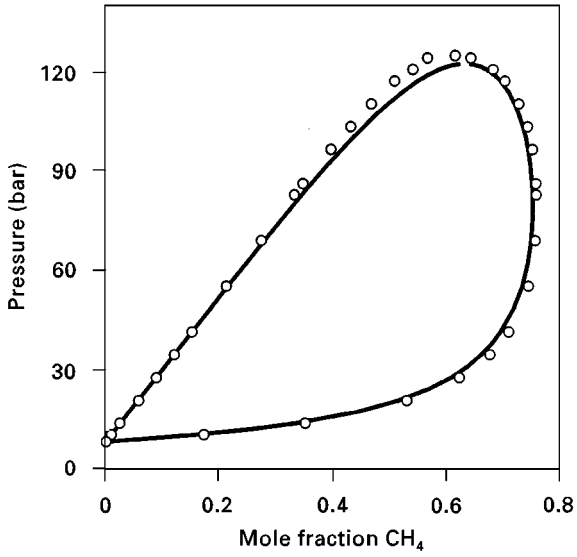

Figure 9 The *P–x–y* behaviour of methane-*n*-butane mixtures at 344 K predicted using the Patel–Teja equation of state with binary parameters obtained from data at 186 K.

parameters ($k_{12} = 0.143$ and $k_{21} = 0.121$) were obtained by fitting data at a much lower temperature of 244 K. The predictions are in excellent agreement with experiment, even though the extrapolation is to a temperature that is above the critical temperature of carbon dioxide (304 K). A similar extrapolation using the Patel–Teja equation of state is shown in **Figure 9** where VLE in the methane-*n*-butane system at 344 K have been predicted using binary parameters ($k_{12} = 0.021$ and $k_{21} = 0.002$) obtained at 186 K. Note that the extrapolation is carried out to a temperature that is well above the critical temperature of methane (190 K). Finally, it should be added that the activity coefficient approach described above cannot be used to correlate or predict supercritical VLE behaviour.

See also: **II/Distillation:** Historical Development; Modelling and Simulation; Multicomponent Distillation; Theory of Distillation; Vapour-Liquid Equilibrium: Correlation and Prediction.

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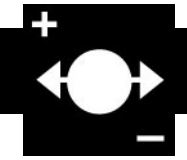
ELECTROPHORESIS



Agarose Gels

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Development

Agarose is a uniquely nonadhesive hydrocolloid that has found many uses in the separation sciences following Araki's preparation of it in 1937 as an apparently sulfate-free component distinct from the sulfate-rich agaropectin in agar. Agar and many of the other hydrocolloids derived from certain species of seaweed had been used mainly in food preparation dating back to the seventeenth century in Japan. Agar was introduced as a medium for immunoelectrophoresis by Grabar and Williams in 1953, and the original technique is occasionally used today. Citrated agar electrophoresis is the current principal method for identification of haemoglobin variants. Agar was

applied by Polson in 1961 for chromatographic separations based on molecular sieving, following which its use gradually gave way to agarose beginning with Hjertén. A word-search of the Medline abstracts indicates that the number of papers per year employing agarose electrophoresis did not surpass agar electrophoresis until 1976.

The sulfate content of grades of agarose supplied for laboratory use is generally below 0.12% compared to 4% in agaropectin (see Armisen, 1997). The ease with which agarose can be cast into gels without additives or cross-linking agents and its inertness towards interacting with proteins and nucleic acids are the principal reasons for its popularity as a separation medium. All that is needed to prepare gels is to dissolve the agarose powder by careful heating to boiling or near boiling temperatures, and let the clear solution cool. Gelation proceeds spontaneously and completely as the solution cools. In addition to the simplicity of gel casting with agarose and its inertness towards interacting with proteins and nucleic acids, it offers many other advantages as a separation medium, such as easy sample recovery, nontoxicity, and amenability to cast and use it in an open faced format. The latter option underlies its unique applicability to numerous immunoelectrophoretic procedures. Agarose is very porous compared to polyacrylamide, and that limits its suitability in unmodified form for sieving-based separations of proteins with molecular masses below 60 kDa. On the other hand, its high permeability makes it, again, superior to polyacrylamide for immunochemical studies.

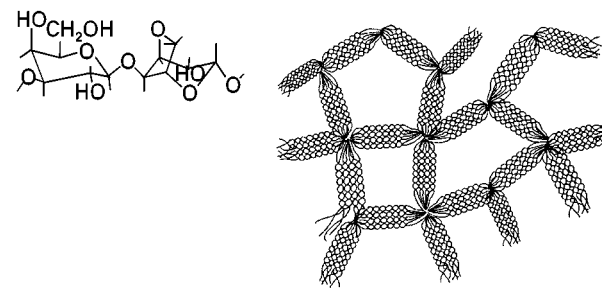

Figure 1 Depiction of structure of the agarose biose unit and the structure of interlocking aggregates of coiled coil dimers of agarose chains based on electron microscopy and X-ray diffraction. (Reproduced with permission from Westermeier, 1997.)

Properties of Agarose Gels

Chemically, agarose is a polysaccharide composed of alternating D- and L-galactose biose (agarobiose) units in chains that are on the order of 400 agarobiose units in length, ~120 kDa. Rees and Arnott each characterized the gelation as proceeding initially through pairing of the 120 kDa chains into 0.8–1.4 nm thickness double helical coils, followed by lateral coalescence of the helical dimers into thick protofibrils with average thickness of the order of 24 nm, although the number of helical dimers can range from 5 to 5000. By contrast, conventional polyacrylamide gels have fibre thickness of only 0.4 nm. Gelation occurs as the thick protofibrils interlink at their loosely coiled ends into a net-like matrix (**Figure 1**). The gels are translucent because of light scattering by the thick fibrils, but become transparent on drying. The ability to form thick, strong fibres enables agarose to form gels at concentrations down to 0.2% as utilized in studies by Serwer. The incorporation of the agarose into thick rather than thin fibres makes the gels highly porous at concentrations up to 6%, the limit to which agarose can be dissolved without resorting to autoclave temperatures. Concentrations up to 16% can be reached by autoclaving.

The high porosity makes agarose superior to polyacrylamide for separation of large proteins, polypeptides, complexes with size ranging from 100 kDa to several megadaltons, and for DNA fragments ranging from 1000 to 23 000 bp. In the interest of retaining the gel casting simplicity of agarose while enhancing its sieving capacity, nonsupercoiling hydroxyalkylated agarose derivatives have been developed which, according to Chrambach, have fibre thicknesses of the order of 0.8 nm, approaching polyacrylamide, but have gel strengths that are low compared to unmodified agarose. Recently, blends of agarose derivatives have been formulated which have improved strength.

The sulfate present in most commercial agarose induces a slight electroendosmosis (EEO) during electrophoresis. Electroendosmosis is the flow of buffer through the gel. In effect it arises because the negatively charged sulfate groups in the matrix cannot move towards the anode which results in a compensatory pumping of buffer to the cathode. The slight effect on mobility of proteins is usually of little consequence; however, it can have pronounced effects on mobilities of buffer boundaries in discontinuous buffer systems used for sharpening bands in the applied samples. It can also induce syneresis and collapse of gels with discontinuous buffer systems due to unequal electroendosmosis across the buffer boundaries. Thus, some of the numerous buffer systems that have been described in the computer output by Jovin for polyacrylamide gels do not work well with agarose gels. Many do, nevertheless. The zero-EEO agarose which is used for isoelectric focusing has its electroendosmotic tendency neutralized by charge-balancing with electropositive groups. The additives in some

zero-EEO agaroses contribute to background staining.

The gelation of agarose depends entirely on hydrogen bonding, and is inhibited by chaotropic agents such as concentrated (~20%) glycerine or urea. Although the high concentrations of these substances usually used in biochemical procedures can block the gelation, they do not appreciably affect gels once solidified. As with all electrophoresis, concentrated ionic chaotropes such as guanidine-HCl and KSCN which are used as protein solubilizing and denaturing agents should be removed and replaced with either urea or SDS prior to electrophoresis. Since the aggregation of agarose is fully reversible, gels can melt under high current. Also, agarose gels are compressible, and tend to drop out of vertical electrophoresis cells unless supported. The ability using weights to mechanically compress agarose gels to within a few per cent of original thickness can be used to advantage. Gels containing more than 2.5% agarose will rehydrate to the original thickness within a fraction of an hour, but gels with 1% or less agarose have a greatly reduced tendency to rehydrate.

Agarose Derivatives

Numerous agarose derivatives have been described for affinity chromatography where addition of charged groups is not critical. All derivatives prepared for electrophoresis involve modifications not imparting electrical charges. As indicated earlier, hydroxyethylated (FMC BioProducts) and hydroxymethylated agarose (Hispanagar, SA) are low melting derivatives which form gels with thin fibres for enhanced sieving. These low melting agaroses have been used to impart sieving during capillary electrophoresis. Allylglycidyl agarose, a very low melting derivative, is frequently used in place of bis-acrylamide for cross-linking acrylamide into stronger gels. All of the commercially available derivatives yield gels with low melting points. That is because the agarose is derivatized in a molten state. As we learned in studies on alkylation of agarose with either glycidol or allylglycidyl ether from preparing glyoxyl agarose and its analogues, the hydroxyl groups involved in the hydrogen bonding functioning in gelation are protected during derivatization of the agarose in the gel state, and the derivatized agarose gel retains much of its original melting and gelling characteristics.

Glyoxyl agarose (oxidized glyceryl agarose) has acetaldehyde substituent groups which can be utilized to form Schiff's base linkages with protein amino groups. At pH < 8.5, these Schiff's base linkages are too dissociated to retard electrophoresis of the proteins. Once separated on the glyoxyl agarose gel, the proteins can be driven to bind to it, either reversibly by immersion in pH 10 buffer to suppress dissociation of the Schiff's base linkages or by immersion in buffer containing sodium cyanoborohydride which rapidly and specifically drives alkylation of amino groups of the protein with the aldehyde groups in the gel matrix. This functionality enables the gel to be used sequentially and repetitively as a protein-separating and immobilizing medium. It can also fix small peptides containing at least a single amino group, but does not fix nucleotides. By compositing it with a removable polyacrylamide filler, it can be used for separations over any molecular weight range.

Agarose/Polyacrylamide Composites

These media were constructed initially to provide a supporting agarose matrix for polyacrylamide at concentrations below 3–4% which do not form firm gels. The combinations can yield exceedingly strong gels (**Figure 2**) and provide facility to achieve a wide range of sieving characteristics. As described by Peacock and Dingman in 1968, strong gels are obtained only when formulated to allow the agarose to gel before the acrylamide polymerizes. By using either non-cross-linked polyacrylamide or cross-linkers such as alkali or periodate degradable 1,2-dihydroxyethylene-bis-acrylamide instead of the usual bis-acrylamide, the poorly-permeable acrylamide filler can be removed leaving a highly permeable agarose matrix to allow direct immunochemical characterization of the fixed components of the electropherogram.

Electrophoresis

Initial applications of agarose electrophoresis focused heavily on diagnostic separations of plasma proteins, particularly lipoproteins, with great improvement over results obtained with paper or with starch which had been used earlier. Straight analytical separations were followed by more definitive immunoelectrophoretic protein identifications. These separations were usually applied to native, undenatured proteins. Following introduction in 1967 of sodium dodecyl sulfate (SDS) by Shapiro, Viñuela and Miazel as a carrier enabling polyacrylamide-based separation of proteins according to molecular weight by imposing overwhelming negative charge over the original and by denaturing conformational differences between proteins, the practice was widely adopted as a rapid means for separating large-sized proteins on agarose. The converse approach of separating proteins according to differences in isolectric points

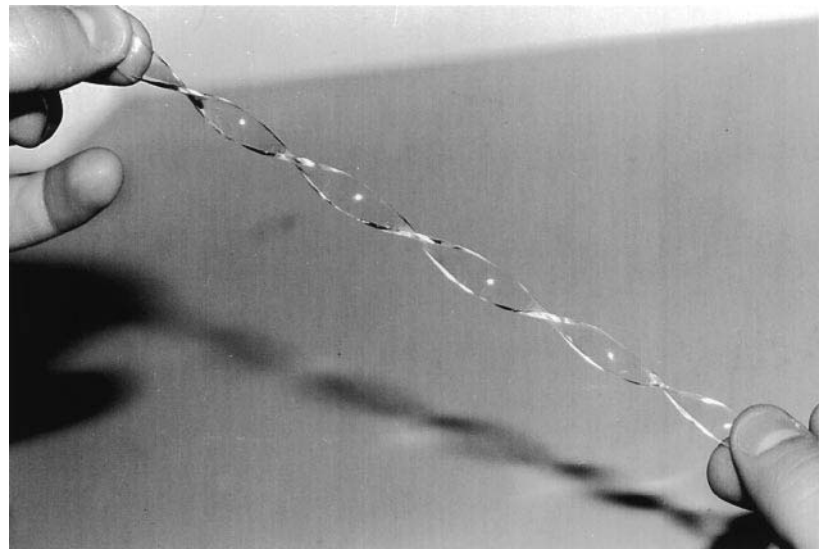

Figure 2 Demonstration of the strength of agarose/polyacrylamide composite gels. (Reproduced with permission from Shainoff, 1993.)

independent of molecular size by establishing a pH gradient across the gel was demonstrated in 1969 by Vesterberg, but a decade passed before the development of zero-EEO agarose enabled this separation method to be used with agarose.

Equipment and Buffer Systems

Agarose gels are usually used for electrophoresis on a horizontal platen on which the gels are placed between anodic and cathodic electrode chambers filled with buffer and connected to the gel with buffer-filled wicks. Except for the usual need to purchase a platen that can be cooled with circulating water, this is a simple arrangement that is easily set up, largely because of the ease with which agarose gels are cast either open faced or in cassettes consisting of two spacer-separated glass plates which can be separated from the gel because the gel is nonadherent. The use of wicks can be troublesome, because voltage is lost across them, and this produces much of the heating and vapour condensation in the chamber. Voltage drop across the gels must be gauged directly from the gel rather than the power supply. Condensation of vapour on the gel is easily prevented by simply laying a vapour barrier, usually GelBond® (FMC BioProducts), over the gel. For nucleic acid electrophoresis the use of wicks has been obviated by a technique known as 'submarine' electrophoresis in which the gel is laid into a buffer-filled platform connecting the electrode chambers. The submarine mode is usually not applicable to protein electrophoresis because proteins tend to diffuse into the surrounding buffer, while nucleic acids which have very low diffusion constants migrate almost entirely due to the applied voltage with little diffusion. Running agarose gels in vertical format with electrode chambers directly above and below the gel-containing cassette, as generally used with polyacrylamide gels, is seldom recommended because of the tendency of agarose to synerese and slip out of the cassette, a problem that can be prevented by use of etched rather than clear glass for the cassette. Slipping can also be prevented by partially immersing the cassette into dilute 0.5% agarose, and letting the agarose drain and dry before pouring the running gel.

Sample applications into agarose gels were initially made by either cutting or punching a slot or hole to receive the solution. This approach continues to be used with nucleic acids which form sharp bands as they migrate out of the nonrestrictive sample solution into the migration-restrictive gel, but comparatively little band sharpening occurs with proteins which easily permeate the gels. Further, the wells induce band distortions of proteins due: (i) to partial permeation into the sides of the wells prior to electrophoresis; and (ii) to uneven voltage drops across the wells. Because of the ease with which solutions can be imbibed into agarose following a temporary compression with blotting paper, samples can be drawn into the gels in nicely demarcated bands through a 'sample application foil', thin plastic overlays with slots through which the samples are drawn into the gels. Large samples need band-sharpening which can be achieved either mechanically by temporarily overlaying a dialysis membrane and thick gel to produce a sharp voltage drop near the origin, or by use of discontinuous buffer systems.

A method for sharpening bands at the beginning of runs at the outset of electrophoresis was devised by Ornstein and Davis using a discontinuous buffer system, 'disc electrophoresis', in which proteins and all but common electrolytes stack in hypersharp bands at the boundary between initial buffer ions producing low conductivity and secondary buffer ions producing high conductivity with a common counter ion. The discontinuous buffer systems worked predictably with over 7000 computer generated buffer systems based on the pK_a values of the leading and trailing buffering ions, as far as known, with polyacrylamide gels which produce no EEO, but only a few have been tested and found to yield operable systems with agarose, as already noted.

The ability to run agarose gels horizontally in a virtual open-faced format is its real advantage. That enables the gels to be cut and manipulated post-electrophoresis to conduct secondary electrophoretic operations with it. This facility has made it the usual medium for immunoelectrophoretic analyses.

Staining

With the exception of silver staining, all commonly used staining methods (Coomassie-based, fluorescent, chemiluminescent, and colloidal-type) work well with agarose, colloidal Coomassie being one of the fastest. An important consideration is to minimize exposure to acid which hydrolytically weakens the gel. Unlike polyacrylamide, agarose gels can be directly immunostained as illustrated (**Figure 3**), but the antibodies should be free of large entrapable aggregates.

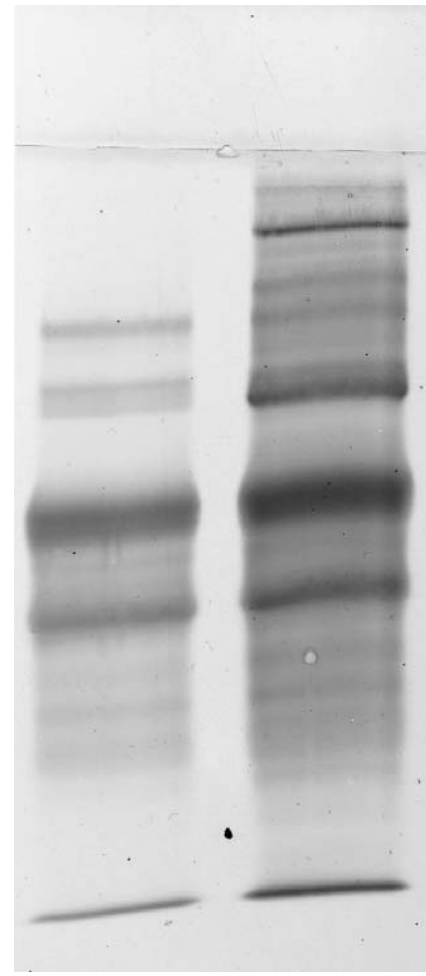

Figure 3 (See Colour Plate 39). Direct dual immunostaining of a polyacrylamide / glyoxyl-agarose composite gel to profile fibrinogen γ-chain (grey), α-chain (amber) cross-linking and hybrid α/γ-chain (umber) cross-linking by plasma transglutaminase (right lane), and the chain composition of plasma fibrinogen. The illustration depicts sieving equivalent to a regular polyacrylamide gel, and subsequent rendering of the gel for antibody permeation by removing the polyacrylamide. (Reproduced with permission from Shainoff *et al.*, 1991, *Journal of Biological Chemistry* 266: 6429.)

Immunoelectrophoresis

These methods are based on immune-precipitate (precipitin) formation at antigen/antibody equivalence, analogous to Ouchterlony immunodiffusional analyses in which antibody and antigen placed in separate wells in agar(ose) form precipitin arcs in the gel at position(s) depending on levels of antigen and antibody in the wells. In the method of Grabar and Williams the proteins under analysis are separated on agar gels, then lateral slots are cut approximately 8 mm to each side of the gel to accommodate antibody. After a day lateral diffusion of the protein bands towards the counter-diffusing antibody one or more precipitin arcs will form in the gel depending on the antibody and antigen heterogeneity. Quantitation of antigen/antibody by this method requires considerable effort and multiple runs. A simpler approach to quantitation was devised by Laurell, using a method known as 'rocket immunoelectrophoresis'.

In the method of Laurell, the gel is poured with antibody added to it at 56°C, and wells are punched in the solidified gel to accommodate the antigen solution. As the antigen moves by electrophoresis out of the well it sweeps soluble immune complexes along with it in a comet-shaped profile until the antigen/antibody levels become equivalent, whereupon the comet-shaped precipitin arc comes to a virtual stop because the antibodies themselves do not migrate at around pH 8.6. The area contained within the arc is usually directly proportional to the antigen level. Because of the ease with which agarose gels can be cut and filled with interposed gels open-faced, this technique is amenable to a myriad of variations, as described by Axelsen and associates.

In one variation of the Laurell method, called crossed immunoelectrophoresis, the proteins are subjected to pre-electrophoresis, and antibody-containing agarose is cast around a strip of the gel. Then, in a crossed electrophoresis the antigens are transferred out of the primary gel into the antibody where they form rocket(s) in line with their initial position. The technique is 'found' useful for quantifying multiple antigens and variant forms of an antigen. If the initial gel contains SDS, it should be quenched by adding nonionic detergent such as Lubrol® to the antibody-containing gel. In the event that the protein becomes insoluble when stripped of SDS, initially it can be fixed, then probed with charge-enhanced carbamylated primary antibody. Retained antibody is measured by electrophoresis into secondary antibody, as illustrated in **Figure 4**. Use of secondary antibodies is also essential when using monoclonal IgG antibodies as primary probes, because these monoclonal antibodies do not form immunoprecipitates on their own unless the epitope is multiply expressed in the antigen.

Direct Immunoprobing

Because of the permeability of agarose gels to antibody it is possible to probe electropherograms directly. Direct immunoprecipitation within the gel is seldom used because of uncertainties of levels or antibody required for substantial precipitation of unknown levels of antigen. The proteins are usually fixed or immobilized, and probed by imbibing labelled antibody into and out of the gel. If secondary antibodies are to be used to report retention of the primary antibody, the primary antibody should in turn be fixed before the secondary probing to avoid dissociative losses of the primary during the secondary probing which takes periods of the order of an hour. Glyoxyl agarose was developed to enable these fixations by chemical immobilization.

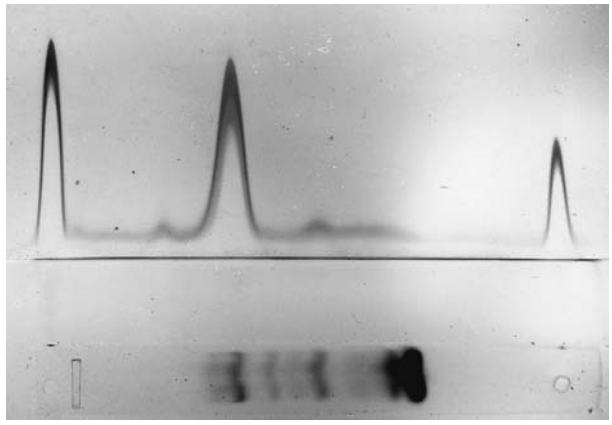

Figure 4 Crossed immunoelectrophoresis to profile plasma fibrinogen derivatives in plasma by: (i) probing the electropherogram with primary antifibrinogen antibodies; and (ii) measuring the retained antibody by displacing to form rockets in secondary gel containing anti-IgG antibodies, with standards for the IgG forming the left- and right-most peaks. This approach was made necessary because insolubility of fibrinogen and its high molecular mass derivatives, once denatured by SDS, cannot be transferred out of the primary electropherogram to form rockets directly. (Reproduced from Dardik *et al.*, 1989, *Cleveland Clinical Journal of Medicine* 56: 451.)

Blotting

This widely used procedure involves transfer of components out of the gel on to a blotting membrane to immobilize them for immunostaining or compositional analysis on an open-faced surface. It is an essential means for probing reactivities of components separated on polyacrylamide because of the low permeability of the gel. Much uncertainty attends this 'blotting', because the transfers are incomplete, and frequently nil with high molecular mass proteins. These proteins not only transfer slowly, but often precipitate within the gel as SDS transfers away from them. Also, very low molecular mass peptides fail to be retained by the blotting membrane.

While blotting from polyacrylamide gels is usually effected by crossed electrophoresis, the transfers out of agarose gels are more simply effected by either compressing them against the membrane supported on filter paper stacks (a method that yields only partial transfer), or by light vacuum suctioning of buffer through the gel on to the membrane supported on a 'gel drier'. However, when SDS is present it must either be quenched by a prior 15 min immersion in buffer containing 1–2% nonionic detergent, or precipitated by immersing the gel in 0.1 M KCl.

Affinity Electrophoresis

These methods use specific ligands, either added to the buffer or immobilized on the gel matrix, to induce shifts in mobilities or apparent concentrations of target proteins. Again, ability to work with open-faced gels to interpose ligand-rich zones makes it an ideal medium for these procedures, as devised with numerous examples from crossed immunoelectrophoresis. With glyoxyl agarose a ligand containing at least a single amino group can be imbibed into the gel through a mask and fixed in place.

Conclusion

Equipment and supplies for general electrophoresis on agarose have undergone little change over the last

two decades. Improved imaging systems and labelling agents for high sensitivity detection and linear quantitation by fluorescence and chemiluminescence offer attractive alternatives to immunostaining and the use of radioisotopes, and have made rapid automated nucleic acid sequencing possible. Except for those involved in nucleic acid work, few laboratories perform electrophoresis on agarose on a day-to-day basis; thus, accessories for many techniques such as immunoelectrophoresis have been dropped by suppliers. There may be a revival because of growing interest in several proteins which are not separable in polyacrylamide matrices, von Willebrand factor multimers for diagnosis of certain types of von Willebrand's disease, and high molecular mass derivatives of fibrinogen as markers of vascular disease.

More efficient cooling and thermal control would allow the application of much higher voltages for high performance agarose electrophoresis above the present limitations imposed by gel meltdown. By using an apparatus in which the gel is enveloped with a membranous bladder for cooling, von Willebrand factor multimers can be separated in 20 min runs producing resolution superior to that obtained with the usual overnight runs widely used through the 1990s.

Precast polyacrylamide gels have become popular, and precast agarose gels are available for submarine electrophoresis. Compressibility of agarose is the principal detriment to precasts for other modes of electrophoresis. Agarose/polyacrylamide composites would not be subject to that drawback, and would probably be marketable in precast form because they offer advantages and are not as easily constructed as gels of agarose itself.

Summarily, agarose gels are simple to construct. They are highly porous and ideal for separating high molecular mass proteins and nucleic acids, and can be modified or composited to extend their utility.

See Colour Plate 39.

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Autoradiography Electrophoresis

See **II / ELECTROPHORESIS / Detection Techniques: Staining, Autoradiography and Blotting**

Blotting

See **II / ELECTROPHORESIS / Detection Techniques: Staining, Autoradiography and Blotting**