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Original Article

Using a Temperature Gradient against the Time in Polyacrylamide Gel Electrophoresis May Eliminate the Need for Stacking Gels

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ABSTRACT

Background and Objectives: **Making stacking gels for polyacrylamide gels in the laboratory by conventional methods is laborious and time consuming. Considering the role of temperature in polyacrylamide gels with respect to electrical resistance and viscosity, we assumed that decreasing the temperature would cause an increase in electrical resistance and viscosity. Ultimately, a downward temperature gradient imposed in the first phase of polyacrylamide gel running time would supposedly improve the migration of macromolecules. This project analyzed the effect of temperature gradient on the migration of macromolecules in the continuous gels (without stacking) and compared it with results obtained using stacking gels.**

Material and Methods: **Electrical resistance was calculated using Ohm's law. Subsequently, to examine the effect of temperature change on macromolecules separation, conformation sensitive gel electrophoresis (CSGE) was used as a model and specimens were run under three different conditions, one of which was prepared with a stacking gel.**

Results: **The electrical resistance showed an inverse relationship with temperature in this study. Separation of the DNA molecules in the continuous gels (with no stacking) was comparable with the conventional method (with stacking).**

Conclusion: **Using a temperature gradient against time may be an alternative method for stacking gels.**

Keywords: Temperature, Polyacrylamide Gel Electrophoresis

Introduction

The main goal of using stacking gel is to compress molecules of different sizes as much as possible into each other to form a thin band just before entering the resolving gel. This causes a reduction in the random distance between the molecules just before entering the wells.

Polyacrylamide gel electrophoresis (PAGE) technique has been developed since its introduction until now. The nature of technique is such that some variables can be changed as required, e.g. length and diameter of gel, the total concentration

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of polyacrylamide and the cross linker (T%) and the ratio of cross linker to polyacrylamide (C%). Temperature is one of the variables, which is usually kept constant during the run; however, it too can become a variable under certain circumstances and there are some applications, which use a temperature gradient along the gel: Denaturing Gradient Gel Electrophoresis, (DGGE) (1) Temperature Gradient Gel Electrophoresis (TGGE) (2), and Temporal temperature gradient gel electrophoresis (TTGE) (3). It has been shown that using stacking gels (part of the gel in sample loading position, which is less concentrated compare to resolving gel) improves the separation of macromolecules in various methods (4). As in the new generations of electrophoresis units, temperature can be easily controlled by a water circulator or thermoelectric units, as is used in newer generations of electrophoresis units. Effect of temperature change can be imposed to gels during the run, which mimics the stacking gel effect in a continuous gel (i.e. with no stacking gel). The hypothesis comes from the observation that in a constant current mode, the applied voltage increases by lowering the temperature. On the other hand, in constant voltage mode lowering the temperature reduces the current. At the same time, the speed of molecular movement decelerates in both conditions. This can be seen in smiling phenomena (5) especially in a vertical electrophoresis apparatus without a temperature-controlling unit. The radiator effect of edges causes the edges to be colder than the mid-portion where the moving dyes run slower. Therefore, lowering the temperature in a single run may mimic the stacking effect in polyacrylamide gel electrophoresis as the molecules move faster in stacking gel compare to resolving in the first time of running.

This project analyzed the effect of temperature gradient on the migration of macromolecules in the continuous gels (without stacking) and compared it with results obtained using stacking gels.

Materials and Methods

Two gels (T%=12.5% and 15%, C%=2, TBE 0.5X, pH 8.3, Diameter = 0.5 mm and Length = 8 cm) were made by a home made cast for MultiphorII® Horizontal Electrophoresis unit (GE-Healtcare) and vertical Electrophoresis Unit (Akhtarian) respectively. Horizontal gel was subjected to 15 minutes pre run at 2 mA constant current mode in 20˚C, the voltage was registered, after then temperature was changed step by step and voltage was registered after one minute in each steps ensuring the gel temperature is constant.

Another gel (vertical) was subjected to constant voltage mode. After 15 minutes pre-run at 50 V in constant voltage mode at 35˚C, the current was registered, after then temperature was changed gradually and current was registered after one minute in each step ensuring the gel temperature is constant.

To examine the real effect of temperature change during electrophoresis, a CSGE (conformation sensitive gel electrophoresis) method (6) was carried on in a constant current mode. The samples were prepared by polymerase chain reaction (PCR) amplification of exon 6 of low density lipoprotein receptor (LDLR) gene from patients with Familial Hypercholesterolemia (FH) based on previously reported methods (7, 8). In brief, PCR reaction conditions consisted of 2 mM Mg, in the following cycles: 96^{o}C 5min x 1, (96^{o}C 1 min, 54^{o}C 1 min, 72^{o}C 1 min) x30 and 72^{o}C 5min. The 25 micro liter reactions contained 10 picograms of following primers: F: TCC TCC TTC CTC TCT CTG GC; R: TCT GCA AGC CGC CTG CAC CG. PCR amplified products (179 bps) were separated on 2.5% agarose gel containing 0.5 micrograms ethidium bromide (GE-Healtcare) in each milliliter and visualized by uv transilluminator (GE-Healtcare).

Following PCR amplification, 10 microliters of the PCR product was mixed with 10 microliters of the loading solution (80% formamide / 0.25% xylene cyanol FF/ 40% sucrose) (GE-Healtcare). Heteroduplex generation was performed by heating and cooling the PCR products (96˚C for 5 minutes, 60˚C for 30 minutes, 50˚C for 2 minutes, 40˚C for 2 minutes). Then 10 microliters of the of heated products were subjected to constant current (10 mA) gel electrophoresis on three similar gels prepared as mentioned above (T%=12.5%) One exception was that it contained 10 percent urea. One of the gels was also prepared with a stacking gel in order to compare the effect of temperature shifting in a gel without stacking to a gel containing stacking (T% 6%). The conditions of electrophoresis were maintained constant but the temperature and stacking gel were changed. Specimens were run once in a gel without stacking at a constant temperature of 5^{o}C, once in a

gel with stacking at 5°C and finally in a gel without stacking at a temperature shifting from 25°C to 5°C for a one hour period. Visualization was performed by staining with silver nitrate as previously reported in literature.

Results

At a constant current, the measured voltage on electrodes reversibly increases with respect to the temperature (i.e. the colder the gel gets, the higher the voltage goes) (Table 1). At a constant voltage, the measured current decreases as the temperature drops (i.e. the colder the gel gets, the lower the current goes) (Table 2).

In this project, the effect of lowering the temperature on double-stranded DNA molecules was tested using three different gels as mentioned above. Seven samples were tested which were taken from FH patients. One of them (No. 4) was taken from a patient with FH who was a known carrier of a heterozygous missense mutation in exon 6 of *LDLR* gene. Fig. 1A shows an additional band in lane 4 which represent heteroduplex formation. The same samples were subjected to the second gel, (Fig. 1B), containing stacking. Additional band is seen in the expected lane, but there are some more bands in all lanes, which were not simply visible on the first gel. Surprisingly, the last gel, which was undergone to temperature shifting not only, showed the expecting bands in lane 4, but a second additional band could be detected. In addition, there are some more bands in all lanes compared to other gels, which show samples are more resolved (Fig. 1C).

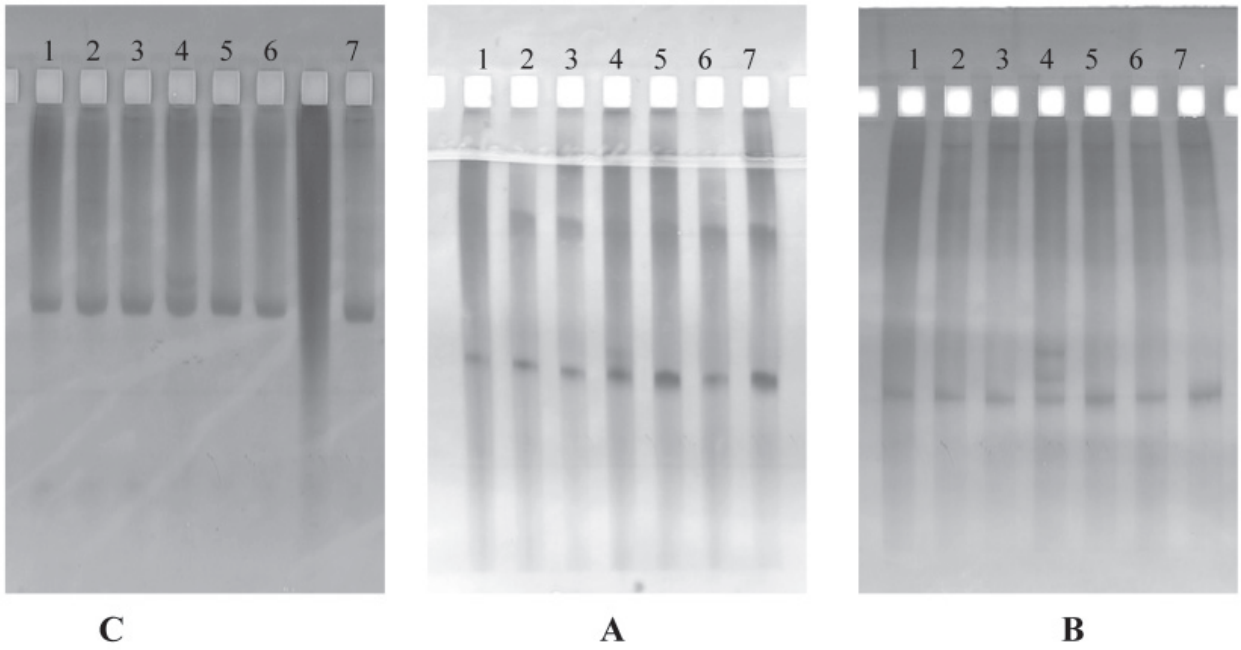

Fig. 1: Same DNA samples in three gels were subjected to conformation sensitive gel electrophoresis (CSGE). (A) Gel without stacking and run at constant temperature (5°C). (B) Gel with stacking and run at constant temperature (5°C). (C) Gel without stacking using temperature gradient from 25°C to 5°C. Arrows show heteroduplexes.

Table 1: Relation between temperature and resistance in constant current mode

Temp°C	Voltage	Current (mA)	Resistance(Ω)	R/cm(Ω)
20	56*(137)**	5(10)	11200(13700)	1400(1712)
16	60(150)	5(10)	12000(15000)	1500(1875)
12	65(196)	5(10)	13000(19600)	1625(2450)
8	71(240)	5(10)	14200(24000)	1775(3000)

* T% = 12.5%, ** T% = 15%

Table 2: Relation between temperature and resistance in constant voltage mode

Temp°C	Voltage	Current (mA)	Resistance(Ω)	R/cm
35	350*(700)**	17(34)	20600(20588)	735(735)
30	350(700)	16(31)	21875(22580)	781(806)
25	350(700)	15(29)	23333(24137)	833(862)
20	350(700)	13(26)	26923(26923)	961(961)
15	350(700)	12(23)	29166(30434)	1041(1086)
10	350(700)	10(20)	35000(35000)	1250(1250)
5	350(700)	8(17)	43750(41176)	1562(1470)

* T% = 12.5%, ** T% = 15%

Discussion

A molecule with a net charge will move in an electric field. This phenomenon, termed electrophoresis, offers a powerful means of separating macromolecules, such as proteins or DNA. The velocity of migration (v) of a protein (or any molecule) in an electric field depends on the electric field strength (E), the net charge on the protein (z), and the frictional coefficient (f):
v=E z / f (9).

The electric force Ez driving the charged molecule toward the oppositely charged electrode is opposed by the viscous drag fv arising from friction between the moving molecule and the medium. The frictional coefficient f depends on both the mass and shape of the migrating molecule and the viscosity (η) of the medium. For a sphere of radius (r) : $\mathbf{f = 6 \pi \eta r}$ (9).

The viscosity of the polymerized polyacrylamide depends on factors in polymerization stage like initiator concentration, polymerization reaction temperature, stirring rate and initiator addition method (batch or dropwise), among which temperature has the main influence (10). In addition to temperature effect on polyacrylamide viscosity in polymerization effect, generally, temperature value has inverse relationship with viscosity in amorphous materials like polymers (11).

On the other hand, the basic equation in electrical science is Ohm's law: **(E = Current x Resistance)**, the mechanism whereby gel electric resistance increases when changing the temperature may be due to viscosity increment of the medium. Macromolecules are larger than anions and cations, therefore the friction coefficient is also much greater in such molecules. The ultimate result is a marked velocity reduction of macromolecules in comparison to ions.

As the temperature decreases during the run time, (e.g. one fourth of total run time), electric resistance of the gel increases. In constant current mode, it may be thought that the movement of the particles should not be influenced, but considering the fact that in electrolytes, electrical conduction happens by full atomic species (ions) traveling, each carrying an electrical charge (12), it can be imagined that each particle should hit the matrix on its route and overcome the obstacle until it arrives to the apposite electrical buffer electrode, so the power supply should compensate this to obtain constant current by increasing the voltage (till reach to power limit which client has set and equals to Voltage X Current). This causes more particles to enter the gel from the buffer electrodes, where at a section of time some are hitting to the matrix and some are passing through. Regardless of the resistance of the gel, the latter remains unchanged in this mode. At the same time, the macromolecules (e.g. DNA, protein) or other molecules like methylene blue and xylene cyanole should challenge with resistance increment. In addition to their larger radius (r) compare to ions, which cause reduction of velocity dramatically, they have no "reserve members" in the buffer to compensate and they should solely overcome it. The net result is movement decrement of macromolecules. This may describe why in the middle of a run, current increment, changes previous sharp bands to broad bands, and after few minutes, reversing back the current, condense the bands again to sharp bands. When the current turns up, movement of the molecules in "one band" gains but the forward molecules of the sharp bands meet

more hitting within the gel matrix. Therefore in this situation "some" backward molecules, in the same band, act as reservoir to compensate and they reach to the front portion with more ease. This makes the front portion of the band denser and moving faster than backward providing a broad band. After turning down the current, revealing the electric pressure decreases passage of the front dense molecules and this gives a chance to backward retarded molecules to reach them resulting sharp bands again.

In constant voltage mode, reducing the temperature increases the electric resistance, so power supply compensates it by reducing the current. This sharpens the band like the effect, which we have mentioned in previous paragraph.

Conclusion

Lowering the temperature in the first portion of the run has been shown to change the electrical resistance of the gel and has caused the macromolecules to assume a relatively regular position before entering into the resolving gel; with bands ultimately showing sharper separation in polyacrylamide gel electrophoresis. Based on this assumption the technique mentioned can be used to replace concentration gradient gels as well. Further research is recommended in order to evaluate if the technique is also applicable to other charged molecules like proteins.

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