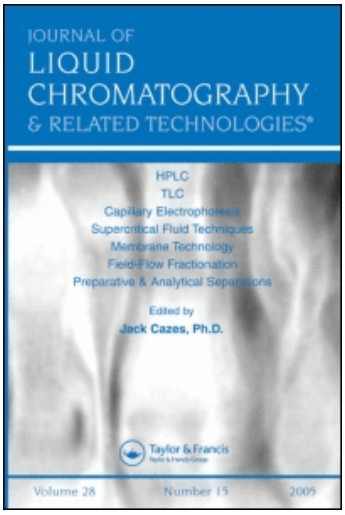

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REHYDRATABLE POLYACRYLAMIDE GELS FOR CAPILLARY ELECTROPHORESIS

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ABSTRACT

A novel separation medium of rehydratable polyacrylamide is described for capillary gel electrophoresis. The dried linear polyacrylamide capable of rehydration to form an aqueous polymeric system which can be filled into a narrow bore capillary column and act as separation matrix. Rehydratable gels provide high resolution and reproducible capillary electrophoresis separations of biopolymers with very long shelf life. This paper relates to separation sieving mediums involving rehydratable gel compositions and methods for using reconstituted linear polyacrylamide solutions as separation mediums in capillary gel electrophoresis of double stranded DNA molecules.

INTRODUCTION

Electrophoresis based separation techniques have been utilized for decades[1] in separating molecules according to differences in their effective charge, and/or molecular size.[2] Traditional electrophoresis separations were mostly conducted in rods or slab gels, typically employing crosslinked polyacrylamide gel or agarose separation matrices.[3] Recently, capillary gel electrophoresis procedures have been evolved, providing extremely high separation power in size separation of biopolymers even with identical or very close mass to charge ratios.[4,5,6] Gel filled capillary columns have mostly been

fabricated in house and recently some supplied directly to researchers by manufacturers.[7] One of the most challenging problem associated with pre-filled gel capillary columns relates to gel stability. Polyacrylamide gels in aqueous environment, at ambient temperatures tend to break down or degrade/hydrolyze under regularly used basic (pH > 6) separation conditions, therefore these columns have limited effective shelf lives and usefulness.

Fabrication of capillaries with the appropriate gels for carrying out electrophoresis analysis involves filling the capillary with a precise mixture of gel monomers, cross-linkers (if necessary), catalyst, and initiator. The gel is then polymerized in situ, inside the capillary column forming the required sieving matrix. Manufacturing of such gel filled capillary columns has the drawback of being very labor intensive and time consuming, and also requires a certain amount of polymerization procedure dexterity, to avoid bubble formation during polymerization and obtain reasonable gel-to-gel performance reproducibility. Additionally, disadvantages relating to the instability of aqueous polyacrylamide gels and the time required to perform polymerization still represent problems.

As an alternative to fixed gel filled capillary columns (crosslinked or chemical gels), replaceable gels (non-crosslinked or physical gels) are also used.[8] When these columns exhibit signs of degradation, users can easily remove the gels from the capillary, utilizing a syringe for home made or a pumping/vacuum systems on automated capillary electrophoresis instruments, to replace separation matrix by forcing fresh gel into the column. This approach allows the gel capillary user extended control over gel mediated electrophoresis separations, since, as soon as, the gel exhibits any deterioration in separation performance, it is a simple procedure to rapidly replace the separation matrix in the column. However, when aqueous gels are stored and intended for use as replaceable gel systems in a later time, similar to pre-filled fixed-gel capillaries, they are also subject to degradation, which is exemplified as rapid changes in flow characteristics and electrophoresis separation performance.[9] This decomposition of aqueous polyacrylamide gels, caused most probably by hydrolysis, is an ongoing process and the degree of degradation increases with time.

Linear polyacrylamide is a hydrophilic polymer,[10] possessing a certain degree of affinity for moisture, which is difficult to completely exclude. However, even when containing trace amount of water, the "dried" polyacrylamide is conveniently stored and reconstituted in any aqueous liquid, and filled into capillary columns for subsequent use for capillary gel electrophoresis separations. Crosslinked gel compositions were reported earlier in rehydratable polyacrylamide slab gel format[11,12,13,14] for separation of

biologically important polymers, such as DNA or protein molecules. Linear polyacrylamide compositions were used recently for capillary electrophoresis based DNA sequencing applications[15] and PCR product identification.[16]

The objective of this paper is to introduce novel, dried linear polyacrylamide compositions, that can be reconstituted in the required buffer system forming a separation matrix which is then used as capillary gel electrophoresis separation medium for biopolymers, with improved handling properties and extended shelf life.

MATERIALS AND METHODS

Apparatus

A P/ACE 5500 capillary electrophoresis system was used during the experiments (Beckman Instruments, Inc., Fullerton, CA) in reversed polarity separation mode (anode on the detection side). The capillary length was 30 cm to the detector (37 cm total) with the internal diameter of 0.1 mm, and a constant running voltage of 11.1 kV (300 V/cm) was applied during the separations. Ultraviolet (UV) detection of the analytes was accomplished at 254 nm. Capillary cartridge running temperature was maintained at 20±0.1°C.

Chemicals

The φX-174 DNA Hae III Restriction Digest (New England Biolabs, Beverly, MA) was diluted with water to 50 μg/mL concentration before injection, and was stored at -20°C when not in use. The molecular weight of the DNA fragments in this mixture ranges from 47,000 - 879,000 Dalton (corresponding to 72-1353 base pairs). Electrophoretic grade acrylamide, Tris, boric acid, EDTA, ammonium persulfate, and tetramethylethylenediamine (TEMED) were used in the experiments (Schwarz/Mann Biotech, Cambridge, MA). All buffer and acrylamide solutions were filtered through a 0.2 μm pore size filter and carefully vacuum degassed (100 mbar).

Procedures

The typical acrylamide polymerization process involved dissolving the sufficient amount of acrylamide monomer (1-12 %), usually in aqueous buffer

solution (100 mM Tris-boric acid, 2 mM EDTA, pH 8.3 at 20°C referred to as TBE buffer), followed by the addition of initiator (ammonium persulfate) and catalyst (TEMED), respectively. During polyacrylamide preparation in aqueous solution, the concentration of initiator and catalyst utilized in the polymerization was varied according to the acrylamide concentration and was generally increased with elevated monomer concentration. As an example, for the preparation of 12% linear polyacrylamide, 1 μL 10% ammonium persulfate and 1 μL TEMED was added to each mL reaction mixture. Vacuum degassing or helium purging of the polymerization reaction mixture is strongly recommended to remove excess oxygen, which inhibits polymerization.[17] Although, room temperature polymerization (20-25°C) is preferred, gentle warming (40-50°C) or cooling (4-10°C) can be utilized to speed up or slow down the polymerization reaction, respectively, in order to obtain the required chain length of the resulting linear polymer.[18] Once the polymerization was complete, typically within 3 to 6 hours, the polyacrylamide solution was subject to dehydration, so the polymer is thoroughly dried until dry polyacrylamide powder was obtained. Application of vacuum (100 mbar) and heat (40-50°C) to the polyacrylamide-water mixture provided a suitable dry composition with the requisite low amount of moisture content. Alternatively, lyophilizing the polyacrylamide solution or precipitating the polyacrylamide would also work. The resulting powdery form linear polyacrylamide can be stored without any decomposition and hydrolysis problems.

Prior to use, the dry, linear polyacrylamide should be reconstituted to the required final separation concentration in the appropriate aqueous buffer system. If denaturants, such as urea, formamide, sodium dodecyl sulfate, or any other additives are needed in the reconstituted composition, it can also be added during this step, in a desired final concentration.

RESULTS AND DISCUSSION

Gel compositions described in this paper are suitable for use in a wide variety of capillary electrophoresis applications in which gels are required as separation medium. The dried linear polyacrylamide formulation is capable of hydrating, to form aqueous polymer matrices of different concentrations, suitable for filling into a capillary electrophoresis columns. Furthermore, different buffer systems and various additives, commonly used in capillary gel electrophoresis are easily added during the rehydration process. It is important to note here, that this also enables addition of materials that otherwise might interfere with the acrylamide polymerization. In contrast to previously published wet polyacrylamide gel compositions for capillary electrophoresis,

which had limited shelf life due to their tendency to degrade resulting in decreasing effectiveness as separation matrices, no such problems are associated with this dry polyacrylamide material.

One of the most important property characteristics of the polyacrylamide composition used during the experiments described in this paper, is the linear (i.e. non-crosslinked) nature of this polymer. Unlike polyacrylamides utilized in conventional rod or slab gel electrophoresis systems, which are prepared with various cross-linkers to provide a polymer matrix with a particular pore size, the polyacrylamide used here was prepared in the absence of any cross-linking reagents. This resulted in a linear polyacrylamide formulation, which can practically be homogeneously dissolved when rehydrated/ reconstituted. Linear polymers appear to be readily dissolved resulting in flow properties which allow solutions of the polyacrylamide to fill into a capillary column with internal diameters of 25 μm and larger. That is the difference if compared to cross-linked polyacrylamide gels, which pick up water by swelling only, while keeping their original shape.[19] Important to note here, that linear polyacrylamide solutions do not possess definite pore sizes, the magnitude of their average dynamic pore structure is subject to physical parameters, such as temperature and buffer ionic strength.[20] Albeit, if necessary, very small amounts of cross-linker (<0.1%) still can be utilized during acrylamide polymerization, with no apparent loss in rehydration kinetics.

The dried, linear polyacrylamide can also include solid additives such as urea, sodium dodecyl sulfate, intercalators, chiral selectors, etc., which are useful for special separations in capillary gel electrophoresis. Also, these additives can be conveniently added during the rehydration process. As an example, typical concentrations of urea in polyacrylamide mediated DNA separation mediums range about up to 9 molar, which can be easily added in the reconstitution process, but would cause crystallization problems during the dehydration. Another example is formamide, which would evaporate during dehydration, but can be easily added up to 70% concentration to the separation matrices during the rehydration process. Frequently used intercalators, such as ethidium bromide, thiazole orange, etc., may also be incorporated in polyacrylamide separation mediums during reconstitution, to enhance separations of double stranded DNA molecules. Cyclodextrins or special affinity additives can be added during the rehydration process of the gel-buffer systems at various concentrations for enabling chiral recognition or affinity interaction based capillary electrophoresis separations. Additionally, a wide variety of pH buffering compounds which are utilized in capillary gel electrophoresis systems, such as phosphate, borate, barbiturate, acetate, etc., all of which are suitable for a number of different separation applications can also be part of the reconstitution solution.

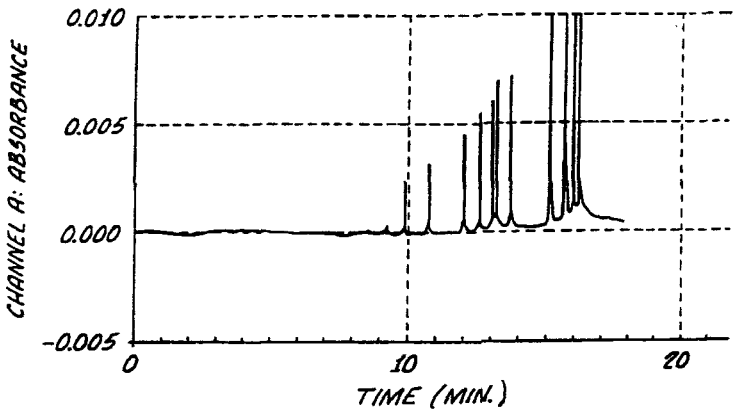

Figure 1. Capillary gel electrophoresis separation of φX-174 DNA Hae III restriction digest fragment test mixture utilizing freshly polymerized 12% linear polyacrylamide gel diluted to 3% and filled into the capillary column. Conditions: Capillary length: 37 cm (30 cm to the detector); I.D.: 0.1 mm; Applied electric field: 300 V/cm; Separation buffer: 100 mM Tris-borate/2mM EDTA, pH 8.3; Temperature: 20°C.

The concentration of polyacrylamide in the final rehydrated form can vary from as little as 0.01% to as high as 10-12% or even above. As an example for the separation of dsDNA molecules, the preferred concentration range is 3 - 6%. Typically the greater the base pair-number of the DNA fragments being separated, the lower the required concentration of linear polyacrylamide. For the φX-174 DNA Hae III restriction digest fragments, (base pairs ranging from MW=47,000 to MW=879,000), linear polyacrylamide concentration of about 3% is preferred. For DNA fragments having lower molecular weights, such as, less than MW=47,000, higher concentrations (5-6%) linear polyacrylamide is preferred. Thus, in selecting the amount of dry polyacrylamide to rehydrate, one should first consider the desired separation application.

Figure 1 exhibits a typical capillary electrophoresis separation of the φX-174 DNA restriction fragment (RF) mixture using a regular pre-filled 3% linear polyacrylamide gel column. This figure indicates baseline separation of all the fragments within 17 minutes. A 12% stock acrylamide solution was vacuum degassed and the polymerization was allowed to proceed for 3 hours at room temperature. After the polymerization was complete, the solution was diluted to a total polymer concentration of 3% with sufficient amount of Tris, boric acid and EDTA added to the solution providing a final buffer concentration of 100 mM Tris-borate, 2mM EDTA (pH 8.3).

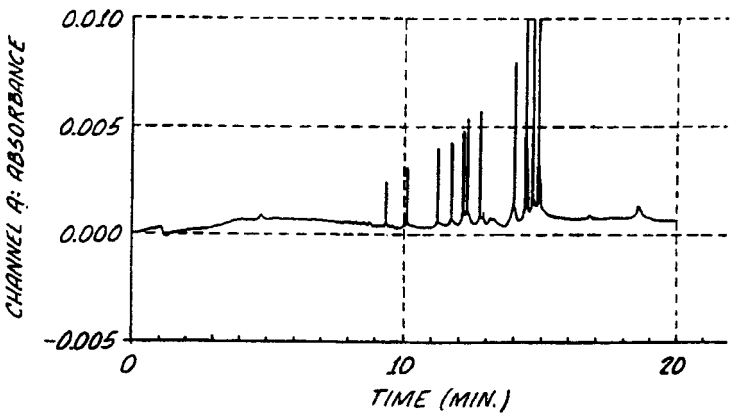

Figure 2. Electropherogram obtained in the separation of φX-174 DNA Hae III restriction digest fragment test mixture using the same conditions as were used to obtain the electropherogram of Figure 1, except that the 12% linear polyacrylamide formulation was completely dried, and then rehydrated in 3% concentration, in 100 mM TBE (pH 8.3) buffer before pumped into the capillary column. All separation conditions are the same as in Figure 1.

The fused silica capillary used in this separation had interior polyacrylamide surface coating[21] which reduced the electroosmotic flow in the column. The capillary was filled by positive pressure pumping with buffered 3% polyacrylamide. The test mixture of φX-174 DNA restriction fragments was separated in the capillary applying 300 V/cm electric field strength and detected at UV 254 nm.

Next, a 12% acrylamide monomer solution was polymerized according to the polymerization procedure described above. After the completion of the polymerization reaction, the water was removed by subjecting the reaction mixture to slight warming (40°C) under a 100 mbar vacuum. The resulting dry polyacrylamide product had powdery consistency. Then, three grams of this dry polyacrylamide was added to 100 mLs of aqueous 100 mM Tris-borate-EDTA buffer (pH 8.3) solution. The mixture was stirred continuously for 1.5 hours until a homogenous gel-buffer system of the rehydrated polyacrylamide was formed. This reconstituted 3% linear polyacrylamide solution was pumped into the coated (see above) capillary column and used for separation. Figure 2 indicates baseline separation of all the double stranded DNA fragments in the φX-174 RF sample mixture in less then 15 minutes, under otherwise similar conditions as described under Figure 1. Upon comparing the electropherograms of Figures 1 and 2, one can observe, that the two separations

are substantially identical. Thus, using rehydrated polyacrylamide provides all the separation performance of fresh gel and at the same time, provides the additional advantages of significantly longer shelf life and user versatility.

CONCLUSION

A number of different gel electrophoresis separation conditions, including those requiring addition of special pH buffering compounds, urea, formamide, SDS, as well as, additives useful in affinity, chiral, etc., separations, can be conveniently used during the rehydration procedure when employing the dried linear polyacrylamide compositions described in this paper. The dry linear polyacrylamide compositions are capable of hydrating to form an aqueous polymer solution, which can be filled into capillary electrophoresis columns, providing fresh gel filled capillaries each time, when necessary.

Advantageously, capillaries filled with reconstituted polyacrylamide have all the functional characteristics of the fresh gel columns without any uncertainty associated with columns filled with standard or replaceable gels and stored for a longer period of time.

Finally, users of dry linear polyacrylamide compositions are assured that rehydrated linear polyacrylamide used in capillary electrophoresis columns will reproducibly support high resolution electrophoretic separations.

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