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Effect of the age of non-cross-linked polyacrylamide on the separation of DNA sequencing samples

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Abstract

Capillaries filled with non-cross-linked polyacrylamide and 7 *M* urea are stable for at least 4 months when stored at room temperature and exposed to the atmosphere. The stability of these capillaries when subjected to four successive DNA sequencing runs at an electric field of 300 V/cm was studied. Capillaries used the day after polymerization showed a dramatic increase in elution time with successive separations. Capillaries used at least 1 week after polymerization showed very reproducible elution times with successive separations. This effect was observed with capillaries where polymerization occurred in situ. Identical results were observed when polyacrylamide was formed in an external vessel and the polymerized material was pumped into the capillary. In all cases, there was a gradual loss in resolution with successive separations; this loss of resolution was independent of the length of time since polymerization. Separations may be made successfully up to at least 115 days after polymerization for fragments less than 300 bases in length.

1. Introduction

Capillary electrophoresis has become a major analytical tool in the field of biological sample analysis. The high separation efficiency of and rapid separations by capillary electrophoresis are particularly powerful in the analysis of DNA sequencing samples [1–22].

Cross-linked polyacrylamide is used to obtain high-resolution separation of sequencing samples. Only a few reports are available on the effect of different parameters (%T, %C) on the separation of DNA [9,10,17]. Usually, only 2–3 separations can be performed on the same gel without a major change in the retention time or the occurrence of bubbles that damage the gel [14].

Non-cross-linked polyacrylamide has been used for the separation of DNA sequencing samples [12,15,20,22]. Non-cross-linked polyacrylamide seems to be less affected by bubble formation than cross-linked polyacrylamide; however, the resolution obtained with non-cross-linked polyacrylamide is lower than that with cross-linked polyacrylamide [17]. At low concentrations, this material can be replaced in a capillary by applying a suitable pressure [23,24]. Refilling the capillary removes the step of realigning the system usually done when changing a capillary. However, after each refill, the capillary needs to be pre-run to remove unpolymerized acrylamide and other small ions. This pre-run time depends on the viscosity of the polymer, and is typically 20–30 min for a 5 %T at 300 V/cm.

Fifty runs or more were reported with the

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same capillary for samples consisting of double-stranded oligonucleotides [25–27]. However, there are only a few reports on multiple runs of DNA sequencing samples on the same capillary. Pentoney et al. [12] used 10 %T 7 *M* urea non-cross-linked polyacrylamide to perform 5–10 separations of DNA sequencing samples. To obtain useful separations, they had to trim 2 mm of the column a few minutes after each injection. This approach has also been used with cross-linked polyacrylamide to reduce the effect of bubbles at the injection end and to perform multiple runs [1,3,25]. Unfortunately, it appears difficult to automate this trimming procedure.

We have studied the effect of ionic depletion at the injection end of non-cross-linked polyacrylamide capillaries [28]. We noticed that the effect of ionic depletion decreases with older gels, which suggests that aged polyacrylamide may be more stable than freshly polymerized material for sequencing applications. In this paper, we describe DNA sequencing with non-cross-linked polyacrylamide (5 %T 7 *M* urea)-filled capillaries used up to 115 days after polymerization. The effects of the age of polyacrylamide on the separation of a DNA sequencing standard were analyzed.

2. Experimental

The Sequitherm Cycle Sequencing kit (Cedarlane Laboratories, Hornby, Canada) and the ΔTaq Cycle Sequencing kit (USB, Cleveland, OH, USA) were used to prepare sequencing samples of fluorescently labeled DNA. The kits' procedures for cycle sequencing were used with the following changes: ROX-labeled M13 universal (−21) primer (ABI, Foster City, CA, USA) was used with M13mp18 single-stranded DNA (USB). Samples were ddATP terminated. The samples were covered with mineral oil and heated for 5 min at 95°C. Cycle sequencing was performed using 30 cycles; each cycle consisting of 45 s at 95°C, 45 s at 47°C and 90 s at 70°C. Samples were precipitated with ethanol, washed and resuspended in 15–25 μl of formamide–EDTA (49:1). Each sample was divided into 5–10 aliquots.

Non-cross-linked polyacrylamide (5 %T) was prepared by taking 1.25 ml of a 20% acrylamide (Bio-Rad, Richmond, CA, USA) stock solution, adding 1.0 ml of 5 × TBE buffer [2.7 g of Tris (ICN Biomedicals, Cleveland, OH, USA), 1.37 g of boric acid (BDH, Toronto, Ontario, Canada) and 1.0 ml of 0.5 *M* EDTA diluted to 50 ml in deionized, filtered water)] and 2.1 g of urea (Gibco BRL, Gaithersburg, MD, USA), and diluting the solution to 5.0 ml with deionized, filtered water. This solution was degassed for at least 20 min by bubbling argon gas through it. The polymerization reaction was initiated and catalyzed by adding 2 μl of N,N,N′,N′-tetramethylethylenediamine (TEMED) (Gibco BRL) and 20 μl of 10% ammonium peroxodisulfate (Boehringer Mannheim, Indianapolis, IN, USA). For in situ polymerization, immediately following the addition of initiators, the solution was injected into fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with typical dimensions of 33 cm × 32 μm I.D. × 143 μm O.D. Capillaries were pretreated for 1 h with a silanizing solution. This solution was freshly prepared by mixing 0.5 ml of water, 0.5 ml of glacial acetic acid and 20 μl of γ-methacryloxypropyltrimethoxysilane (Sigma, St. Louis, MO, USA). All chemicals were of electrophoretic grade. The day of preparation was taken as day zero. Non-cross-linked polyacrylamide-filled capillaries older than day zero were left to age on the bench and trimmed before use.

For capillaries that were refilled with polyacrylamide, the polymerization occurred in a disposable centrifuge tube. On the day of analysis, the polymerized material was pumped into a capillary using a gas–tight syringe and a locally constructed connector.

The capillary electrophoresis system and fluorescence detection system used for these experiments were an in-house design [6,17]. The high-voltage power supply used in this experiment was a Spellman (Plainview, NY, USA) CZE1000R. Fluorescence was excited with a yellow (8 mW, λ = 594 nm) He–Ne laser (PMS, Electro-Optics, Boulder, CO, USA). Fluores-

cence was collected at right-angles to the laser beam with a 125 × microscope objective (Leitz, Weizlar, Germany). The fluorescence was imaged on to an iris, passed through a 630DF30 band pass filter (Omega Optical, Brattleboro, VT, USA) and detected with an R1477 photomultiplier tube (Hamamatsu, Middlesex, NJ, USA).

3. Results and discussion

Separation of sequencing samples was performed on non-cross-linked polyacrylamide-filled capillaries from 1 to 115 days after polymerization. Fig. 1 presents four subsequent sequencing runs done on a 1-day-old non-cross-linked polyacrylamide-filled capillary at −300 V/cm. The migration times increased from run to run; from the first to the fourth run, the migration time for base 91 increased from 23.7 to 39.4 min (66% increase in retention time), and the migration time for base 252 moved from 43.3 to 70.8 min (63% increase in retention time). Also, the peak heights decreased by a factor of 10 after four sequencing runs, despite the use of a fresh sample for each run. At the same time, the current in the capillary decreased from 1.21 μA for the first run to 0.70 μA for the fourth sequencing run.

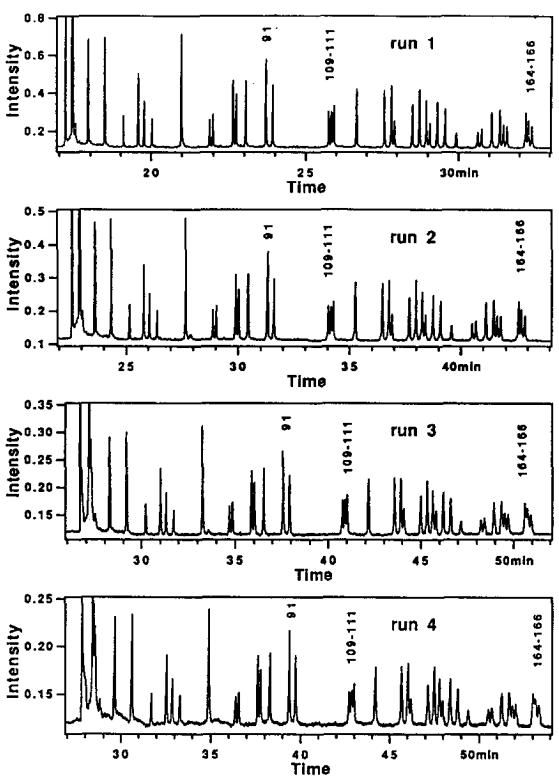

Fig. 1. Four subsequent separations of M13mp18 ddATP-terminated sequencing samples at −300 V/cm on a 35 cm long capillary filled with 1-day-old non-cross-linked polyacrylamide. The same, arbitrary, intensity scale was used for all runs. Fragments 91, 109–111 and 164–166 bases in length are noted.

The decrease in current is due to depletion of ions at the injection end [28]. This depletion of ions causes a localized increase in the electric field near the injection end, and a decrease of the electric field in the rest of the capillary. In addition, this depletion of ions extends further into the capillary with time, thus increasing the asymmetry of the electric field in the capillary. Depletion of ions causes the migration times to increase from run to run and also decreases the amount of sample loaded on to the capillary. Depletion of ions has been described by various workers for conventional slab gel experiments [29–34].

Fig. 2 presents separations performed with a capillary used 20 days after polymerization. After four runs, the migration time for base 91 changes from 22.2 to 22.3 min (0.04% increase in retention time), and for base 252 from 38.9 to 39.1 min (0.6% increase in retention time). The current was consistently 1.55 μA for the four runs. The peak heights change by less than 10%. While we have noted earlier the current stability for aged polyacrylamide capillaries [28], this is the first observation of highly stable migration times in aged non-cross-linked polyacrylamide capillaries.

The improved stability of polyacrylamide with age was also noticed in a different set of experiments, in which the polyacrylamide was polymerized in an external vessel and then pumped into the capillary. A capillary was refilled with a 1-day-old non-cross-linked polyacrylamide and three subsequent sequencing runs were performed (Fig. 3). The change in migration time is

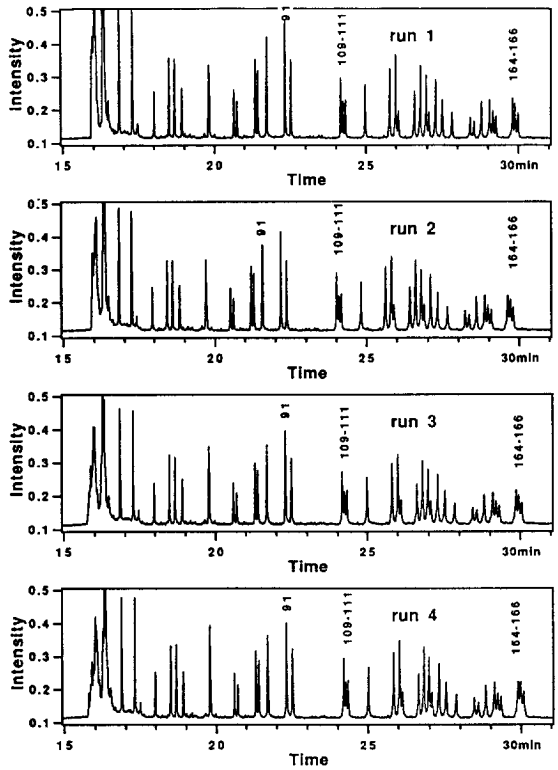

Fig. 2. Four subsequent separations of M13mp18 ddATP-terminated sequencing samples at −300 V/cm on a 32.1 cm long capillary filled with 20-day-old non-cross-linked polyacrylamide.

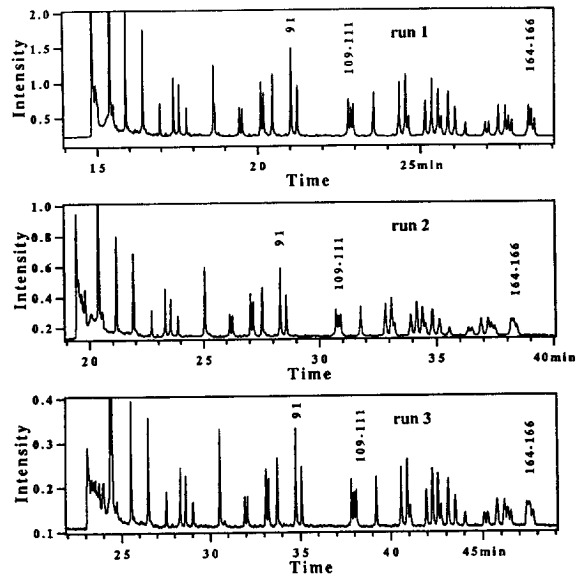

Fig. 3. Three subsequent separations of M13mp18 ddATP-terminated sequencing sample at −300 V/cm on a 36 cm long capillary. The capillary was filled with 1-day-old non-cross-linked polyacrylamide before the first run, using a gas-tight syringe.

similar to Fig. 1. When the capillary was refilled with a 20-day-old non-cross-linked polyacrylamide and four subsequent sequencing runs were performed (Fig. 4), the migration time was stable to 5% R.S.D. These results indicate that the change in the migration times with age is not due to a problem with the capillary wall. Instead, the change in migration time is due to changes in the properties of aging polyacrylamide.

Migration times were measured for bases 91 and 252 for capillaries ranging from 1 to 115 days old. Four successive runs were performed on each capillary. For non-cross-linked polyacrylamide less than about 2 days old, the mobilities (Fig. 5), there was a decrease by 40% for base 91 and 39% for base 252 from the first to the fourth sequencing run. In the case of non-cross-linked polyacrylamide 7 to 34 days old, the mobilities decreased by an average of 6.2% for base 91 and 4.5% for base 252 from the first to the fourth run. In the case of the 115-day-old non-cross-linked polyacrylamide-filled capillary, the mobilities increased by 29% for base 91 and 35% for base 252 from the first to the fourth run.

There was no detectable electro-osmosis in these capillaries. Because of the design of our system, any electro-osmotic pumping will displace the polyacrylamide matrix into the path of the laser beam of our detector. No such displacement was noted in any of our experiments.

A non-linear least-squares routine was used to fit a four-parameter Gaussian function to the peaks. From the values of migration time and peak width, the resolution, R, is calculated for peaks separated by a single base. The resolution (Fig. 6) for any particular run was weakly dependent on the age of the capillary; however, the resolution decreased significantly with the number of sequencing runs. This decrease could be caused by a decrease in peak spacing and/or an increase in peak width from run to run. Mobility, and hence peak spacing, are stable

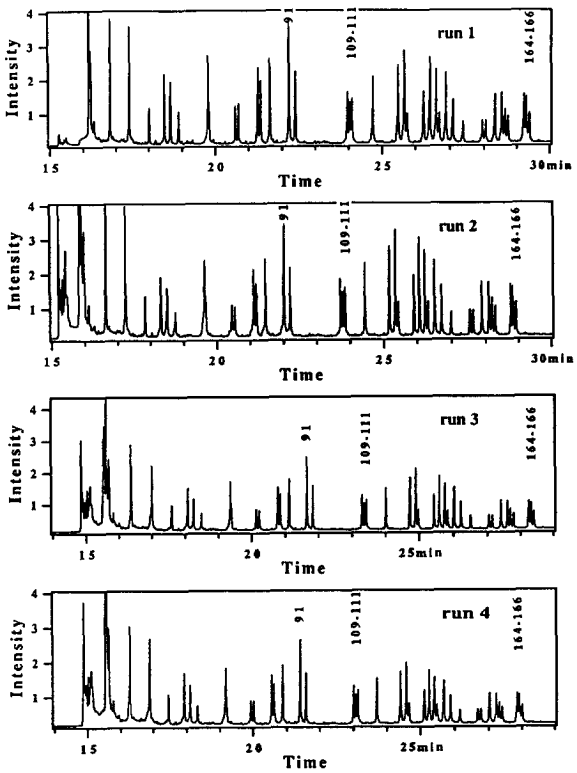

Fig. 4. Four subsequent separations of M13mp18 ddATP-terminated sequencing sample at −300 V/cm on a 36 cm long capillary. The capillary was filled with 20-day-old non-cross-linked polyacrylamide before the first run, using a gas-tight syringe.

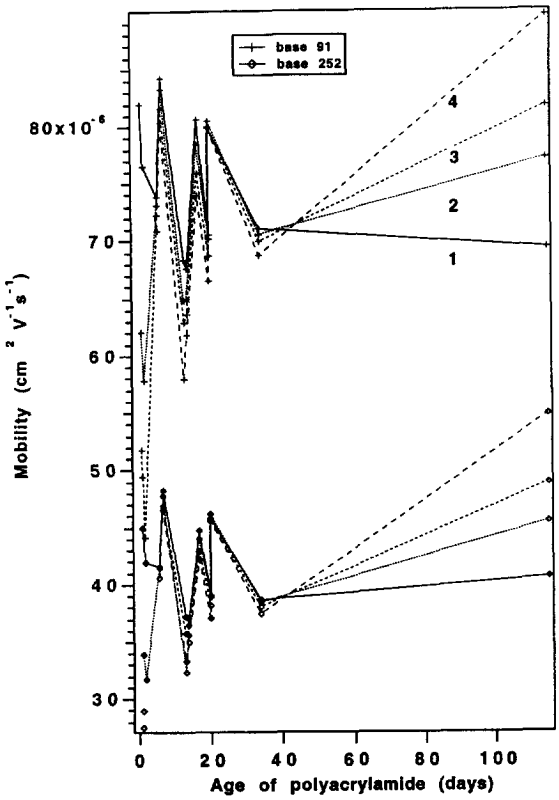

Fig. 5. Mobilities for bases 91 and 252 versus the age of non-cross-linked polyacrylamide for four subsequent sequencing runs. The run number is indicated beside each line.

within the four runs for most of the different ages of non-cross-linked polyacrylamide; however, in the case of the 115-day-old non-cross-linked polyacrylamide, there is a decrease in peak spacing from the sequencing run one to four, and in the case of capillaries less than 2 days old, there is an increase in peak spacing from the sequencing run one to four.

Fig. 7 shows the number of theoretical plates for base 91 versus the age of non-cross-linked polyacrylamide. In all cases, except for the 115-day-old polyacrylamide, the plate count decreases from run to run. In the case of fresh non-cross-linked polyacrylamide (less than about 2 days old), the migration time increases by 66% and the peak widths increase by 200%, resulting in a decrease by a factor of three in the number of theoretical plates from the first to the fourth sequencing run. Non-cross-linked polyacrylamide between 6 and 34 days old has migration times that increase by an average of 8% and peak widths that increase by 70%. This results in a decrease by a factor of 2.5 in the number of theoretical plates. Finally, in the case of the 115-day-old non-cross-linked polyacrylamide, the migration times decrease by an average of 25% and the peak widths increase by 1%, resulting in a decrease by a factor of 1.8 in the number of theoretical plates.

4. Conclusion

Non-cross-linked polyacrylamide can be used to perform subsequent sequencing runs on the same capillary if the non-cross-linked poly-

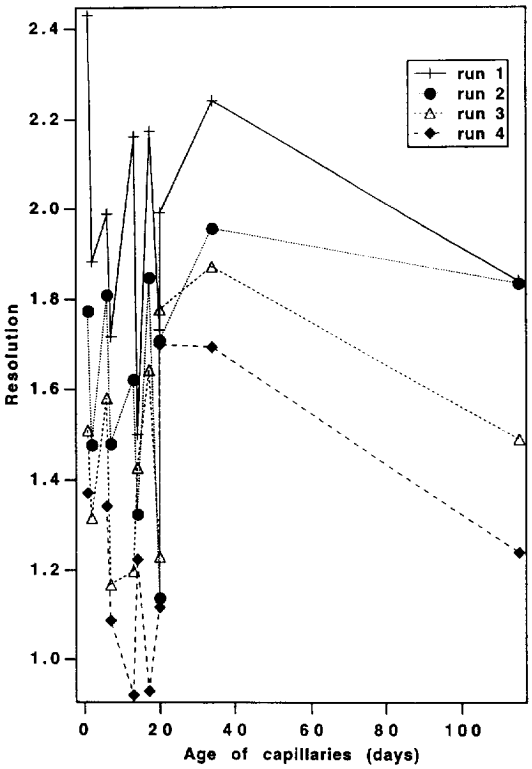

Fig. 6. Resolution for base 91-2 versus the age of non-cross-linked polyacrylamide for four subsequent sequencing runs. The run number is indicated beside each line.

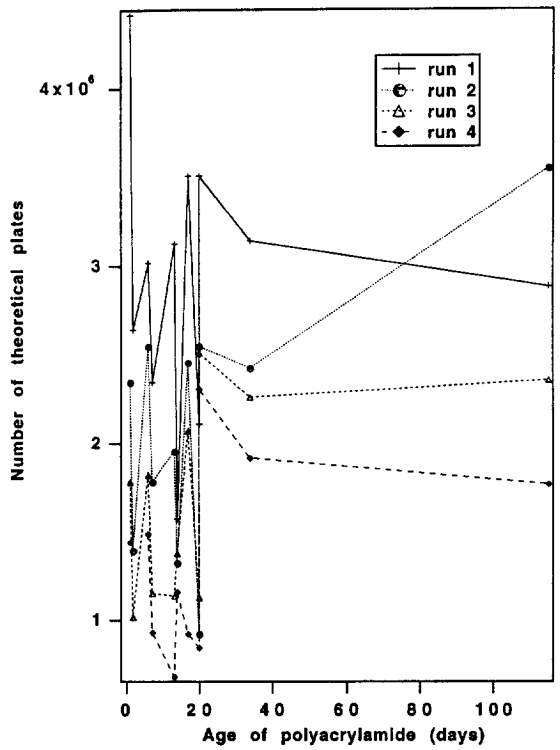

Fig. 7. Theoretical plates for base 91 versus age of polyacrylamide for four subsequent sequencing runs. The run number is indicated beside each line.

acrylamide is aged properly before use. This behavior occurs whether polymerization is performed in situ or if polymerization occurs in an external vessel and the polymerized material is pumped into the capillary. The migration times for aged non-cross-linked polyacrylamide change by less than 8% for base 91 and 5.5% for base 252 within four sequencing runs. Sufficient resolution was obtained for DNA sequence determination for fragments less than 300 bases in length for at least four sequencing runs and for capillaries containing polyacrylamide that was used at least 2 weeks after polymerization. This sequence read length is similar to that observed earlier at 300 V/cm; we have demonstrated that operation of these capillaries at lower electric fields leads to longer sequencing read length.

In an accompanying paper [35], we describe how aged non-cross-linked polyacrylamide may be used to obtain many separations of sequencing samples. This approach requires the minimization of template concentration in the sequencing sample, the use of aged polyacrylamide and the reversal of the electric field between separations to relax ionic depletion at the injection end of the capillary.

Acknowledgements

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