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Journal of Chromatography A, 744 (1996) 325–331

JOURNAL OF CHROMATOGRAPHY A

Pseudo-coulometric loading in capillary electrophoresis DNA sequencing

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Abstract

While injection volumes in capillary electrophoresis are typically in the nanoliter range, it is difficult to physically prepare and manipulate samples much smaller than a microliter. As a result, only a small fraction of the analyte contained with the sample volume is transferred to the capillary. This problem is particularly acute in DNA sequencing applications, where on-column stacking is difficult and where the sequencing sample is relatively expensive to prepare. We report a method that transfers 75% of the DNA contained within a 3 μl sample onto a capillary for DNA sequencing. This method relies on the use of very low ionic strength formamide to resuspend the DNA after an ethanol precipitation. The use of low ionic strength formamide achieves two tasks. First, it produces a very high resistance sample, which increases the voltage drop across the sample and decreases the field across the capillary. This electric field manipulation ensures that DNA fragments do not migrate down the capillary during the loading process, allowing long injection periods without excessive band-broadening. Second, the low ionic strength of the formamide increases the transference number of the DNA; more of the current passing through the injection tip of the capillary is carried by DNA fragments. In the limit of complete elimination of impurity ions from the loading solvent, current passing through the sample is carried only by DNA fragments and loading becomes a coulometric process.

Keywords: Injection methods; DNA

1. Introduction

Very small sample volumes are injected in capillary electrophoresis. However, it is often difficult to take advantage of this property simply because it is difficult to manipulate small volume samples. While micromachining technology offers the potential for automated manipulation of nanoliter samples [1,2], current technology is unable to exploit the nanoliter injection volume capabilities of capillary electrophoresis.

Burgi and Chien have presented a series of papers that describe the use of stacking conditions in zone electrophoresis [3,4]. A large plug of analyte is injected onto the capillary. If the ionic strength of the sample is sufficiently low, the majority of the electric field will drop across the high resistance sample buffer. Sample ions are stacked at the front of the sample plug, ready for separation by zone electrophoresis in the higher ionic strength running buffer.

Capillary electrophoresis is a particularly powerful technique for DNA sequencing. Modern separations are based on noncrosslinked polyacrylamide and have generated sequencing read lengths of over 600 bases in 2 h [5–8]. Efficient sample loading would be useful in DNA sequencing applications because it could reduce the significant cost of enzymes, fluorescently labeled primers, and dideoxynucleotides used

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0021-9673/96/$15.00
PII S0021-9673(96)00438-4

in the sequencing reaction. Unfortunately, on-column stacking is usually difficult in DNA sequencing applications, where the viscous separation medium obstructs the physical loading of large sample volumes onto the capillary. The only hope for successful on-column stacking will arise from the use of low viscosity, short-chain polymers, such as those described by Gelfi et al. [9].

In this manuscript, we document a very efficient method of loading DNA onto sequencing capillaries. To understand the method, it is necessary to understand the makeup of the sequencing sample itself. DNA sequencing fragments are relatively short single-stranded oligonucleotides, ranging up to a few thousand bases in length, that incorporate a fluorescent tag. A much larger template molecule is associated with the sequencing fragments; this template may be either single stranded or double stranded, depending on its origin. The DNA sequencing fragments are complimentary to the template and will hybridize to it. The fragment–template hybrid has low mobility and will not generate useful sequencing information. To minimize hybrid formation, the sequencing samples are ethanol precipitated, excess buffer is decanted, and the samples are resuspended in a mixture of formamide–0.5 *M* EDTA (49:1) at pH 8.0. Formamide acts as a denaturing agent, disrupting intrastrand hydrogen bonds, freeing the sequencing template. A small amount of EDTA is often added to the formamide to complex divalent metal ions that might be associated with the DNA. A fair amount of salt also tends to be present in the sequencing sample, particularly if a thermostable DNA polymerase and cycle sequencing are used to generate the DNA fragments. Finally, formamide slowly hydrolyzes to ammonia and formic acid, which further increases the concentration of ions in the sample. Because of the presence of these ions, DNA sequencing fragments represent a low fraction of the total ionic content of the sample solution.

Efficient loading of DNA onto the capillary requires that the DNA be a significant fraction of the total ionic strength of the solution. Conventional loading buffers contain 10 m*M* EDTA plus impurity ions present in formamide and miscellaneous ions associated with the sequencing reaction. As a result, only a small fraction of the DNA contained with the sample is loaded onto the capillary. However, if contaminating ions are rigorously excluded, then a very large fraction of the DNA can be transferred from the sample to the capillary. In this manuscript, we document this coulometric loading procedure.

2. Experimental

Formamide (Fluka) was deionized by passing it on a mixed bed ion-exchange resin (Bio-Rad, AG501-x8). The conductivity of formamide was measured with a General Radio Corporation model 1650-A impedance bridge.

The Sequitherm Cycle Sequencing Kit (Cedarline Labs., Hornby, UK) was used to prepare sequencing samples of fluorescently labeled DNA. The manufacturer's protocol for cycle sequencing was used with the following changes: ROX-labeled M13 universal (−21) primer (ABI, Foster City, CA, USA) was used with M13mp18 RF1 single stranded DNA (USB, Cleveland, OH, USA). Samples were ddTTP 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 ethanol precipitated, washed and resuspended in 10 μl deionized formamide. Then the sample was diluted again in deionized formamide or a mixture of 0.5 *M* EDTA in formamide (49:1).

The silica capillaries (Polymicro, Phoenix, AZ, USA), with typical dimensions of 33 cm×50 μm I.D.×143 μm O.D., were treated for 1 h with a silanizing solution. This solution was freshly prepared by mixing 0.5 ml of glacial acetic acid, 0.5 ml of water, and 5 μl of γ-methacryloxypropyltrimethoxysilane (Sigma, St. Louis, MO, USA). Then a 3%T (acrylamide with the appropriate catalyst) solution was introduced into the capillary to coat the wall with a layer of linear polyacrylamide. Once the capillary was filled with the silane solution, the reaction was allowed to proceed for 20 min. Vacuum was then used to draw ethanol through the capillary to rinse residual silane solution. The vacuum was then used to pass air through the capillary.

Before using and whenever it was necessary, the capillary was refilled with 5%T 0%C 7 *M* urea polyacrylamide. Filling the capillary was done by introducing the injection end of the capillary in a 200

μl gas tight syringe (Hamilton, Reno, NV, USA) with a Valco fitting and then applying pressure using a low pressure syringe pump (Razel model A-99, Stamford, CA, USA) for 10 min. The noncrosslinked polyacrylamide sieving matrix (5%T, 0%C) was prepared by mixing 1.25 ml of a 20% acrylamide (Bio-Rad) stock solution, 1 ml of 5×TBE buffer [2.7 g Tris (ICN Biomedicals, Cleveland, OH, USA), 1.37 g boric acid (BDH, Toronto, Canada) and 10 ml 0.5 *M* EDTA diluted to 50 ml in deionized water], and 2.1 g of urea (Gibco BRL, Gaithersburg, MD, USA) and making the solution up to 5.0 ml in deionized and filtered water. This solution was degassed for 20 min by bubbling argon gas through it. The polymerization reaction was initiated by adding 2 μl N,N,N′,N′-tetramethylethylenediamine (TEMED) (Gibco BRL) and 20 μl of 10% ammonium persulfate (Boehringer Mannheim, Indianapolis, IN, USA). The solution of 5%T 0%C 7 *M* urea was used 5 days or more after polymerization.

The capillary electrophoresis system and fluorescence detection system used for these experiments are an in-house design [10,11]. In this system, one end of the capillary is kept at high voltage using a Spellman CZE 1000R (Plainview, CA, USA) high voltage power supply, while the other end is held in a grounded sheath flow cuvette. Excitation of sequencing samples is achieved using a 5 mW yellow, $\lambda = 594$ nm, He-Ne laser (PMS, Electro-Optics, Boulder, CO, USA) focused on the sheath flow cuvette below the tip of the capillary. Fluorescence is collected at right angles to the laser beam with a 125X microscope objective (Leitz, Weizlar, Germany). The light collected is imaged onto 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). The signal from the photomultiplier tube was converted to voltage, filtered, and digitized by a Macintosh computer.

The free solution mobility of fluorescently labeled single-stranded DNA was run in 1×TBE buffer at a potential of 29 000 V in a 73.8 cm long capillary. The electroosmotic mobility was determined by changing the ionic strength of the buffer by 10% to 1.1. The electroosmotic mobility was $9.9 \pm 0.1 \cdot 10^{-4}$ $cm^2\ V^{-1}\ s^{-1}$. Electrophoretic mobility is obtained by subtracting electroosmosis from total mobility. The mobility of DNA sequencing samples was $-5.1 \pm 0.1 \cdot 10^{-4}$ $cm^2\ V^{-1}\ s^{-1}$. The free solution mobility of the primer was slightly lower, $\mu_e = -5.0 \cdot 10^{-4}$ $cm^2\ V^{-1}\ s^{-1}$.

3. Results and discussion

DNA sequencing samples are loaded onto the capillary by application of a potential to the solution for a period, *t*. The voltage induces a current, *I*, through the capillary. This current is carried by ions from the sample through the capillary; the total charge that passes through the capillary during the loading period is $t \times I$ Coulombs. Typical sample loading conditions are the use of 7800 V potential for 30 s. This voltage induces a current of 2 μA, which transfers a total of $6 \cdot 10^{-5}$ Coulombs or $4 \cdot 10^{14}$ unit charges (600 pmol of charge) onto the capillary.

The transference number of an ion is simply the fraction of charge carried by that ion. The transference number for DNA is given by

$$T_{DNA} = [DNA] \cdot N_{DNA} \cdot \mu_{DNA} / \sum [ion] \cdot N_{i\ ion} \cdot \mu_{i\ ion} \quad (1)$$

where N_{iion} is the charge on ion i and μ_{iion} is the free solution mobility of ion i. We have measured the free solution mobility of DNA in 1×TBE buffer, $\mu_{DNA} = 5 \cdot 10^{-4}$ $cm^2\ V^{-1}\ s^{-1}$, which is essentially independent of the fragment size. This value is close to the free solution mobility of most small ions. Assuming equal mobility for all ions in the solution, then the transference number for DNA is simply equal to its concentration divided by the total ionic strength.

The amount of DNA loaded during an injection is maximized by maximizing the transference number. Elimination of EDTA from the formamide loading solution, along with use of deionized formamide, minimizes the total ionic strength. Further reduction in ionic strength is produced by desalting the DNA sequencing sample through, for example, the use of a size exclusion membrane.

3.1. Injection

Fig. 1 shows the effect of deionizing formamide on the injection of ROX-primer. A $4 \cdot 10^{-12}$ *M*

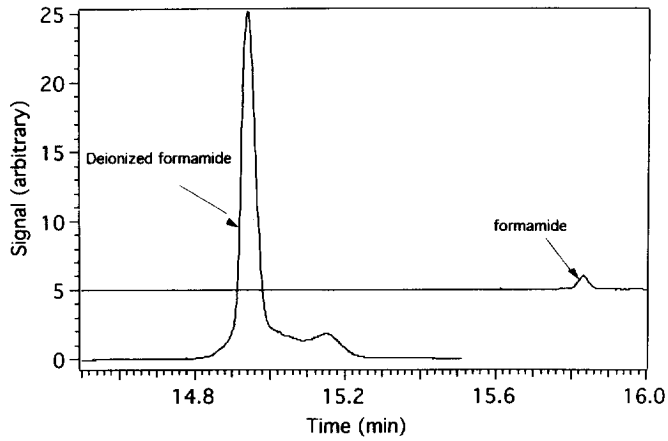

Fig. 1. Peaks generated by injection of fluorescent primer. The formamide curve corresponds to the injection of DNA from a sample resuspended in formamide–EDTA. The curve was offset for clarity. The deionized formamide corresponds to the injection of DNA from a sample re-resuspended in deionized formamide.

solution of ROX-primer was injected onto the capillary for 30 s at a constant current of 2 μA. The primer was prepared in a deionized formamide resuspension solvent. An 8 kV potential was then applied for 1.5 min. A second injection was made for 30 s, this time with the primer resuspended in the conventional formamide–EDTA solution. Peak heights were 20 to 40 times higher for samples resuspended in deionized formamide compared with samples resuspended in EDTA–formamide. This increase in peak height is inversely proportional to the conductivity of the formamide re-suspension buffer. The deionized formamide had a resistivity of 480 KΩ-cm while the conventional formamide–EDTA mixture had a resistivity of 23 kΩ-cm. The same current was applied for both injections; in both cases, ions containing $3 \cdot 10^{14}$ units of charge were loaded onto the capillary. In the case of deionized formamide, DNA fragments have a higher transference number, resulting in larger sample loading.

Another way of increasing the amount of sample loaded during the injection is to increase the injection time. Injection can be done in two modes: constant voltage and constant current. Peak height is not linearly related to injection time for constant voltage mode. This nonlinearity is due to a decrease in conductivity at the injection end of the capillary; we have described the phenomenon elsewhere [12]. It is due to a difference in transference number of ions between the free solution and the polymer filled capillary. The decreased conductivity at the capillary tip causes the current to drop during a constant voltage injection, which decreases the amount of sample injected per unit time. In constant current injection mode, the intensity versus the injection time is nearly linear, Fig. 2, $r > 0.999$. During injection, the electric field increases to keep the current constant.

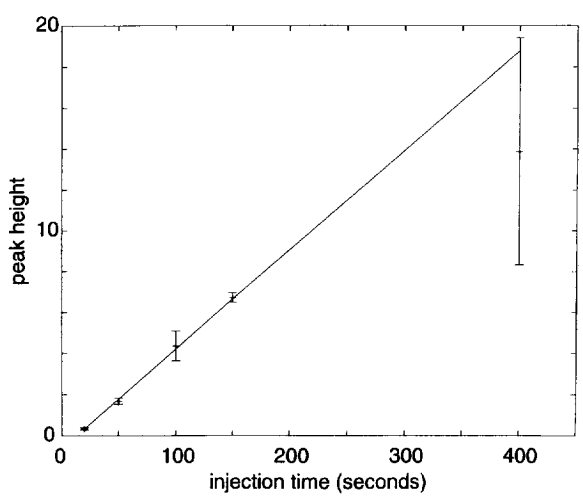

Fig. 2. Peak height versus injection time for samples resuspended in deionized formamide. Samples are injected at constant current of 2 μA. Between two and four replicate injections were made for varying periods. The data are plotted ± 1 standard deviation. The line is the weighted linear least squares fit.

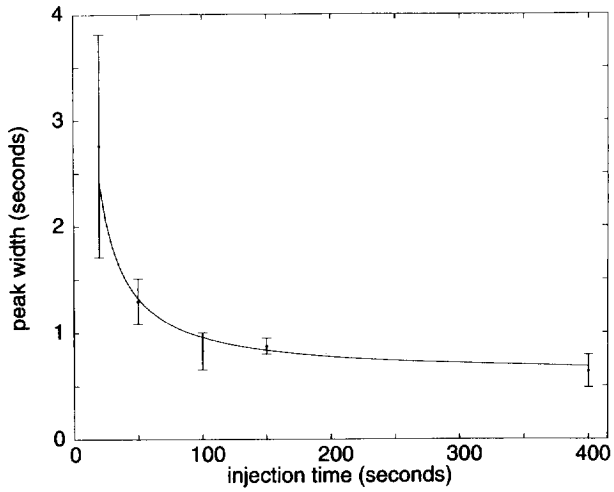

Fig. 3. Peak width versus injection time for the samples of Fig. 2. The data are plotted ±1 standard deviation. The smooth curve is the least squares fit to Eq. 2.

The peak width decreases inversely with injection time, Fig. 3, from 20 to 400 s,

$$w = 0.6 + 37/t \quad (2)$$

where t is the injection time. The fit is excellent, with $\chi^2 = 1.00$. At first blush, this result is surprising; while DNA fragments are being loaded onto the capillary, they are also being driven through the separation medium; peak width should increase with loading time. Instead there presumably is an isotachophoretic focusing effect at the capillary tip. More work will be required to characterize this phenomenon.

3.2. Depletion of the sample

The use of a low ionic strength DNA sample leads to rather high loading efficiency. Loading efficiency is determined from replicate injections from the same sample. We used a dilute solution of ROX-primer (2 amol/μl) and a small volume of solution (3 μl) in deionized formamide for subsequent injections; a total of $3.6 \cdot 10^6$ DNA molecules are contained within the sample. The first two injections generate peaks of roughly comparable height; subsequent injections produce an exponential decrease in height with injection number.

Fig. 4 shows the peak height versus the injection number for two different injection times (75 and 150 s). A nonlinear least-squares regression analysis fits an exponential function to the peak height

$$\text{Height} = A + B \cdot e^{-n/n_c} \quad (3)$$

where A is a residual offset, B is the initial peak height, n is the injection number, and n_c is the number of injections necessary to decrease the peak height to e^{-1} of the initial value. The results for the regression analysis are shown as the smooth curve in Fig. 4

$$\text{Height} = 0.03 \pm 0.02 + 1.8 \pm 0.2 \cdot e^{-t/(2 \pm 0.2)} \quad (4)$$

$\chi^2 = 0.03$, $N = 8$ for 75 s injection

$$\text{Height} = 0.06 \pm 0.04 + 7.8 \pm 2.2 \cdot e^{-t/(1 \pm 0.2)} \quad (5)$$

$\chi^2 = 0.005$, $N = 5$ for 150 s injection.

In both equations, the residual offset is zero within experimental error, which means the amount of DNA remaining in the sample goes to zero after many injections. The peak height remained roughly constant for the first few injections and then droped exponentially with subsequent injections. In the large volume sample, several injections are required to reduce the impurity ion concentration below the electrolysis level. Until the ionic strength drops to that level, the transference number of DNA will remain roughly constant and the number of moles of

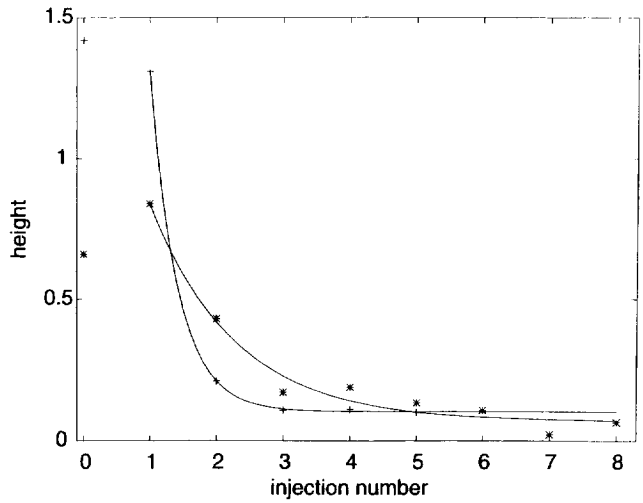

Fig. 4. Peak height versus the number of injections from the same sample. Samples are injected at constant current of 2 μA from a sample volume of 3 μl. The data denoted by "+" are injected for 150 s, while the data denoted by "*" are injected for 75 s. The smooth curves are the least squares regression analysis of offset exponential decays to the data. The first injection was not used in the fit.

DNA loaded onto the capillary will not change in subsequent injections. Once the ionic strength drops to the electrolysis level, the transference number of DNA will drop exponentially with subsequent injections. The rate at which DNA is removed from the capillary is given by n_c; in the case of a 150 s injection, the peak height for a subsequent injection is $e^{-1} = 37\%$ of the height of the preceding injection; roughly 2/3 of the DNA is loaded in each injection. This exponential decrease in DNA concentration with succeeding injections is surprising. The amount of DNA removed from the sample is in proportion to the fraction of charge carried by the DNA. This fraction should remain constant after injection, because injection removes both DNA and impurity ions from the sample. The overall conductivity of the sample should drop with subsequent injections but the fraction of charge carried by DNA should be constant. Electrolysis of the solvent creates additional ions to match the number of solvent ions lost during injection. The transference number of DNA decreases exponentially with subsequent injections because DNA is depleted while the total ionic strength remains constant.

The rate of removal of DNA from the sample should be related to the sample volume. Larger volume samples, at equal concentration, will contain more DNA; injection will remove a smaller fraction of DNA from larger samples. To investigate the depletion of DNA from large volume samples, a series of experiments was performed with constant injection time, 150 s, but increasing sample volume, Fig. 5. The results of the regression analysis are included as the smooth curve in the figure.

$$\text{Height} = 0.03 \pm 0.01 + 10.5 \pm 1.6 \cdot e^{-i/(0.7 \pm 0.1)} \quad (6)$$

$\chi^2 = 0.0002$, $N = 5$ for 3 μl sample

$$\text{Height} = 0.06 \pm 0.04 + 7.8 \pm 2.2 \cdot e^{-i/(1.3 \pm 0.3)} \quad (7)$$

$\chi^2 = 0.026$, $N = 6$ for 9 μl sample

$$\text{Height} = 0.06 \pm 0.02 + 1.5 \pm 0.3 \cdot e^{-i/(5 \pm 2)} \quad (8)$$

$\chi^2 = 0.116$, $N = 12$ for 20 μl sample.

The residual offset for these equations is close to zero, which indicates that there is no long term retention of DNA in the sample vessel. The characteristic injection number increases roughly in proportion to the sample volume; five injections are required to decrease the DNA content of the 20 μl sample by 2/3 while less than one is required for the 3 μl sample.

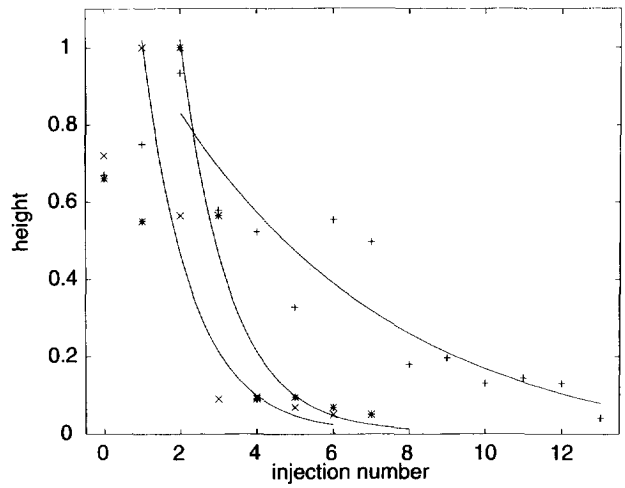

Fig. 5. Peak height versus the number of injections from the same sample. Samples are injected at constant current of 2 μA for 150 s. The data denoted by an "x" are injected from a 3 μl sample, the data denoted by an "*" are injected from a 9 μl sample, and the data denoted by a "+" are injected from a 20 μl sample. The smooth curves are the least squares regression analysis of offset exponential decays to the data. The first injection was not used in the fit.

4. Conclusions

DNA samples that are resuspended in low ionic strength formamide are efficiently loaded onto a separation capillary. This efficient transfer of sample implies that dilute DNA sequencing samples are required for analysis. As a result, it should be possible to modify the DNA sequencing reaction to use a lower concentration template, enzyme, nucleotides, and primer. Decreased consumption of these reagents will be particularly important in large scale sequencing efforts, such as the human genome initiative and large scale genomic disease screening efforts, where a significant cost savings could be achieved.

Acknowledgments

This work was supported in part by the Department of Energy-Human Genome Initiative (USA) grant number DE-FGO2-91ER61123. Support by DOE does not constitute an endorsement of the views expressed in this article. This work was also supported by both the Canadian Bacterial Diseases Network and the Canadian Genetic Diseases Network. NJD acknowledges a McCalla professorship from the University of Alberta.

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