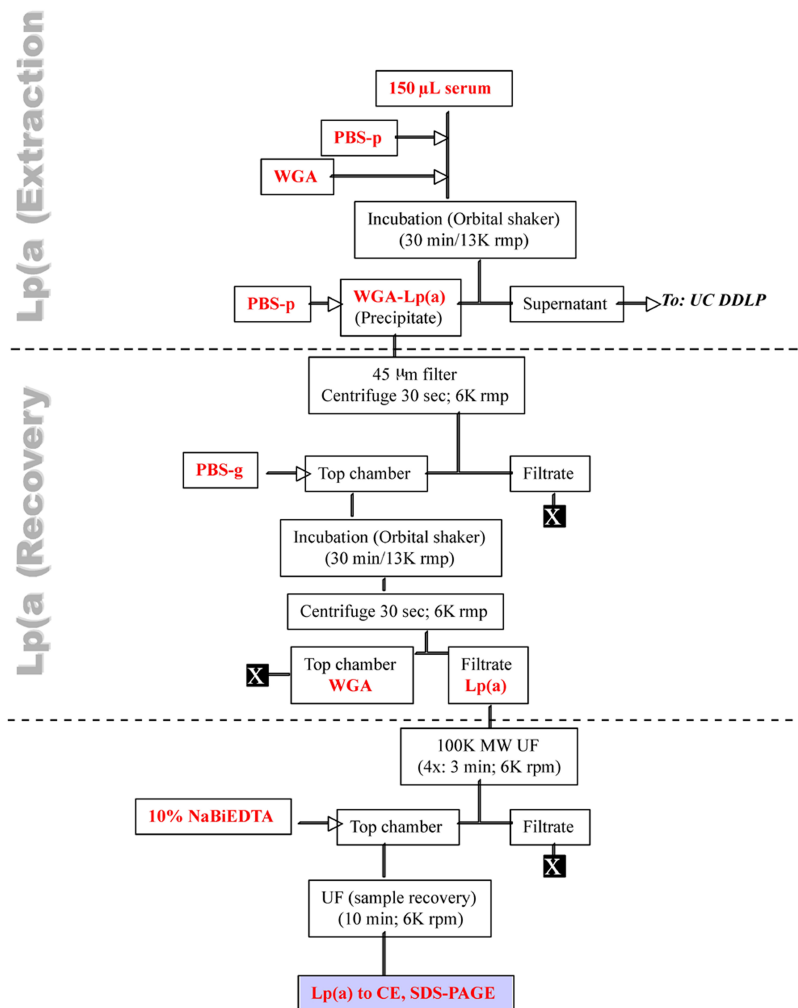


Fig. 58. Flow diagram for Lp(a) Differential Density Lipoprotein Profiling

Exporting CE Electropherograms into Origin 7.0

This protocol explains how to export CE electropherograms from P/ACE to obtain the electropherogram in Origin 7.0.

Setting P/ACE for Exporting Electropherograms

1. In the offline feature go to *Method* then *Export Options*. Select **Channel A** and check on **Electropherogram**.
2. Select to which drive/file the electropherogram is going to be exported (bottom of the export window).
3. Click OK.

The electropherogram will be exported as a TEXT file to the drive/file specified by the user. Exporting of the file will take place every time the *Analyze* icon is clicked, however the analysis (peaks time, mobilities, area, etc.) **WILL NOT** be exported.

The file name of the exported electropherogram will be the same as the file name given to the Analysis being exported.

Importing Electropherograms into Origin 7.0 and Analysis

1. Open a new origin worksheet.
2. Click on the *Import ASCII* icon.
3. Browse for the file containing the exported CE file and import it. This will result in two filled columns. One is labeled *Version(X)* and the other *B(Y)*.
4. Create a new column between *Version(X)* and the other *B(Y)*.

5. Right click on the top of the *Version* column. Select the *Properties* option at the bottom of the list.
6. Change the *Plot Designation* to **Disregard**. Click *Next*.
7. Change the newly inserted **column A** *Plot Designation* to **Y**. This will be the Y-axis of the electropherogram.
8. Change the **column B** *Plot Designation* to **X**. This will set this column as the X-axis in the electropherogram.
9. Click OK to exit that window.
10. Set the *Column Values* of column **A(Y)** to: $col(Version)/1000$. This will scale the Y-axis to mAU.
11. Set the *Set Column Values* of column **B(X)** to $i*0.01668$. This will scale the X-axis to time in minutes.
12. Plot columns **A(Y)** and **B(X)**.
13. Check if the peak EOF peak time is the same as in the original CE electropherogram. Correct the scale if necessary by adjusting the column B Values (see step 11).
14. Go to *Tools*, then *Baseline* and create a baseline. You can create it automatically or using the *User Define Equation* setting *y* to the y-axis value wanted as baseline. Finally click on *Subtract*.
15. Go to the second tab in the *Baseline* window and click on *Set Peaks*. This will label each peak with a value corresponding to their position related to the X-axis (peak time).

16. Go to the bottom of the Origin window and double click on the file labeled *BsPeak*.
17. Scroll to find the column labeled **PosPkL**. This column contains the peak times generated in the previous step. Delete any values before the EOF. The last two digits of every value can be deleted as well.
18. Go to **Layer 1** in the window where the electropherogram was plotted. Remove the *Baseline* and *bspeak_negpk* values.
19. Double click on one of the peak labels. A window will open, rotate the peak 90°, and set the *Y offset* to 170. Click OK

The electropherogram generated in Origin can be exported as a Figure file.

Protocol for Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

This protocol was elaborated for the separation of apo(a) from other serum proteins after extraction from serum by WGA. A NuPAGE Tris-Acetate SDS-PAGE gel (3-8%) was used. The power unit was set at 150 V constant. NuPAGE sample buffer and running buffer were used for electrophoresis. All products except for the samples were purchased from Invitrogen. The XCell SureLock Mini-Cell was used for all SDS-PAGE experiments (Invitrogen, Catalogue numbers EI0001, EI0020 and EI0002).

1. Prepare the samples for electrophoresis by mixing 10 μL of sample with 2.5 μL of sample buffer and 3 μL 10% mercaptoethanol. Heat the samples at 90°C for 7 minutes.
2. Remove gel from package, remove sticker at the bottom (foot) of the gel and place it in the electrophoresis unit.
3. Fill the upper buffer chamber (chamber formed behind the gel), with 200 mL of running buffer. Make sure to completely cover the sample wells.
4. Make sure that the upper buffer chamber is not leaking.
5. Load samples to the gel wells. Use a long pipette tip for this purpose. Lower the tip to the bottom of the sample well and slowly pipet sample into well without contaminating another well with the sample. Load sample buffer in all wells, whether or not they contain sample.
6. Fill the Lower Buffer Chamber (anode) with 600 mL of running buffer.
7. Close the electrophoresis system. Plug the connections to the power unit.

8. Start program #5. This program was set for apo(a) electrophoresis.
9. Stop the run at 1.20 hours or when the dye front is close to the foot of the gel.
10. Remove the gel from the electrophoresis unit, rinse with water. Remove the gel from the gel cassette.
11. Stain with Coomassie Blue (follow the bottle instructions) or prepare the gel for Western Blot.

Protocol for Western Blot Transfer [126]

This protocol was used for the transfer of 1-D electrophoresed gels using the XCell II-Blot Module. Nitrocellulose membranes were purchased from Invitrogen (Catalogue number LC2001) with pore size of 0.45 μm .

1. Prepare the transfer buffer by mixing 50 mL NuPAGE Transfer Buffer (50X) with 100 mL methanol and 850 mL deionized H_2O .
2. Soak the blotting pads until saturated in the transfer buffer. Remove air bubbles by gently pressing with the finger.
3. Soak two filter papers and the transfer membrane in transfer buffer.
4. Remove the gel from the electrophoresis unit and plastic cassette. Remove wells on gel.
5. Place a pre-soaked filter paper on top of the gel. Lay just above the foot at the bottom of the gel without covering it with the filter paper. Remove any trapped air bubbles by carefully rolling a finger over the surface of the filter paper.
6. Turn the plate over so the gel and filter paper are facing downwards over a clean surface.
7. Carefully remove the gel from the plate.
8. Cut the foot of the gel.
9. Perform transfer immediately! Wet the surface of the gel with the transfer buffer and place a pre-soaked transfer membrane on the gel. Remove air bubbles.
10. Place a pre-soaked filter paper on the top of the transfer membrane. Remove air bubbles.

11. Place 2 soaked blotting pads into the cathode (-) core of the blot module (dark gray colored, deeper of the two cores).
12. Assemble the the gel membrane in the following order from bottom of the cathode to top: Blotting pads, filter paper, gel, transfer membrane, filter paper. Figure 59 shows the order described here.
13. Add two blotting pads on the top of the second filter paper, and place the anode (+) on the top of it.
14. Position the gel membrane sandwich, blotting pads, and cores vertically in the XCell II-Blot module. Secure it in place with the gel tension wedge.
15. Fill the blot module with transfer buffer until the gel/membrane sandwich is covered in transfer buffer. Do not fill al the way to the top.
16. Fill the outer chamber with deionized water.
17. Close the system, and plug it to the power unit.
18. Run program #7 for 1 hour (Set to 30 V constant).

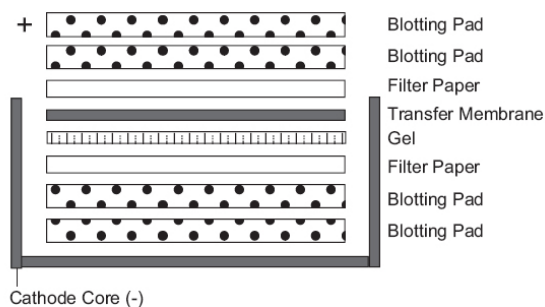


Fig. 59. Western transfer arrangement for the XCell II-blot Module

Protocol for Immunodetection of Apo(a) after Western Blot [120]

This protocol was used for the immunodetection of apo(a) following Western transfer. Invitrogen's WesternBreeze Chromogenic Western Blot Immunodetection Kit was used (Invitrogen, Catalog nos. WB7103, WB7105, WB7107). DiaSorin's monoclonal anti-Lp(a) was used as primary antibody.

Prepare the following solutions prior to immunodetection for nitrocellulose membranes:

- **Blocking Solution** (14 mL water, 4 mL Blocker/Diluent (Part A) and 2 mL Blocker/Diluent (Part B))
- **Primary Antibody Diluent** (10 μ L anti-Lp(a) and 10 mL Blocking Solution)
- **Antibody Wash** (150 mL water and 10 mL Antibody Wash Solution (16X))

Immunodetection Protocol

1. Remove membrane from gel/membrane sandwich.
2. Wash membrane for 5 minutes in water. Repeat twice.
3. Place the membrane in 10 ml of **Blocking Solution**. Incubate for 30 minutes on a rotary shaker set at 1 revolution/sec. Decant the **Blocking Solution**.
4. Rinse the membrane with 20 ml of **water** for 4 minutes. Decant and repeat once.
5. Incubate the membrane with 10 ml of **Primary Antibody Solution** for 1 hour. Decant.
6. Wash membrane for 5 minutes with 20 mL of **Antibody Wash**, decant. Repeat three times.

7. Incubate membrane in 10 mL of **Secondary Antibody Solution** for 30 minutes. Decant.
8. Wash membrane for 5 minutes with 20 mL of **Antibody Wash**, decant. Repeat three times.
9. Rinse the membrane with 20 ml of **water** for 2 minutes. Repeat twice.
10. Incubate the membrane in 5 mL of **Chromogenic Substrate** until purple bands develop on the membrane (10 minutes to 1 hour).
11. Rinse the membrane with 20 ml of **water** for two minutes. Repeat twice.
12. Dry the membrane on a clean piece of filter paper to open air. Scan membrane to record results

VITA

Irma Leticia Espinosa Garcia received her B.A. in chemistry at Instituto Tecnológico y de Estudios Superiores de Monterrey (ITESM), Campus Monterrey, México in June 2000.

In 2000 she was accepted at Texas A&M University as a Ph.D. student in analytical chemistry. In that same year she joined Professor Ronald D. Macfarlane's Cardiovascular Chemistry Lab at Texas A&M University. During her graduate studies, she developed methods for the analysis and characterization of lipoprotein(a) in human serum, including separation by carbohydrate affinity, purification by ultracentrifugation and characterization by SDS-PAGE and capillary electrophoresis.

In 2005 she was awarded a Business Certification from Mays School of Business at Texas A&M University. In that same year she successfully completed a summer professional internship at Procter & Gamble Co. in Cincinnati, Ohio.

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- Martell Travel Award. Department of Chemistry, Texas A&M University. February 2006
- Graduate Student Council. Graduate Research Week award. Poster presentation. Texas A&M University, March 2005
- Graduate Student Council. Graduate Research Week award. Oral presentation. Texas A&M University, Spring 2004

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