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A REVIEW ARTICLE ON GEL CHROMATOGRAPHY

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Abstract:
Seminal fluid is the liquid component of sperm, providing a safe surrounding for spermatozoa. The seminal plasma has the feature common to many other body fluid, characterized by a high dynamic range of proteins, which visualizes physiological and biochemical processes of semen. As in other body fluids, it is convenient to distinguish in the seminal plasma between proteins and non-proteins. The molecular exclusion chromatographic technique presents important points to constitutional proteins preservation, in addition to the conventional phenomenon; witch exclusion hydrophobic interactions can provide a higher resolution in the chromatogram and do not provide non-specific interactions between protein structures. The aim was the chromatography profile to simple study of proteins seminal plasma domestic animals (Stallions, Canine, and Goat), by the technique of gel filtration chromatography. The samples (seminal plasma proteins) were chromatographed in a Superose 12 HR 10/30, equilibrated with 25 mMTris-HCl (Sigma) with 0.15 M NaCl (Sigma), pH 7.4 at room temperature in a fast performance liquid chromatography (FPLC-system), using a flow rate of 0.5 mL.min-1. There was a molecular separation of significantly different molecular weights, which makes possible the logarithmic relationship between molecular weight and the elution volume. Calibration of the gel filtration column resulted in an equation y = ax + b, where the values of a and b were 5.43 and-2.175, respectively, and the corresponding errors were 0.115 and 0.256, respectively. The experimental error was less than 5% for most of the protein molecular masses. The work showed that the gel filtration chromatography technique provided an excellent analytical repeatability, and could therefore, be a valuable tool to the study preliminary of seminal plasma.
Key words: Chromatography, Principle, advantages and disadvantages, instrumentation Methodology, applications

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INTRODUCTION:

In previous reports we discussed lignin fractionation gel permeation chromatography (GPC) on Sephaex LH-20 and LH-60 with dimethyl-formamide as by d solvent (DMF), and the use of ligninmodel compounds and polystyrene molecular weight standards for calibration of these columns (Connors et al. 1978; Connors 1978).From results obtained with Sepahdex LHand from publishedmolecular weight figures for kraft lignin from spruce as determined by the shortcolumn sedimentation techniques in an ultracentrifuge (McNaughton et al. 1967), it appeared that a gel which would fractionate up to about 100,000 molecular weight would be adequate for chromatographic of lignins in organic solvents. A major point established from chromatographic experiments in organic solvents for kraft and synthetic lignins and' lignin isolated from wood degraded by a brown-rot fungus is that these Lignins exhibit a bimodal molecular size distributionas judged by their elution profiles (Brown et al. 1968; Wayman and Obiaga 1974; Hüttermann 1978; Lindstrom 1979). We have found the same bimodal pattern of molecular weight distributionin our current experiments on kraft, synthetic, and Brauns. native lignin from aspen when chromatographed on gel columns or high-pressure liquid chromatography (HPLC) columns in organic or aqueous organic solvents. In experiments -reported here, in which lithium chloride (LiCl) was added to DMF, the bimodal elution pattern was changed to a single broad peak, indicating that the bimodality of lignin was due to molecular association. Material and Methods Gel Permeation Chromatography with Organic Solvents The gel materials used in this work are commercial prod ucts available from Pharmacia Fine Chemicals (Piscataway, N.J.). Polystyreneswere purchased from Pressure Chemical Co. (Pittsburgh, Pa.). Chemicals used were reagent gradeand the DMF was redistilled prior to use. Kraft lignin (Indulin AT) was obtained from the Westvaco Co. (North Charleston, S. C.). The Brauns native lignin from aspen was a gift of Dr. F.

E. Brauns. Preparation of the synthetic lignin has been The bulk of our work was performed witha 1.3 i. d. × 115 cm Octylsepharose CL-4B. We found that Sepharose CL-4B gel was softened byDMF and thus not suitable for prolonged use, although a bimodal elution pattern was noted for kraft lignin when this gel was used with DMF, DMSO, or 50% aqueous DMSO. In GPC experiments with controlled pore glass 2000 (Electro-Nucleonics, Inc., Fairfield, N. J.), we foundthat kraft lignin was absorbed on the gel in experiments with DMF and DMSO. One-milliliter samples were collected and the fractions monitored either manually or by use of a spectrophotometer equipped with flow cells. In general, approximately 1/2-mg lignin samples were used. Lignin samples were monitored at 280 nm and polystyrenes at 272 nm. We found no absorption effects on the Sephadex or Sepharose gels with the lignins used in these experiments as evidenced by color being retained on the gel. An exact check of this with Indulin AT showed that 100.35% of the material was eluted from Octylsepharose CL-4B. We have found that this gelis stable over a several-month period with only a very small compacting with time which results in a 1 to 2 ml shift in the void volume. High-Pressure Liquid Chromatography HPLC experiments were under conditions — with four µ-styragel columns with (106, 106, 105, and 104 A porosities) — described by Minor (1979) for chromatography of cellulose derivatives. The lignin samples were used at a concentration of 1 mg per milliliter of DMSO. Kraft Lignin Dialysis Kraft lignin (4.0 g Indulin AT) was dissolved in 200 ml 0.1 N NaOH and dialyzed for 72 hours against 2 l 0.1N NaOH with two changes of alkali. The sample was then further dialyzed for 72 hours against 2l distilled water with four changes of water. The solution in the dialysis bag was then acidified to pH 5 with hydrochloric acid, warmed to 6o°C for 30 minutes and the precipitate isolated by filtration. The retentate was washed with water and then stirred with 100 ml methanol and filtered.The brown powder was washed further with 100 ml methanol then airdried to yield kraft lignin dialysis fraction I (17.5%). The methanol fractions were combined and evaporated to 50 ml volume, allowed to stand in the freezer overnight, and the precipitate filtered to give kraft lignin dialysi Gel Permeation Chromatography with Sephade G-75 and 0.1 N NaOH Kraft lignin and dialysis fractions I and II were chromatographed on a 2.7 × 69 cm Sephadex G-75 column with carbonate-free O.1 N NaOH as eluant and monitored at 280 nm. The raw chromatographic data were digitized and transformed, using a flexible Fortran IV program, to plots of absorbance versuscolumn retention volume, VR . VR is defined as Ve — Vo/Vo, +where Ve is the elution volume and Vo the interstitial volume of the column. Ultracentrifuge Experiments Weight average molecular weight (Mw) values were obtained with a Beckmann Spinco Model E analytical ultracentrifuge by the short-column sedimentation technique. Each sample was scanned at 280 and313 nm at rotor speeds of 12,000 and 17,000 rpm. Equilibrum was reached in 60 hours at 12,000 rpm and 24 hours at 17,000 rpm. The sample size was approximately 2.5 x 10-2 g/l in

pH 9.5 NaOH with NaCl of o.35-o.45 M added for ionic strength. The partial specific volume was taken to be 0.653 ml/g (McNaughton et al. 1967). Results and Discussion In efforts to find a simple method for determining the molecular weight and molecular weight distribution of various lignins by chromatographic methods using organic solvents, we have used various carbohydrate gels for GPC, and μ-styragel columns for HPLC. With GPC we employed polystyrenes, and with HPCL we used dextrans, for characterizing the fractionating capacity of the columns in terms of molecular weights. Our experiments with these systems resulted in bimodal molecular weight patterns .

PRINCIPLE:

Nucleic acid molecules are separated by applying an electric field to move the negatively chargedmolecules through a matrix of agarose or other substances. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. This phenomenon is called sieving.[2] Proteins are separated by the charge in agarose because the pores of the gel are too small to sieve proteins. Gel electrophoresis can also be used for the separation of nanoparticles.

Gel electrophoresis uses a gel as an anticonvective medium or sieving medium during electrophoresis, the movement of a charged particle in an electric current. Gels suppress the thermal convection caused by the application of the electric field, and can also act as a sieving medium, slowing the passage of molecules; gels can also simply serve to maintain the finished separation so that a post electrophoresis stain can be applied. DNA gel electrophoresis is usually performed for analytical purposes, often after amplification of DNA via polymerase chain reaction (PCR), but may be used as a preparative technique prior to use of other methods such as mass spectrometry, RFLP, PCR, cloning, DNA sequencing, or Southern blotting for further characterization.

ADVANTAGES:

As a separation technique, GPC has many advantages. First of all, it has a well-defined separationtime due to the fact that there is a final elution volume for all unretained analytes. Additionally, GPC can provide narrow bands, although this aspect of GPC is more difficult for polymer samplesthat have broad ranges of molecular weights present. Finally, since the analytes do not interact chemically or physically with the column, there is a lower chance for analyte loss to occur.] For investigating the properties of polymer samples in particular, GPC can be very advantageous. GPC provides a more convenient method of determining the molecular weights of polymers. In fact most samples can be thoroughly analyzed in an hour or less.Other methods used in the past were fractional extraction and fractional precipitation. As these processes were quite labor-intensive molecular weights and mass distributions typically were not analyzed. Therefore, GPC has allowed for the quick and relatively easy estimation of molecular weights and distribution for polymer samples

DISADVANTAGES:

There are disadvantages to GPC, however. First, there is a limited number of peaks that can be resolved within the short time scale of the GPC run. Also, as a technique GPC requires around at least a 10% difference in molecular weight for a reasonable resolution of peaksto occur. In regards to polymers, the molecular masses of most of the chains will be too close for the GPC separation to show anything more than broad peaks. Another disadvantage of GPC for polymers is that filtrations must be performed before using the instrument to prevent dust and other particulates from ruining the columns and interfering with the detectors. Although useful for protecting the instrument, there is the possibility of the pre-filtration of the sample removing highermolecular weight sample before it can be loaded on the column. Another possibility to overcomethese issues is the separation.

INSTRUMENTATION:

Gel permeation chromatograph is conducted almost exclusively in chromatography columns. The experimental design is not much different from other techniques of liquid chromatography. Samples are dissolved in an appropriate solvent, inthe case of GPC these tend to be organic solvents and after filtering the solution it is injected onto a column. The separation of multi-component mixture takes place in the column.

The constant supply of fresh eluent to the column is accomplished by the use of a pump. Since most analytes are not visible to the naked eye a detector is needed. Often multiple detectors are used to gain additional information about the polymer sample. The availability of a detector makes the fractionation convenient and accurate.

Gel:

Gels are used as stationary phase for GPC. The pore

size of a gel must be carefully controlled in order to be able to apply the gel to a given separation. Other desirable properties of the gel formingagent are the absence of ionizing groups and, in a given solvent, low affinity for the substances tobe separated. Commercial gels like PLgel & Styragel (cross-linkedpolystyrene-divinylbenzene), LH-20 (hydroxypropylated Sephadex), Bio-Gel (cross-linked polyacrylamide), HW-20 & HW-40 (hydroxylated methacrylic polymer), agarose gel and are often used based on different separation requirements.[

Column:
The column used for GPC is filled with a microporous packing material. The column is filled withthe gel.

Eluent:
The eluent (mobile phase) should be a good solvent for the polymer, should permit high detectorresponse from the polymer and should wet the packing surface. The most common eluents for polymers that dissolve at room temperature GPC are tetrahydrofuran (THF), *o*- dichlorobenzene and trichlorobenzene at130–150 °C for crystalline polyalkynes and hexafluoroisopropanol (HFIP) for crystalline condensation polymerssuch as polyamides and polyesters.

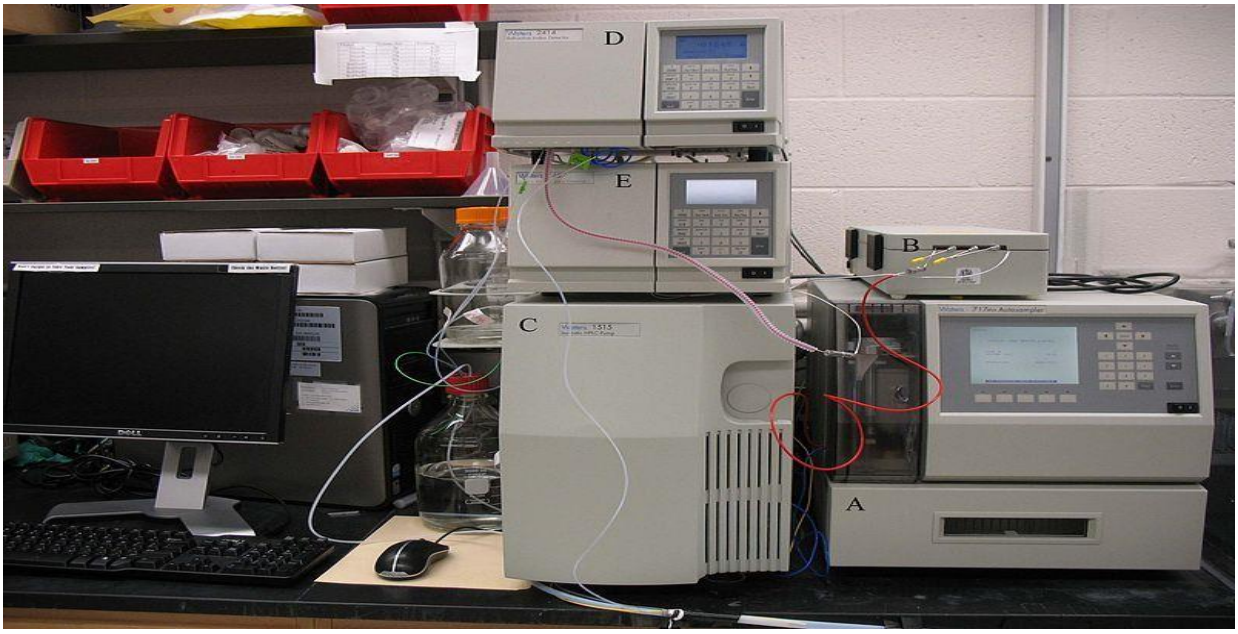
Pump:
There are two types of pumps available for uniform delivery of relatively small liquid volumes forGPC: piston or peristaltic pumps.

Detector:

In GPC, the concentration by weight of polymer in the eluting solvent may be monitored continuously with a detector. There are many detector types available and they can be divided intotwo main categories. The first is concentration sensitive detectors which includes UV absorption, differential refractometer (DRI) or refractive index (RI) detectors, infrared (IR) absorption and density detectors. The second category is molecular weight sensitive detectors, which include low angle light scattering detectors (LALLS) and multi angle light scattering (MALLS). The resulting chromatogram is therefore a weight distribution of the polymer as a function of retention volume.

The most sensitive detector is the differential UV photometer and the most common detector is the differential refractometer (DRI). When characterizing copolymer, it is necessary tohave two detectors in series. For accurate determinations of copolymer composition at least two of those detectors should be concentration detectors.The determination of most copolymer compositions is done using UV and RI detectors, although other combinations can be used.

Data analysis :
Gel permeation chromatography (GPC) has become the most widely used technique for analyzingpolymer samples in order to determine their molecular weights and weight distributions. Examples of GPC chromatograms of polystyrene samples with their molecular weights and dispersities are shown on the left.

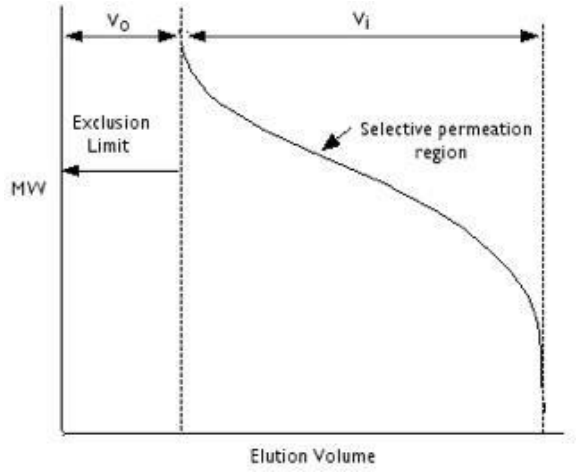


A typical GPC instrument including: A. Autosampler, B. Column, C. Pump, D. RI detector,E. UV-vis detector [fig-1]

Inside of an autosampler for running several samples without user interaction, e.g.overnight [fig-2]

Benoit and co-workers proposed that the hydrodynamic volume, V_η, which is proportional to the product of [η] and M, where [η] is the intrinsic viscosity of the polymer in the SEC eluent, may beused

as the universal calibration parameter. If the Mark–Houwink–Sakurada constants K and α areknown (see Mark–Houwink equation), a plot of log [η]M versus elution volume (or elution time) for a particular solvent, column and instrument provides a universal calibration curve which can be used for any polymer in that solvent. By determining the retention volumes (or times) of monodisperse polymer standards (e.g. solutions of monodispersed polystyrene in THF), a calibration curve can be obtained by plotting the logarithm of the molecular weight versus the retention



time or volume. Once the calibration curve is obtained, the gel permeation chromatogramof any other polymer can be obtained in the same solvent and the molecular weights (usually Mn and Mw) and the complete molecular weight distribution for the polymer can be determined. Atypical calibration curve is shown to the right and the molecular weight from an unknown samplecan be obtained from the calibration curve.

METHODOLOGY:

GPC separates based on the size or hydrodynamic volume (radius of gyration) ofthe analytes. This differs from other separation techniques which depend upon chemical or physical interactions to separate analytes.[3] Separation occurs via the use of porous beads packed in a column (see stationary phase (chemistry)).

The smaller analytes can enter the pores more easily and therefore spend more time in these pores, increasing their retention time. These smaller molecules spend more time in the column and therefore will elute last. Conversely, larger analytes spend little if any time in the pores and are eluted quickly. All columns have a range of molecular weights that can be separated.

Range of molecular weights that can be separated for each packing material

If an analyte is too large, it will not be retained; conversely, if the analyte is too small, it may be retained completely. Analytes that are not retained are eluted with the free volume outside of the particles (V_O), while analytes that are completely retained are eluted with volume of solvent held in the pores (V_i). The total volume can be considered by the following equation, where V_g is the

volume of the polymer gel and V_t is the total volume:[3]

As can be inferred, there is a limited range of molecular weights that can be separated by each column, and therefore the size of the pores for the packing should be chosen according to the rangeof molecular weight of analytes to be separated. For polymer separations the pore sizes should beon the order of the polymers being analyzed. If a sample has a broad molecular weight range it may be necessary to use several GPC columns in tandem to resolve the sample fully.

Range of molecular weights that can be separated for each packing material [fig-3]

Applications of Gel-Filtration Chromatography:

GPC is often used to determine the relative molecular weight of polymer samples as wellas the distribution of molecular weights. What GPC truly measures is the molecular volume and shape function as defined by the intrinsic viscosity. If comparable standards are used, this relativedata can be used to determine molecular weights within ± 5% accuracy. Polystyrene standards with dispersities of less than 1.2 are typically used to calibrate the GPC.[4] Unfortunately, polystyrene tends to be a very linear polymer and therefore as a standard it is only useful to compare it to other polymers that are known to be linear and of relatively the same size.

This technique, however, is not without its disadvantages. When separating proteins by gel-filtration chromatography, for example, proteolysis becomes an increasing problem, since the target protein frequently becomes the abundant substrate for proteases also present in the mixture, consequently

reducing recovery of activity. Because of the large size of gel-filtration columns, large volumes of eluent are usually required for their operation, often creating excessive running costs. Gel filtration also has an inherent low resolution compared to other chromatographic techniques because none of the molecules are retained by the column and nonideal flow occurs around the beads. In addition, this technique has a low sample-handling capacity dictated by the need to optimize resolution.

Despite these disadvantages, gel-filtration chromatography still occupies a key position in the field of biomolecule separation because of its simplicity, reliability, versatility, and ease of scale-up. A brief overview of its main applications is given below.

Separation of Proteins and Peptides:
Because of its unique mode of separation, gel-filtration chromatography has been used successfully in the purification of literally thousands of proteins and peptides from various sources. These range from therapeutic proteins and peptides, which together constitute a multibillion euro world-wide market, to enzymes and proteins for industrial applications; some examples are outlined below.

Recombinant human granulocyte colony stimulating factor (rhG-CSF) was refolded from inclusion bodies in high yield, with great suppression of aggregates formation, by urea-gradient size-exclusion chromatography on a Superdex 75 column A similar technique was used to purify human interferon-gamma, solubilized from inclusion bodies by 8 M urea, to a specific activity of12,000,000 IU/mg with protein recovery of 67 % Luteinizing hormone (LH) was purified 46-fold from crude pituitary extract by gel filtration on two Sephacryl S-200 columns. The method exploited differential binding of LH (in the crude extract) to blue dextran for the first chromatography step. Before the second step, addition of high salt released LH from the blue dextran, enabling effective purification . Fusion ferritin (heavy-chain ferritin plus light-chain ferritin) has also been purified by urea-gradient gel filtration. In this case, fusion ferritin solubilizedfrom inclusion bodies with 4 M urea was applied to the column. Refolding enhancers were included in the urea-diluent buffer subsequently applied to the column to produce properly foldedfusion ferritin multimers .

A continuous rotating annular size-exclusion chromatography system permitted the purification of crude porcine lipase with productivity of approximately 3 mg lipase per mg gel per hour and an activity recovery of almost 99 % .

Among food-use proteins, hen egg lysozyme has been successfully refolded using both acrylamide- and dextran-based gel columns (Sephacryl S-100 and Superdex 75, respectively) . Gel filtration has also proven useful for the purification of the whey proteins alpha-lactalbumin and beta-lactoglobulin from aqueous two-phase systems .

Protein engineering techniques enable the design of self-assembling multimeric protein cages for applications in nanotechnology . Grove et al. describe a gel-filtration method to examine the metal ion-mediated assembly of protein cages.

Size-Exclusion Reaction Chromatography: Protein PEGylation:
Covalent attachment of PEG (polyethylene glycol; “PEGylation”) to a protein can attenuate its antigenicity and/or extend its biological half-life or shelf life. Size-exclusion reaction chromatography (SERC) permits one to control the extent of a reaction (such as PEGylation) thatalters molecular size and to separate reactants and products. In SERC, injection of reactants onto a size-exclusion chromatography column forms a moving reaction zone. Reactants and products partition differently within the mobile phase leading to different flow rates through the column.

Thus, products are removed selectively from the reaction zone, shortening their residence time inthe reaction zone and separating them into the downstream section of the column. In PEGylation, addition of PEG groups to the protein significantly increases molecular size, allowing the use of SERC to obtain a dominant final PEGylated protein size in high yield. The principle was successfully demonstrated using two model proteins, alpha-lactalbumin and beta-lactoglobulin.

Separation of Nucleic Acids and Nucleotides:
Gel-filtration chromatography has for many years been used to separate various nucleic acid species such as DNA, RNA, and tRNA as well as their constituent bases, adenine, guanine, thymine, cytosine, and uracil. Linear phage lambda DNA and circular double stranded phage M13 DNA, for example, can be completely separated from chromosomal DNA and RNA by gel filtration on Sephacryl S-1000 Superfine .

Plasmid DNA can also be purified by gel filtration, although modern commercial kits often use a centrifugal spin column format for greater convenience. Limonta et al. describe the novel use of two gel-filtration steps, one before and one after a reverse-phase operation, to purify plasmid DNA from a clarified alkaline *E.* cell lysate.

Endotoxin Removal:

The presence of bacterial endotoxin is unacceptable in injectable recombinant biologicals, since endotoxin in the bloodstream can induce a pyrogenic response. Good manufacturing practice (GMP) will effectively remove endotoxin, but preclinical biologics may be produced under non- GMP conditions. London et al. investigated various means of endotoxin removal from preparations of a recombinant human protein. Endotoxins typically form aggregates, which may be quite large. A Superdex 200 size-exclusion column (1.75 L bed volume) removed most of the "spiked" endotoxin from an applied sample of monomeric monoclonal antibody, which was obtained in good yield Absolute Size-Exclusion Chromato-graphy (ASEC):

Absolute size-exclusion chromatography (ASEC) is a technique that couples a dynamic light scattering (DLS) instrument to a size-exclusion chromatography system for absolute size measurements of proteins and other macromolecules as they elute from the chromatographic system. Dynamic light scattering (DLS; also known as photon correlation spectroscopy or quasi- elastic light scattering) is a technique that uses light scattering patterns (usually from a laser source)to determine the size distribution profile of small particles in suspension, or of polymers (such as proteins) in solution . DLS can also be used to probe the behavior of complex fluids such as concentrated polymer solutions.

The sizes of the macromolecules are measured as they elute into the flow cell of the DLS instrument from the size-exclusion column. It should be noted that the technique measures the hydrodynamic size of the molecules or particles and not their molecular weights. For proteins, a Mark–Houwink type of calculation can be used to estimate the molecular weight from the hydrodynamic size.

A big advantage of DLScoupled with SEC is the ability to obtain enhanced DLS resolution. BatchDLS is quick and simple to perform. Using SEC, the proteins and protein oligomers are separated, allowing oligomeric resolution. ASEC can also be used for aggregation studies: although the aggregate concentration may not be calculated, the size of the aggregate will be measured, being limited only by the maximum size eluting from the SEC columns. Limitations of ASEC include flow rate, concentration, and precision. Because a correlation function requires anywhere from 3 to 7 s to properly build, only a limited number of data points can be collected across the peak.

Molecular Mass Estimation:

Gel-filtration chromatography is an excellent alternative to SDS-PAGE for the determination of relative molecular masses of proteins, since the elution volume of a globular protein is linearly related to the logarithm of its molecular weight . One can prepare a calibration curve for a given column by individually applying and eluting at least five suitable standard proteins (in the correct fractionation range for the matrix) over the column, determining the elution volume for each protein standard, and plotting the logarithm of molecular weight versus V_e/V_0. When a protein ofunknown molecular weight is applied to the same column and eluted under the same conditions, one can use the elution volume of the protein to determine its molecular weight from the calibration curve.

Group Separations:

By selecting a matrix pore size which completely excludes all of the larger molecules in a samplefrom the internal bead volume, but which allows very small molecules to enter this volume easily,one can effect a group separation in a single, rapid gel-filtration step which would traditionally require dialysis for up to 24 h to achieve. Group separation can be used, for example, to effect buffer exchanges within samples, for desalting of labile samples prior to concentration and lyophilization, to remove phenol from nucleic acid preparations and to remove inhibitors from enzymes(*see*, for example

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