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Review

Advances in agarose gel electrophoresis of serum lipoproteins

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Abstract

Agarose gel electrophoresis has been extensively employed by researchers to gain a greater understanding of lipoprotein biology and its relationship to cardiovascular disease. Advances in this technique have been made in the visualization and quantitation of separated lipoproteins, in the use of agarose gel electrophoresis for detection and quantitation of apolipoproteins of the separated lipoproteins, and in the detection of lipoprotein heterogeneity. Agarose gel electrophoresis has been employed for two-dimensional electrophoretic analysis of lipoproteins as well as in several different methods which probe the immunological properties of lipoproteins. Agarose gel electrophoresis has thus become an important tool in the study of serum lipoproteins in both clinical and basic science laboratories.

Contents

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1. Introduction

Electrophoresis in agarose gels is a universally employed technique for the characterization of serum lipoproteins. This review is intended to familiarize the reader with some of the newer applications of this methodology. It does not include the vast literature in which agarose gel electrophoresis is used to diagnose hyperlipidemias or to detect "novel" lipoproteins. For this information, the interested reader is referred to the classic text on hyperlipidemias [1]. Also, this review does not discuss in detail the refinements of methodology that permit better visualization and quantitation of lipoproteins in agarose gels.

2. General principle of agarose gel electrophoresis

In 1968, Noble [2], Rapp and Kahlke [3], and McGlashan and Pilkington [4] introduced the use of agarose gel electrophoresis for the separation and characterization of serum lipoproteins. Briefly, Noble [2] electrophoresed serum lipoproteins for 2 h in a gel composed of 0.6% agarose, 0.5% bovine serum albumin in 50 m*M* sodium barbital buffer, pH 8.6, and spread on a Cronar polyester film strip. The inclusion of albumin was to broaden the lipoprotein bands and eliminate the irregularities in their shape. In other experiments, agar was added to the agarose to help harden the gel. After the electrophoretic period, the gel strip was fixed and dehydrated in a solution containing 5% glacial acetic acid and 75% ethanol, and finally dried in an 80°C oven. The pattern of lipoprotein bands was visualized after staining with the fat stains, Sudan Black B and Oil Red O, and lipoprotein content was determined by densitometry [2,5–7]. Alternatively, the individual lipoprotein bands could be cut from the gel and analyzed either for dye content [8] or cholesterol [9]. In most cases, the resulting electropherograms showed three distinct bands; a β (low-density lipoprotein, LDL), a pre-β (very-low-density lipoprotein, VLDL), and an α band (high-density lipoprotein, HDL), all migrating anodically with the α band moving the greatest distance (Fig. 1). (The migration of LDL and HDL in agarose has recently been shown to be directly related to their surface charge [10]). Chylomicrons did not enter the gel and stayed at the origin. Agarose gel was preferred to paper as medium for electrophoresis because the pre-β band in the former was clearly defined [2]. While numerous changes in the methodology of agarose gel electrophoresis (including changes in the type of buffer, agarose, and even the solvent for the lipid dye) [11–14] have been suggested over the last 25 years, the work of Noble [2] still forms the basis for the agarose gel electrophoresis of serum lipoproteins.

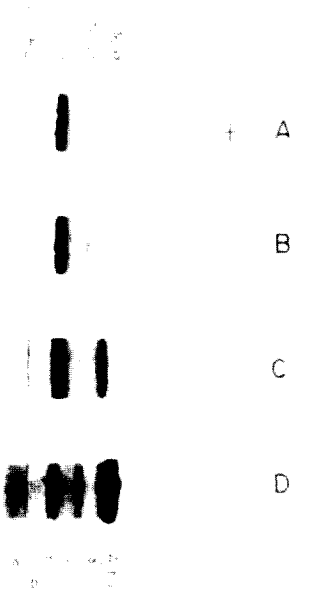

Fig. 1. Lipoprotein patterns of normal human serum by agarose gel electrophoresis. (A, B) adult fasted males, (C) adult nonfasted female and (D) normal serum stained for protein. A–C were stained with Oil Red O. From Ref. [56].

Application of agarose gel electrophoresis to the study of LDL was stimulated by the hypothesis that modification of LDL is important in atherogenesis [15,16]. Acetylation [15] and oxidation [16] of LDL have been shown to produce a lipoprotein that, unlike native LDL, is capable of being avidly endocytosed by macrophages.

One characteristic which is seen with modified LDL is increased migration upon agarose gel electrophoresis. This enhanced electrophoretic mobility is caused by the binding of adducts to the lysine residues of the LDL protein forming a more negatively charged lipoprotein [16]. The need to monitor the degree of LDL modification has increased the use of agarose gel electrophoresis in lipoprotein research.

3. Methods to detect and quantitate lipoprotein and cholesterol content after agarose gel electrophoresis

Since Noble [2] employed Oil Red O and Sudan Black B to stain lipoproteins, there have been several other methods introduced to visualize and quantitate serum lipoproteins in situ after agarose gel electrophoresis.

3.1. Precipitation of lipoproteins in gels with polyanions

In 1973 Wieland and Seidel [17] described an ingenious method in which lipoproteins that have undergone electrophoresis can be precipitated with polyanions in situ to facilitate their visualization under oblique light. It was found that in the presence of heparin, magnesium chloride and sodium chloride, VLDL and intermediate-density lipoprotein (IDL) can be selectively precipitated (LDL and HDL do not form precipitates under these conditions). This method allowed for the direct determination of Type III hyperlipoproteinemia, a dyslipoproteinemia in which VLDL co-migrates with the β band. To visualize LDL and HDL, these lipoproteins were selectively precipitated by treating gels with a solution of calcium chloride and dextran sulfate [17]. Using selective precipitation, Wikinski et al. [18] were able to separate IDL and β-VLDL lipoproteins from LDL and electrophorese LDL from the β-zone. (LDL, but not β-VLDL and IDL, is still able to migrate in the agarose gel since the polyanion solution does not neutralize the electrical charge of this particle). Lipoprotein bands were cut out of the gel and cholesterol was quantitated in each lipoprotein band by enzymatic assay [19] or the ferric chloride method [9,18]. A commercial product has been developed (Lipidophor system) to precipitate the major lipoprotein classes with phosphotungstate, magnesium chloride and sodium chloride. Quantitation is performed by measuring turbidity in a densitometer and converting these measurements by algorithms to lipoprotein cholesterol values [20].

3.2. Determination of glycated lipoproteins

Non-enzymatic glycation of serum lipoproteins occurs in diabetic patients. LDL isolated from diabetic patients has an enhanced electrophoretic mobility on agarose gels [21]. To visualize and quantitate glycated lipoproteins, Kobayashi et al. [22] overlaid the electrophoresed agarose gel with a cellulose acetate membrane that had been soaked with nitroblue tetrazolium, a dye which has been extensively used as an indicator of the reducing activity of glycated proteins. The resulting blue-stained lipoprotein bands were quantitated by densitometry at 545 nm. The concentrations of glycated HDL, LDL and VLDL lipoproteins were found to be greater in diabetic patients when compared to normal subjects. This technique provides a new way to monitor the glycation of lipoproteins in diabetes.

3.3. Staining of lipoproteins with nile red

Nile red, a fluorescent lipid dye is known to fluoresce in the presence of lipoproteins [23]. Nile red, when added to the lipoproteins prior to electrophoresis, does not alter their electrophoretic migration. An important benefit of nile red staining is that the stained lipoproteins fluoresce red under UV light and can be easily visualized with the aid of an UV transilluminator. The electrophoretic run can be interrupted and the pattern of the lipoproteins monitored by placing the gel under UV light. Should additional separation of lipoproteins be required, the electrophoretic run can be resumed. A similar prestaining of lipoproteins with Sudan Black B has also been reported [9]. The major drawback to vis-

ualizing lipoproteins by nile red is that this dye also fluoresces in the presence of hydrophobic proteins. For this reason, nile red is best suited for analyzing the pattern of isolated lipoproteins in gels free of serum albumin. The best application of this method is in the determination of the electrophoretic mobility of modified LDL.

3.4. Determination of cholesterol content of lipoproteins in agarose gels by enzymatic assays

In 1979, Conlon et al. [24] and Stein et al. [25] reported that HDL cholesterol can be quantitated in serum subjected to agarose gel electrophoresis. This technique was developed from a similar method using cellulose acetate electrophoresis [26]. Serum is first electrophoresed in an agarose gel and then the gel is treated with a solution containing cholesterol esterase, cholesterol oxidase, horseradish peroxidase, phenol and aminoantipyrine. A quinoneimine dye is generated by the enzymic cholesterol reagent and stains the HDL band which then becomes clearly discernible and measurable by densitometry. While Conlon et al. [24] found an excellent correlation between this methodology and a standard technique for determining HDL-cholesterol, Stein et al. [25] found that this methodology, though promising, cannot replace the standard heparin–manganese precipitation procedure. An alternate enzymatic staining procedure for quantitating cholesterol in all lipoprotein classes (employing cholesterol esterase, cholesterol dehydrogenase, phenazine methosulfate and 4-nitroblue tetrazolium chloride to form a purple formazan compound) was reported several years ago [27].

4. Quantitative transfer of electrophoresed lipoproteins from the agarose gel to nitrocellulose

A recent advance in lipoprotein methodology has been in the quantitative transfer of individual lipoprotein fractions from agarose gel electropherograms for the measurement of apolipoproteins. The electrophoretically separated lipoproteins are quantitatively transferred to nitrocellulose by either electrophoresis [28] or by capillary blotting [29]. The nitrocellulose blots are then treated with an antibody to a specific apolipoprotein. Apolipoproteins are measured in these blots from the binding of radiolabeled protein A [28] or conjugated secondary antibody [30]. Quantitation of the blot can also be performed by chemiluminescence [29]. This transfer technique has been used to detect pre-β-migrating HDL containing apolipoprotein A-I [28,30] and to understand the role of apolipoprotein A-I in cholesterol efflux from cells [31]. Ishida and coworkers [28,30] also analyzed plasma apolipoprotein A-I by two dimensional electrophoresis and immunoblotting. The molecular size of apolipoprotein A-I of the pre-β migrating band could be determined by first subjecting plasma to agarose gel electrophoresis followed by electrophoresis in a second dimension into a linear non-denaturing polyacrylamide gradient gel.

The transfer of lipoproteins to nitrocellulose has been employed to detect and quantitate serum lipoprotein *a* [Lp(a)], a lipoprotein which has been shown to be a risk factor for coronary atherosclerosis. Duvic et al. [32] demonstrated the combined use of agarose gel electrophoresis and immunoblotting in detecting the pre-β migrating Lp(a). Recently, Kawakami et al. [33] electrophoresed serum lipoproteins in an agarose gel containing 0.6% agarose, 3% sucrose and 0.05% albumin and found a distinct extra band between the classical β and pre-β zones. When the lipoproteins were transferred to a nitrocellulose membrane and analyzed for Lp(a) content, this band stained for Lp(a) as well as reacted with anti-LDL and anti-plasminogen antibodies.

4.1. Electrophoresis in presence of sodium dodecyl sulfate

In a newly described variant of the classical methodology, a high-resolution method has been developed to screen apolipoprotein *a* polymorphism and different apolipoprotein B species [34,35] using immunoblotting after agarose gel electrophoresis. To achieve resolution in both cases, sodium dodecyl sulfate (SDS)-treated samples were subjected to electrophoresis in a 1.5% agarose containing 0.1% SDS [35] or 0.2%

SDS [34]. This technique resolved 23 apolipoprotein *a* isoforms [35] and the different apolipoprotein B species [34]. This recently introduced technique is potentially an important tool for the analysis of apolipoprotein heterogeneity.

In addition to the electrophoretic transfer of lipoproteins from an agarose support to nitrocellulose, electroelution of lipoproteins from agarose gels has been performed to isolate specific lipoproteins [28], though the recovery from the gel may be unsatisfactory. Finally, though beyond the scope of this review, apolipoprotein phenotypes can be also be determined by electrofocusing and immunoblotting/immunofixation techniques employing agarose gels [36–38].

5. Electrophoresis of lipoproteins in gradient acrylamide–agarose gels

Single density polyacrylamide gel electrophoresis has been used to separate serum lipoproteins [39,40]. In addition, human serum lipoproteins have been reproducibly separated into as many as 15 bands on continuous (3.5–8%) gradient gel columns using acrylamide concentration gradient electrophoresis [41,42].

The use of a discontinuous acrylamide–agarose gel to separate lipoproteins has also been reported [43]. Employing a 2% acrylamide stacking gel and 3% acrylamide resolving gel (both gels containing 0.7% agarose), Moulin et al. [43] found that VLDL was retarded from entering the resolving gel where LDL and HDL were separated. Using a refinement of this method, Lee et al. [44] developed an exponential gradient gel of 0–10% acrylamide and 0.5% agarose for resolving the major lipoprotein classes (VLDL, IDL and LDL) into three separate bands which can be further analyzed by immunoblotting. This hybrid electrophoretic method may prove to be extremely useful for examining the heterogeneity of lipoproteins.

6. Immunoelectrophoresis using agarose gels

Several immunoelectrophoretic methods have been developed using agarose gels to quantitate content of apolipoproteins as well as to examine lipoprotein heterogeneity. Some examples of these techniques are discussed below.

6.1. Immunoelectrophoresis of lipoproteins

This method is performed by first electrophoresing the lipoproteins in agarose gels and then placing immune sera to the specific apolipoprotein in troughs that run in the direction of the electrophoresis. The formation of immunoprecipitation lines locates the apolipoprotein among the resolved lipoproteins [45,46]. In the case apolipoprotein A-I, this technique was employed to detect the presence of apolipoprotein A-I in lipoproteins with pre-β electrophoretic mobility [46] and to characterize the apolipoprotein A-I containing lipoprotein produced by a hepatoma-derived cell line [47].

6.2. Electroimmunoassay

This method depends on the electrophoresis of plasma or isolated lipoproteins into a gel which is composed of agarose (approximately 1–2%), dextran, and an antibody to a specific apolipoprotein. Dextran is added to the gel to enhance the immunoprecipitation process. After electrophoresis, the immunoprecipitates are stained for protein with Coomassie Brilliant Blue and are quantitated by measuring the height of the peak or rocket. Curry et al. [48] employed a rocket electroimmunoassay to measure serum apolipoproteins A-I and A-II without adding detergents or delipidating the samples. Although some investigators may prefer pretreatment of lipoproteins prior to electroimmunoassay, this technique remains a standard laboratory procedure for the quantitation of many different apolipoproteins.

6.3. Two-phase electroimmunoassay

In this method, plasma or lipoproteins are electrophoresed in a 1% agarose gel which contains an antiserum to one apolipoprotein in the lower half of the gel (closer to the origin) and another antiserum to a different apolipoprotein in the upper half of the gel. The resulting

precipitin lines can be used to identify heterogeneity of lipoproteins. Atmeh et al. [49] used this technique to identify subpopulations of HDL by placing anti-apolipoprotein A-I in the upper zone and anti-apolipoprotein A-II in the lower zone. The results demonstrated that certain HDL species did not contain apolipoprotein A-II.

6.4. Two-dimensional immunoelectrophoresis

This technique has been employed to examine the heterogeneity of lipoproteins [50–53]. Lipoproteins are first electrophoresed in agarose gels [50,51] or in polyacrylamide gels [52]. The gel is then usually cut and an agarose gel containing an appropriate antiserum is cast around the selected segment of the cut gel. After electrophoresis in the second direction, the gels are visualized by protein staining and can be quantitated by measuring peak height. This method has been used to demonstrate the presence of "free" apolipoprotein A-I in serum [54]. While apolipoprotein A-I is associated with HDL in normal fasting sera, individuals with hypertriglyceridemia have a significant portion of their apolipoprotein A-I associated the pre-β band (free A-I) [54].

In another type of "cross immunoelectrophoresis", the lipoproteins are electrophoresed in the second direction into zones of agarose composed of different antisera. By changing the order of the zones which are in proximity to the initial electrophoresed gel, it is possible to demonstrate the heterogeneity of lipoproteins based on apolipoprotein content. In the case of HDL, it was possible to identify lipoprotein particles which contain only apolipoprotein A-I and particles containing both apolipoprotein A-I and A-II [51]. A modification of this technique has been used to examine the size heterogeneity of Lp(a) [53].

In summary, the electrophoresis of lipoproteins has been widely employed to quantitate apolipoprotein content both of whole serum as well as of specific lipoprotein fractions. In addition, the heterogeneity of lipoproteins has been probed with immunochemical techniques using agarose gel electrophoresis. Other techniques will also continue to facilitate investigation of lipoprotein biology. Capillary electrophoresis [55] is an important new method for the analysis of LDL and HDL. This isotachophoretic procedure with a discontinuous electrolyte system gives rapid (less than 12 min) resolution and clear detection of serum lipoproteins.

7. Conclusions

This review was written to familiarize the reader with some of the various ways agarose gel electrophoresis of lipoproteins have been employed to investigate serum lipoproteins. Its importance is demonstrated by the many uses developed for lipoprotein research during the past 25 years. It is our hope that the cataloging of these methodologies may assist the reader to choose and adapt those appropriate for their research area. In recent years, agarose gel electrophoresis has been especially helpful in examining lipoprotein heterogeneity. Agarose is by far the most popular medium for separating lipoproteins and it will probably continue as the most important medium for this purpose. Using the past as a guide, it is easy to predict that derivative methods will continue to flow from the basic methodology.

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