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Application of spherical and other polymers in capillary zone electrophoresis: separation of antiviral drugs and deoxyribonucleoside phosphates by different principles

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Abstract

Soluble polymers of linear chains with limited branching and spherical polymers (limit dextrins and sucrose, such as Dextran and Ficoll (Pharmacia Chemicals), yielding lower viscosities, are examined here for the separation of different nucleotides and several anti-AIDS drugs by capillary zone electrophoresis (CZE). The linear polymer forms a network, but spherical polymers appear to create a second pseudo-phase. In general, they tend to enhance the solute mobility and reduce peak width; thus, they improve the column efficiency. We observe that the beads of a spherical polymer produce a pseudo-phase even in a very low polymer concentration. The proposed method involving a spherical polymer yields the best separation for twelve deoxyribonucleoside mono-, di- and triphosphates in ca. 10 min. Common anti-AIDS drugs (ddA, ddC, ddI, d4T, AZT) and an AZT metabolite (AZT-glucuronate) are resolved by using conventional micellar electrokinetic capillary chromatography (MEKC). These results not only offer fast and highly sensitive detection techniques for the pharmacokinetics of nucleotides, drugs, and their metabolites, but they also demonstrate an application of the proposed second pseudo-phase involving spherical polymer beads in CZE separations.

Keywords: Polymer phases; Pseudo phases; Nucleotides; Anti-AIDS drugs; Antiviral drugs

1. Introduction

To study the metabolic turnovers of different nucleotides in research and clinical laboratories, sensitive separations of nucleoside mono-, di- and triphosphates are required. Separation of nucleic acid bases, ribo- and deoxyribonucleosides and nucleotides have been achieved using high-performance liquid chromatography (HPLC) in the past [1–4]. Some of these methods offer very fast analyses, but they lack sensitivity of CZE [5]. CZE offers excellent mass sensitivity, but yields poor UV detection because its light path is shorter than that of HPLC (typically 75 μm versus 5–10 mm). Cohen et al. initially described the separation of bases, nucleosides and oligonucleotides with the use of sodium dodecyl sulfate (SDS) micelles and noted that the more uncharged hydrophobic species exhibit larger partition coefficients and longer migrations [6]. Using anionic micelles (micellar electrokinetic capillary chromatography, MEKC), Novotny and coworkers have described fast separations of nucleoside mono-, di- and triphosphates with a picomo-

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lar detection limit; they also noted the favourable effect of cationic surfactants on these separations [7]. Huang et al. have also described separation of ribonucleotides, but by using a cross-linked polyacrylamide gel capillary with a 5 m*M* detection limit [8]. Recently, optimal separations of purine bases and ribonucleosides by CE methods have been reported in somewhat basic pH conditions (20 m*M* borate buffer, pH 9.4) and with a sensitivity of 0.5 μ*M* [9]. The comparative stability of thymidine and its analogs has been monitored also in a tetraborate buffer of pH 10.5 by employing CZE [10]. The electrophoretic behaviour of isomers of ribonucleoside monophosphates has been studied using CZE while using a complex forming reaction with β-cyclodextrin and borate [11]. Adenine and cytosine derivatives undergo base catalyzed deamination under very basic conditions, producing inosine and uracil compounds, respectively. Analytical methods are needed for resolving nucleosides and nucleotides, preferably under slightly acidic conditions.

Different nucleotides fractionate in three major groups according to their net charge in conventional CZE. We have investigated CZE methods with different polymer additives to obtain high sensitivity and accuracy for the separation of nucleosides, their analogs, and their mono-, di- and triphosphates, while avoiding chemical modification procedures. Large charged species such as oligonucleotides tend to separate in the presence of linear polymers by a sieving effect or by entanglement; however, small charged species, such as nucleotides, are not influenced. Different types of spherical polymers are examined here to offer a novel separation mechanism, i.e. a second pseudo-phase for the separation of small charged molecules.

A polymer of smaller chains, yielding a smaller mesh size, should be appropriate for the fractionation of different deoxyribonucleotides. Soluble polymers of limited branching, such as polyethylene glycol and hydroxyethyl cellulose are studied here. Other polymers, which may produce relatively lower viscosity, such as spherical polymers, are also examined. They are limit dextrins (Dextran T-70) and polysucrose polymers (Ficoll 70 and 400), typically used for cell separations (Pharmacia, Uppsala, Sweden). Differences in solution homogeneity should cause changes in the molecular charge and conformation. Such differences should promote variance in the electrophoretic behaviour of the solutes. In this system, three major forces are recognized: (a) electrophoretic mobility (μ_{em}) in one direction, (b) electroosmotic flow (EOF or μ_{eo}) in the reverse direction, and (c) molecular filtering due to the mesh size.

Separation and sensitive analysis of antiviral drugs, shown in Fig. 1, are needed to study their pharmacokinetics in biological fluids. Conventional CZE offers very sensitive separations, but fails to resolve uncharged molecules, such as the nucleosides which move with the bulk solution. MEKC offers solute partitioning between micelles, formed by SDS, and the bulk solution. The MEKC method with other additives is studied for the separation of these drug molecules (Fig. 1).

2. Experimental

2.1. Materials

The 2′,3′-dideoxyribocytidine (ddC), 3′-azido-2′,3′-dideoxyribothymidine (AZT), 5′-O-glucuronide-3′-azido-2′,3′-dideoxyribothymidine (Glu-AZT), 5′-deoxyribonucleoside mono- (5′dAMP, 5′dCMP, 5′dTMP, 5′dGMP), di-, (dADP, dCDP, dGDP, dTDP), and triphosphates (dATP, dCTP, dGTP and dTTP) were obtained from Sigma Chem. (St. Louis, MO, USA); 2′,3′-dideoxyriboinosine (ddI), 2′,3′-dideoxyriboadenosine (ddA) and 2′,3′-dideoxy-2′,3′-didehydroribothymidine (d4T) were gifts from Dr. C. Sapino, Site Director, Bristol-Myers Co., Syracuse, NY, USA. Poly(ethylene glycol)s (PEG M_r 200, and M_r 10 000) were purchased from Aldrich Chem. (Milwaukee, WI, USA), Dextran T-70 and Ficoll 400 from Pharmacia (Uppsala, Sweden), ethylene glycol (EG) from Fisher Scientific (Fair Lawn, NJ, USA), and SDS of electrophoresis grade from Polysciences (Warrington, PA, USA). All buffers were made according to published procedures by using appropriate weak acid and its sodium salt [12]. Buffer pH values were adjusted after incorporation of additives, if any. Buffer solutions were filtered through a 0.2-μm pore filter (Gelman Sciences, Ann Arbor, MI, USA).

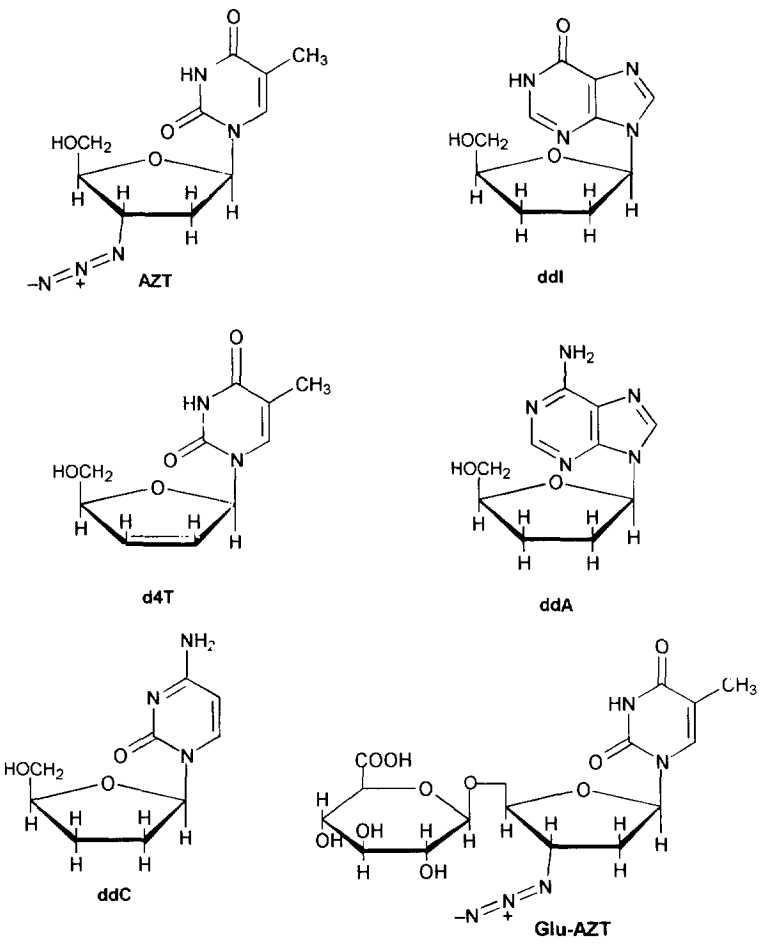

Fig. 1. AZT, 3′-azido-2′,3′-dideoxyribothymidine; ddI, 2′,3′-dideoxyriboinosine; d4T, 2′,3′-didehydro-2′,3′-dideoxyribothymidine; ddA, 2′,3′-dideoxyriboadenosine; ddC, 2′,3′-dideoxyribocytidine; Glu-AZT, 5′-glucuronide-3′-azido-2′,3′-dideoxyribothymidine.

2.2. Preparation of dideoxyribonucleoside and nucleotide solutions

Concentrated stock solutions of dideoxyribonucleosides, deoxyribonucleosides and deoxyribonucleotides were prepared in 1.0 ml of 20 m*M* succinate buffer, pH 5.5. To enhance the solubility of the purine derivatives and that of d4T, 0.1 ml of methanol was added to the solution. The solutions were filtered through a 0.2-μm filter tip-syringe assembly (Millipore, Bedford, MA, USA), appropriately diluted (0.1 *M* HCl, 0.1 *M* NaOH or succinate buffer pH 5.5), and their spectra were analyzed in the running buffer using a Gilford spectrophotometer (Ciba Corning, Oberlin, OH, USA) [3]. For the capillary electrophoresis work, standard solutions of known concentrations of six nucleosides and twelve nucleotides were prepared by mixing an appropriate quantity of the compound. Samples were stored at −20°C until ready for column injection.

2.3. Capillary zone electrophoresis system and operation

The CE equipment designed in this laboratory consisted of four basic components [3]: (a) a fused-silica capillary tubing (80 cm×75 μm I.D. and 280 μm O.D., 52 cm effective length) and an anodic reservoir, electrically grounded, (b) a specially de-

signed on-line Z-shaped flow cell with a light path of 3 mm (LC Packing, San Francisco, CA, USA), (c) variable-wavelength UV monitors, a Spectroflow 757 (Kratos Analytical Instruments, Ramsey, NJ, USA) modified to accommodate the above-mentioned flow cell and a CV^4 Model (Isco, Lincoln, NB), and (d) a high-voltage power supply (0–30 kV) with features of programmable, reversible-polarity output (Bertan Assoc., Hicksville, NY, USA). The direction of the solution flow during the CE could be changed by simply reversing the electrode polarity. The electric current was monitored using a digital multimeter (John Fluke, Everett, WA). An HPLC elution pump (Water Assoc., Milford, MA, USA) was used for column washing and to initiate the sample injection. The detector signals, fed to a strip chart recorder for instant monitoring, were also acquired with an IBM-compatible PC by using an analog-to-digital interface and were retrieved with the help of software (Nelson Analytical, Cupertino, CA, USA). A sample was normally injected from the anode side and the detector was installed on the cathode side. The operation temperature was controlled at 20±0.5°C.

2.4. Diverse methods

The electroosmotic flow of the capillary was measured by using a neutral marker [13]. The specific viscosity of the solutions containing polymers were measured with reference to pure water (20±0.5°C) by using an Ostwald viscometer [14,15]. The migration time of the micelles in MEKC was determined according to the method of Terabe and co-workers [16,17] by using Sudan Black as a marker. The results in the different figures are presented showing migration time, similar to the retention volume in HPLC, except that we report the results based on the time difference between the migration of the solute and that of a neutral marker which appears very early in the chromatogram (similar to $t_R - t_o$ in HPLC). Hence, it is termed here as the relative migration time but it should not be confused with the ratio of t_m to t_o. This value, in our opinion, is more practical, because it eliminates variation from one experiment to another and it is also not an abstract number, such as a ratio which is difficult to visualize.

3. Results and discussion

3.1. Separation of deoxyribonucleosides and anti-AIDS drugs

Usually, nucleosides do not exhibit significant charge differences; hence, they fail to resolve using conventional CZE and emerge in the breakthrough peak. We find, as others have noted, that the nucleosides can be resolved by MEKC, that is by adding SDS to the buffer in order to form micelles. A typical separation of five dideoxyribonucleoside (anti-AIDS) drugs and glucuronate derivative of AZT (Glu-AZT) is shown in Fig. 2. The separation is caused by differences in size and hydrophobicity among different species. Small nucleosides (d4T and

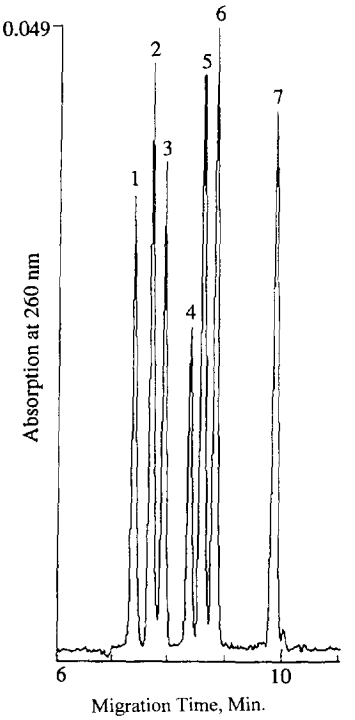

Fig. 2. Separation of anti-AIDS drugs and Glu-AZT by MEKC. A mixture of five dideoxyribonucleosides and AZT derivative was applied to an uncoated capillary column (80 cm×75 μm, 52 cm effective length) using a positive flow injection method and electrophoresis is carried out in a 50 m*M* phosphate buffer containing 40 m*M* SDS (pH 6.5) using 20 kV (69 μA) and monitoring at 260 nm. Peaks: 1 = impurity; 2 = d4T; 3 = ddC; 4 = ddI; 5 = AZT; 6 = ddA; 7 = Glu-AZT.

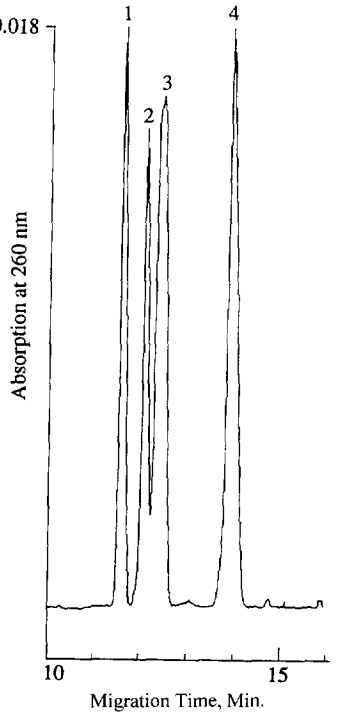

Fig. 3. Separation of deoxyribonucleosides by MEKC. A mixture of six deoxyribonucleosides are separated under the conditions of Fig. 1, except that 15 kV was applied. Peaks: 1 = dC + dT; 2 = m^5dC; 3 = dA + dG; 4 = m^6dA.

ddC) exit before the larger ones (AZT and ddA), indicating differences in their partition coefficients. Though dideoxyribonucleosides are satisfactorily resolved, deoxyribonucleosides under these conditions can only be resolved partially. For example, common deoxyribonucleosides and their two methylated species resolve in four peaks, as shown in Fig. 3. Between pyrimidine (dC+dT) and purine (dA+dG) nucleosides, each group emerges in one peak, while pyrimidine ones emerge before the more hydrophobic purine deoxyribonucleosides. The eukaryotic minor component, m^5dC emerges immediately after the pyrimidine deoxyribonucleoside peak and the prokaryotic minor component of DNA (m^6dA) exits very late in the electrophorogram because of its strong hydrophobic character. Both minor components are found in DNA in minute amounts (1% of dC or dA). They generally elute with their parent compounds in HPLC methods [3]. Here, these two species are well resolved. A modification of these separation conditions can perhaps also resolve the major components of the mixture.

3.1.1. Influence of buffer additives

The influence of different additives, Ficoll (M_r 400 000), ethylene glycol (EG, M_r 28), poly(ethylene glycol)s (PEG, M_r 200 and 10 000), and a dextrose polymer (Dextran T-70, M_r 70 000) are examined in this study. The results in Table 1 and Fig. 4a indicate that all additives (except for PEG of M_r 10 000, not shown) tend to reduce both peak migration and peak width, resulting in greater separation efficiency. Dextran in 2.5% concentration offers the best resolution of the five drugs among this group of the

Table 1
Separation of antiviral drugs: effect of different additives and electrical potentials in MEKC

Additive	M_r	Relative viscosity	15 000 V		20 000 V	
			Plates[a]	μ_{eo}^b	Plates[a]	μ_{eo}^b
None[c]		1.00	73 200	4.0	48 000	4.5
Ficoll 2.5%	400 000	1.20	95 700	3.5	77 900	4.0
Ficoll 7.5%	400 000	2.60	99 200	2.8	88 300	3.3
PEG[d] 2.5%	200	1.00	96 000	3.7	79 400	4.0
Dextran 2.5%	70 000	1.40	192 000	3.9	131 200	4.2
EG[e] 2.5%	28	1.00	138 100	4.0	117 400	4.3

[a] Theoretical plate numbers per meter, derived from ddC and ddA.
[b] Electroosmotic migration is expressed in $cm^2\ V^{-1}\ s^{-1}$ (10^{-4}).
[c] Separations performed by MEKC using a 50 m*M* phosphate buffer, pH 6.5 containing 40 m*M* SDS and an additive as indicated.
[d] PEG, poly(ethylene glycol).
[e] EG, ethylene glycol.

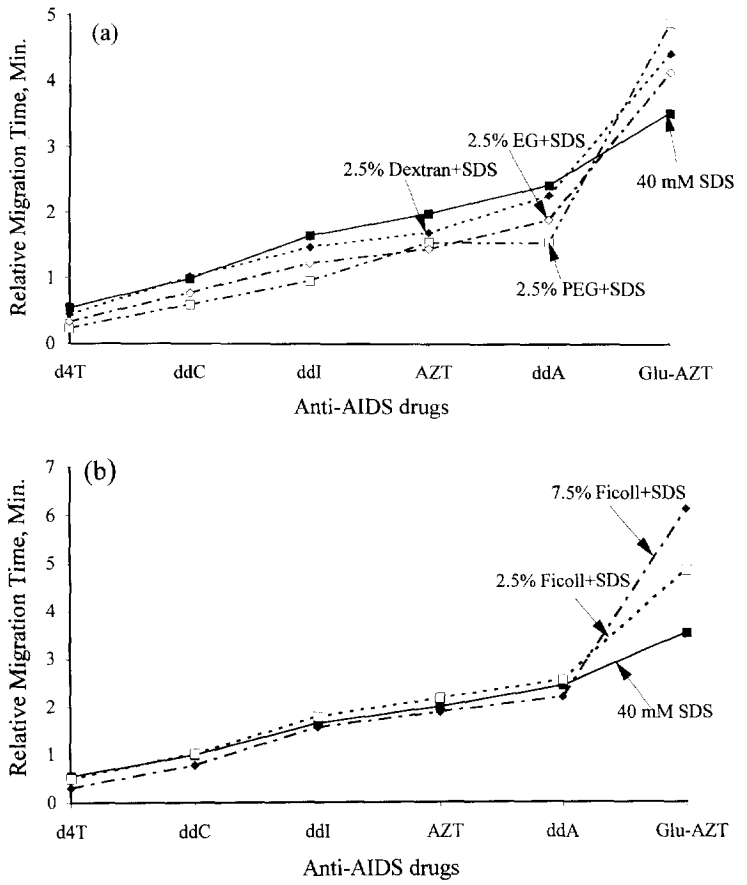

Fig. 4. Emergence of the anti-AIDS drugs and Glu-AZT in MEKC. The buffer contains different types of additives, in addition to SDS: (a) EG, PEG M_r 200 and Dextran; (b) Ficoll in different concentrations. For other experimental details, see Fig. 2 caption. The relative migration time refers to the migration time of the peak minus the reference peak time.

additives (Fig. 5). An increase in the voltage equally enhances the emergence of these drugs, but lowers the separation efficiency.

Most additives slightly increase the buffer viscosity without changing the elution order of the drugs. However, they also significantly increase the migration of Glu-AZT because of the larger size. Only a marginal separation between ddA and AZT can be observed with PEG (2.5%, M_r 200). An application of a larger PEG polymer (M_r 10 000) yields the worst results. The influence of Ficoll, shown in Fig. 4b, indicates that while a 2.5% concentration of Ficoll somewhat increases the relative migration, a higher concentration (7.5%) of Ficoll decreases their emergence from the capillary.

3.1.2. Role of additives in MEKC separations

Substances other than a detergent may be added to the run buffer to alter selectivity. Such modifiers may affect the electrical charges on the micelles or on the solute, thus altering the solubility (partitioning) of a solute in the micelles. Modifiers may also serve as a second pseudo-phase providing solute partitioning between the micelles and other hydrophobic phase, see Fig. 6a. Separation can be achieved based on the differences in the distribution of the solutes between the hydrophobic and the charged micellar phase.

An introduction of a polymer (linear, partial branched or highly branched) to MEKC increases the aqueous-phase viscosity and reduces opposite migra-

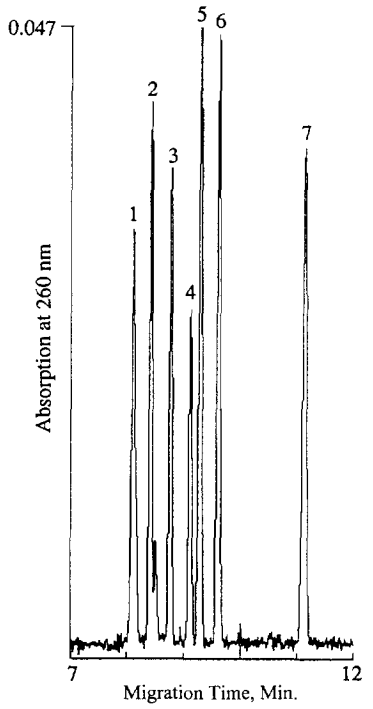

Fig. 5. Separation of the anti-AIDS drugs and Glu-AZT by MEKC with polymer additives. Separation was according to the conditions in the caption of Fig. 1, except that Dextran T-70 (2.5%) was added to the buffer. Peaks: 1 = impurity; 2 = d4T; 3 = ddC; 4 = ddl; 5 = AZT; 6 = ddA; 7 = Glu-AZT.

tion of micelles, thus resulting in sharper and early peaks (Fig. 4). The small chain polymers [ethylene glycol, poly(ethylene glycol) of M_r 200] tend to decrease hydrophobic interactions between nucleosides and micelles; they reduce partitioning between micelles and the aqueous solution and increase separation efficiency (Table 1). PEG has strong interactions with the inner capillary surface, while the dextran seems to increase the buffer viscosity. In this study, we have attempted to create a second pseudo-phase by adding highly soluble spherical polymers of different kinds. A spherical polymer, such as Ficoll, appears to form a second pseudo-phase and influences interactions between micelles and solutes, reduces the micellar migration and offers improved separation.

Migration behaviour in MEKC is governed by hydrophobicity, i.e. more hydrophobic solutes interacting strongly with the micellar phase move more slowly than the hydrophillic solutes. Terabe et al. [18] proposed a "migration time window" for the migration of solute that interacts with the micelles. Accordingly, the migration of a typical solute (t_M) falls between the migration of a hydrophillic solute exhibiting no interaction with the micelles (such as water, t_o) and a strong hydrophobic solute interacting effectively with the micelles, i.e. completely solubil-

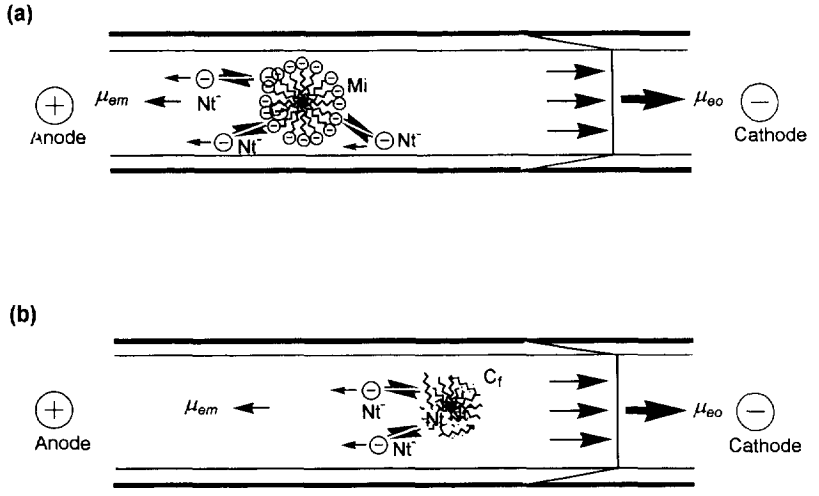

Fig. 6. Diagram representation of proposed separation mechanisms of ribonucleotides by: (a) MEKC, and (b) PS–CZE with Ficoll. Mi, micelle formed by SDS; Nt^-, nucleotides; C_f, coiled structure of Ficoll.

ized by the micelles (t_{mc}). Thus, the time interval permitting the separation is: $t_{mc}-t_o$. A spherical polymer appears to increase the t_{mc}/t_o ratio. Hence, the capacity factor of the neutral solute, such as a nucleoside, should increase with a parallel increase in the t_{mc} value or a decrease in the t_o value:

$$k' = \frac{t_M - t_o}{t_o(1 - \frac{t_M}{t_{mc}})}$$

This argument is supported by the fact that the μ_{eo} value progressively decreases as more Ficoll is added to the run buffer, as shown in Table 1 and Fig. 4b.

3.1.3. Behaviour of the nucleosides in MEKC

Hydrophobic and neutral dideoxyribonucleosides (ddA and AZT) reside more easily inside the micelles; therefore, they emerge later than their relatively hydrophillic species (ddC and d4T); the latter ones perhaps move with the bulk solution (Fig. 2). This phenomenon is also applicable in the case of the separation of typical deoxyribonucleosides. The results indicate that pyrimidine nucleosides elute prior to the purine ones and the methylated (hydrophobic) species appear after their parent nucleosides (Fig. 3). In addition to the hydrophobicity, the size of the nucleosides also plays an important rule in these separations. Bulkier molecules, such as Glu-AZT, exhibiting lower diffusion rates, are trapped easily in the micelles and elute slowly. In addition, sugar hydrophillic groups in Glu-AZT can also reduce interactions with the micelles resulting in a lower migration time. However, the sugar carboxylic group, fully ionized at pH 6.5, can be expected to contribute substantially to the electrophoretic mobility (μ_{em}) in the opposite direction, thus resulting in a net loss in the migration of this molecule ($\mu_{eo}-\mu_{em}$). The separation and identification of anti-AIDS drugs by MEKC provides a fast and highly sensitive detection technique for monitoring the drugs and their metabolites (Fig. 1).

3.2. Separation of deoxyribonucleotides

3.2.1. Separation by capillary zone electrophoresis

The separation of deoxyribonucleotides by conventional CZE is not practical because these charged species exhibit electrophoretic migration in the opposite direction and may also interact with the capillary surface producing broad peaks and low column efficiency, as shown in Fig. 7. The deoxyribonucleotides emerge in increasing order of their anionic charge, i.e. deoxyribonucleoside monophosphates, followed by diphosphates, and then triphosphates. As expected in CZE, the hydrophobic purine deoxyribonucleotides (dG before dA) emerge prior to the hydrophillic pyrimidine ones (dC after dT) (see below).

In CZE, migration of the nucleotides depends on their net electrical charge (negative phosphate less positive base charges) and molecular size. Though the nucleotides basically exhibit a net negative charge, they move downstream with the predominant electroosmotic flow against their negative charges

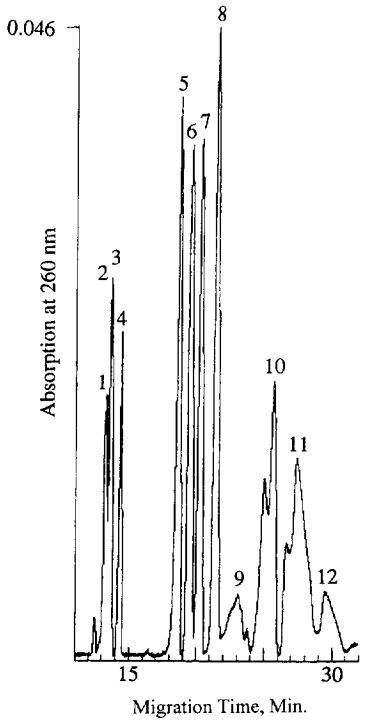

Fig. 7. Separation of deoxyribonucleotides by CZE. A mixture of twelve deoxyribonucleotides is applied to an uncoated capillary column (80 cm×75 μm, 52 cm effective length) using a positive flow injection method and electrophoresis is carried out in a 50 m*M* phosphate buffer (pH 6.5) by applying 20 kV (60 μA) and monitoring at 260 nm. Peaks: 1 = dGMP; 2 = dAMP; 3 = dTMP; 4 = dCMP; 5 = dGDP; 6 = dADP; 7 = dTDP; 8 = dCDP; 9 = dGTP; 10 = dATP; 11 = dTTP; 12 = dCTP.

(cathodic end). They emerge in CZE in the order of nucleoside mono-, di- and triphosphates. Among the nucleotides having the same charge, but different sizes, larger purine nucleotides encounter less hindrance in migrating upstream because they easily move with the bulk solution (Fig. 7). If the capillary surface is capped by dynamic coating (PEG), the nucleotides migrate (electrophoretic) to the anodic end by their electrophoretic migration and emerge in the reverse order, i.e. nucleoside triphosphates exit before diphosphates and the monophosphates fail to emerge from the column (results not shown).

3.2.2. Evidence of second pseudo-phase formation by a spherical polymer

The typical influence of different Ficoll concentrations on the migration velocity of bromophenol blue and on the solution viscosity is shown in Fig. 8. The hydrophobic bromophenol blue dye (pK_a of 4.0) dissolves very poorly in water and exhibits a net negative charge at pH 6.5. This dye binds with the Ficoll polymer, but not completely. Therefore, it should not be considered as a true marker. In polymer solution–capillary zone electrophoresis (PS–CZE), the migration of the dye in an uncoated capillary should not change when the polymer is added (low viscosity). However, bromophenol blue apparently interacts strongly with Ficoll micelles, forming a second pseudo-phase, as observed by a decrease in its migration (see below).

The migration time is not linearly related to the viscosity; however, the migration velocity is inversely proportional to the viscosity. Therefore, the migration velocity of blue dye, as well as the solution viscosity are plotted against different concentrations of the Ficoll polymer. In Fig. 8, while the viscosity increases constantly, the velocity changes with the polymer contents in the solution. When a small amount of Ficoll is added (1%), the migration velocity of the marker increases considerably, and continues to increase until the Ficoll concentration reaches 3% of the solution. Between 3% and 8% of Ficoll, the marker's velocity does not change until the relative viscosity doubles. A Ficoll concentration in excess of 8% tends to decrease the migration velocity of the marker, and is inversely proportional to the buffer viscosity.

These changes in dye migration offer evidence in

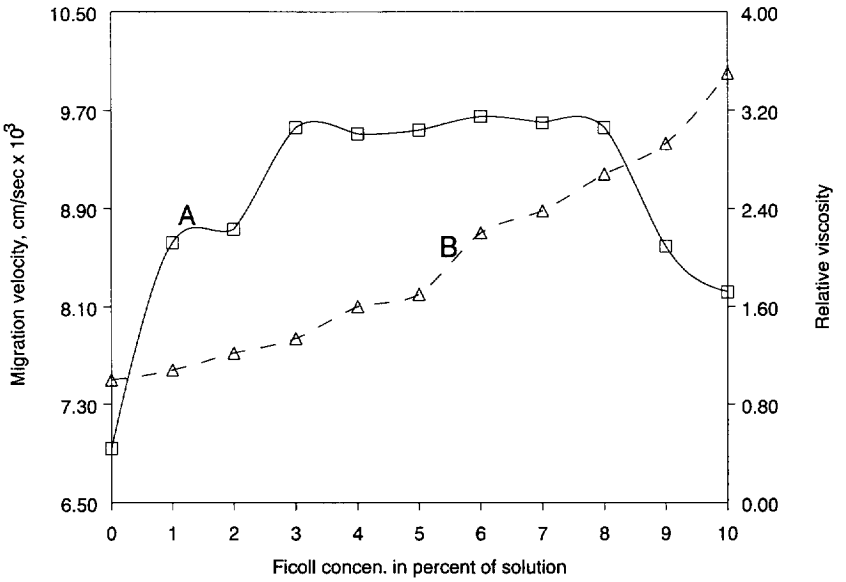

Fig. 8. Relation between the solution viscosity and the migration time of bromophenol blue in the presence of different concentrations of Ficoll additive: (a) migration velocity of bromophenol blue, and (b) relative viscosity of the buffer. The marker is applied to an uncoated capillary (50 cm×75 μm, 30 cm effective length) and electrophoresis was carried out in a 50 m*M* phosphate buffer with different Ficoll concentrations, pH 6.5, and monitored at 260 nm.

support of a micellar-type pseudo-phase formation by the Ficoll polymer. Two opposing factors (polymer phase movement and viscosity pull) appear to contribute to the movement of bromophenol blue. The Ficoll polymer phase moves the dye with the flow of the aqueous solution, but an increase in the solution viscosity by this polymer tends to lower the migration. Initially with a low concentration of Ficoll (1–3%) the polymer phase appears to dominate the solute movement, thus producing an early emergence of the dye. With increased Ficoll contents (3–8%), both factors (polymer phase movement and viscosity pull) cancel each other out, thus producing an unaltered dye movement. However, at a very high concentration of the Ficoll (8–10%), the solution viscosity causes a considerable migration of the dye (Fig. 8). The Helmholtz equation indicates that the electroosmotic flow is inversely proportional to the solution viscosity [19]. This theoretical prediction is confirmed by the migration behaviour of bromophenol blue in the higher percentage range. In this study, the dynamic driving force ($\mu_{eo} - \mu_{em}$) linearly decreases with increased solution viscosity as a result of Ficoll addition. A 5% concentration of Ficoll minimally affects the electroosmotic flow (about 8% value) and increases the relative viscosity by 0.6 units (results not shown).

3.2.3. Spherical polymer and separation efficiency

Characteristics of Ficoll polymer

Ficoll is a synthetic polymer of sucrose with a high molecular mass (M_r 400 000). These beads are highly branched and compactly coiled with a Stokes' radius of about 10 nm. It has an intrinsic viscosity about three times lower (17 ml g^{-1}) than that of a linear polysaccharide (Dextran, 49 ml g^{-1}) of the same molecular mass [20]. Apparently, the coiled Ficoll structure in solution behaves like a micelle with a hydrophobic interior and a hydrophillic exterior, see Fig. 6b. Unlike a micelle, however, this coiled structure is very stable, and exhibits only a weak surface polarity. Unlike the strong sulfate anions in SDS, Ficoll can only offer undissociable polyhydroxy groups. The charge difference between the interior and the exterior of the Ficoll micelles is not well established.

A study of the effect of Ficoll concentration on the column separation efficiency for several nucleotides showed that a direct relationship exists between the Ficoll concentration (viscosity increase) and the separation efficiency in both CZE and MEKC separations (results not shown). Both plate number (N) and solution viscosity are inversely proportion to the solute diffusion [19]. Hence, our observation of an increase in efficiency with a parallel increase in viscosity confirms the theory. However, this parallel holds good only within a specific and practical range of solution viscosities. Excessive viscosity will greatly reduce the solute movement at a given temperature.

The effect of Ficoll on nucleotide separation, studied for several Ficoll concentrations (1–8%) and shown in Fig. 9a–b, indicates that a lower or a higher concentration than 5% Ficoll does not yield satisfactory resolutions. Hence, this amount can be considered as a critical concentration of the spherical polymer required for these separations. This additive enhances the peak resolution between the purine and the pyrimidine group of nucleotides. The migration pattern of the nucleotides changes proportionally with the amount of the Ficoll added. Fig. 10 shows comparisons of the relative migrations of the nucleotides in three different modes, i.e. polymer solution CZE (three Ficoll concentrations), MEKC (SDS) and CZE (no additives).

Fig. 11 shows a comparison of the migration of guanosine mono-, di- and triphosphates in the presence of different Ficoll concentrations (0–10%). The most significant decrease in the migration of dGDP and dGTP is observed between 1% and 3% of Ficoll in solution, while dGMP is least affected. Their migration is hardly influenced by a higher concentration of Ficoll (3% to 9%) although it is significantly increased with a 10% concentration of Ficoll. Interestingly, these results are in line with those observed for bromophenol blue in Fig. 8, confirming the need for a critical concentration of the spherical polymer for optimal solute migration.

Influence of other polymer additives

The influence of other polymers (Dextran T-70 and PEG) and additives (ethylene glycol and SDS) is compared with simple CZE (buffer) in Fig. 12, while using uncoated capillaries. An addition of a linear polymer (hydroxyethyl cellulose, HEC) (results not

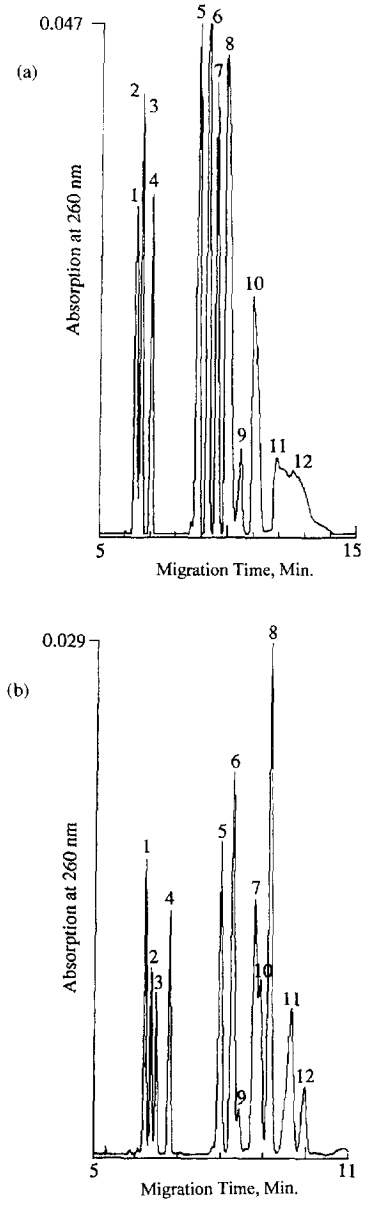

Fig. 9. Influence of Ficoll concentration on the resolution and elution order of deoxyribonucleotides in polymer solution – CZE. Ficoll in different concentrations is added to the buffer: (a) 1%, and (b) 5% of Ficoll. A mixture of twelve deoxyribonucleotides is applied to an uncoated capillary (50 cm×75 μm, 30 cm effective length) and electrophoresis was carried out in a 50 m*M* phosphate buffer with different concentrations of Ficoll, pH 6.5, and monitored at 260 nm. Peaks: 1=dGMP; 2=dAMP; 3=dTMP; 4=dCMP; 5=dGDP; 6=dADP; 7=dTDP; 8=dCDP; 9=dGTP; 10=dATP; 11=dTTP; 12=dCTP. Note triphosphates of G and T emerge before diphosphates of T and C monomers, respectively.

shown) or a partially branched polymer (Dextran) fails to influence the nucleotide separation. They are too small to interact with the polymer network. The results indicate that all polymers including SDS appear with lower relative migrations of different nucleotides, although their elution order remains unchanged. The effect of different additives on buffer viscosity, electroosmotic flow and separation efficiency is listed in Table 2.

An addition of 2.5% of PEG (M_r 10 000) to the solution allows the emergence of the deoxyribonucleotides only towards the cathodic end. The order of their appearance is also reversed. For example, nucleoside triphosphates elute prior to their diphosphates and monophosphates fail to emerge from the column during a 50 min electrophoresis (results not shown). This particular polymer appears to form a dynamic coating over the capillary surface, and, in effect, significantly reduces the electroosmotic flow (about 47%). The nucleotides with PEG (M_r 10 000), therefore, appear to move more by their electrophoretic migration rather than the electroosmotic flow.

3.2.4. Separation of deoxyribonucleotides by MEKC

Under the MEKC conditions (Fig. 13), i.e. with the addition of SDS to the solution, most deoxyribonucleotides are satisfactorily resolved. The peaks are symmetrical and they emerge early, but in the same order as in CZE. Though the addition of SDS does alter the electroosmotic flow, the column efficiency improves marginally. SDS micelles carry with them the charged nucleotides, thus reducing their electrophoretic migration. Micelles with the nucleotides move together with the electroosmotic flow because of their large size, resulting in symmetrical peaks. SDS in MEKC serves two functions: it reduces the capillary surface (i.e. surface interactions) and it forms charged micelles, yielding solute partitioning. The nucleotides emerge in the order of nucleoside, mono-, di- and then triphosphates as observed in CZE. Unexpectedly, hydrophobic purine nucleotides emerge before the hydrophillic pyrimidine species (Fig. 13).

A combination of SDS with other additives improves the column efficiency, but yields poor separations, as shown in Table 3. In this combination of

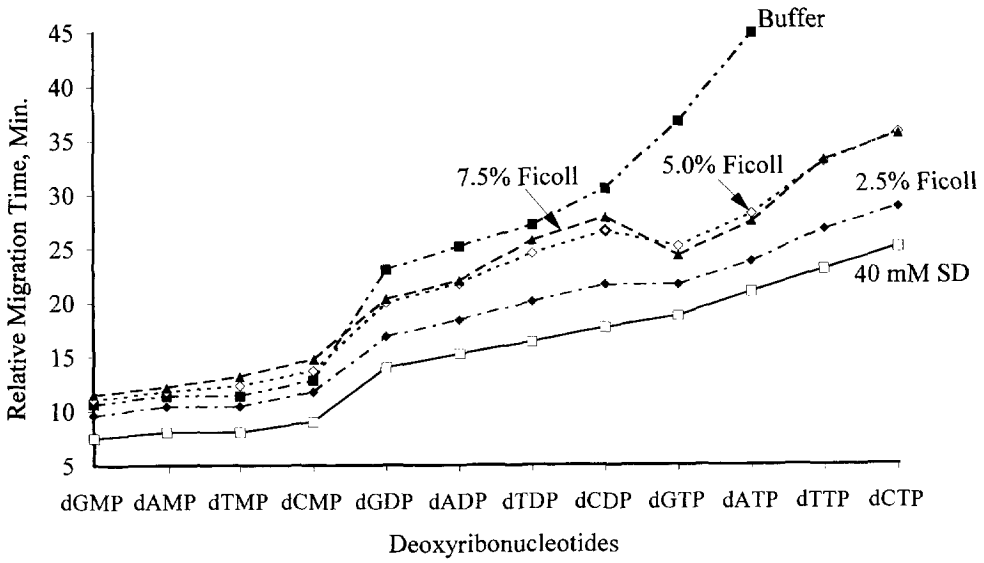

Fig. 10. Emergence of deoxyribonucleotides in the presence of Ficoll (or SDS) additive. A mixture of twelve deoxyribnucleotides is separated by using different concentrations of Ficoll (or SDS) under the experimental conditions given for Fig. 7. The relative migration time refers to the migration time of the peak minus the "dead" time.

two additives, two different separation mechanisms are visualized. For example, the micelles produced by SDS offer partitioning, while the linear and partially branched polymers yield a second pseudo-phase for the relative distribution of the solutes. Because of the small size of the nucleotides, the latter interactions for certain polymers may be insignificant. A spherical polymer, such as Ficoll,

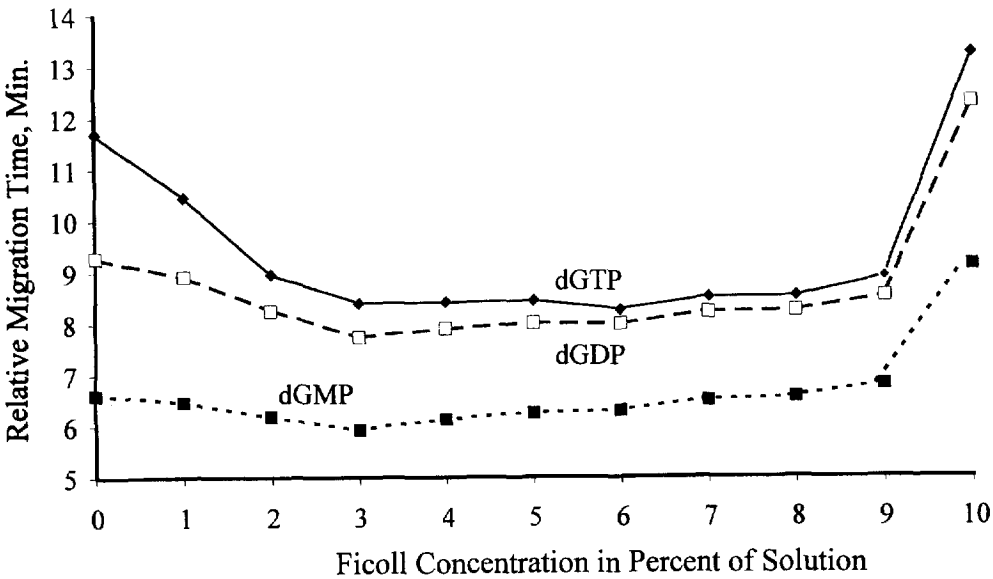

Fig. 11. Emergence of guanine deoxyribonucleoside phosphates in polymer solution–CZE by using Ficoll as an additive polymer. For experimental conditions, see the Fig. 9 caption.

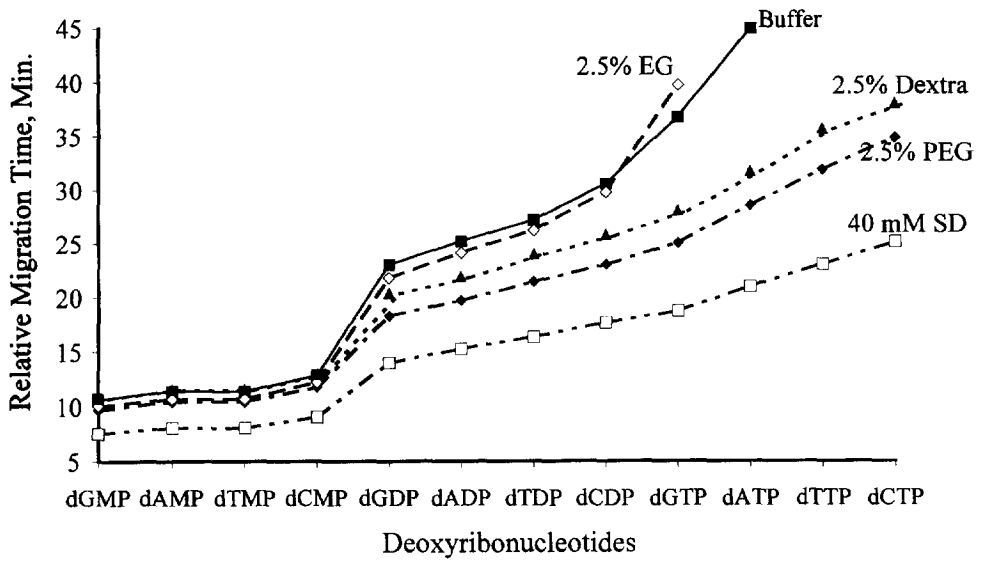

Fig. 12. Emergence of deoxyribonucleotides in the presence of different additives. A mixture of twelve deoxyribonucleotides is separated by using different additives under the experimental conditions of Fig. 7. (PEG, polyethylene glycol of M_r 200.)

appears to yield the best separations for the nucleotides even in the absence of SDS.

Application of MEKC for the nucleotide separation

CZE can best offer separations of neutral molecules based on very small differences in their viscous pulls. MEKC combines the separation mechanism of chromatography with the electrophoretic and electroosmotic movement of the solutes [18,21]. This method provides for the separation of electrically neutral species. In addition to neutral molecules, MEKC can be employed to resolve ionic compounds [21–24]. The micelles form a pseudo-phase and provide partitioning of solutes between the micelles and the aqueous phase. Negatively charged micelles are attracted by the anode, but they move to the cathode by strong electroosmotic flow. Electrophoretic mobility of the micelles is less than that of the

Table 2
Separation of nucleoside phosphates: Effect of different additives and electrical potentials in CZE and PS–CZE

Additive	M_r	Relative viscosity	15 000 V		20 000 V	
			Plates[a]	μ_{eo}[b]	Plates[a]	μ_{eo}[b]
None[c]	–	1.00	18 400	4.2	22 400	4.7
Ficoll 2.5%	400 000	1.20	58 800	3.7	30 700	4.5
Ficoll 5.0%	400 000	1.90	63 200	3.5	52 500	4.3
Ficoll 7.5%	400 000	2.60	106 900	3.2	104 600	3.8
PEG[d] 2.5%	200	1.00	35 800	4.2	n/d	n/d
Dextran 2.5%	70 000	1.40	49 100	3.8	65 900	4.0
EG[e] 2.5%	28	1.00	28 500	4.2	24 200	4.3

[a] Theoretical plate numbers per meter, derived from dCMP, dGDP, and dGTP.
[b] μ_{eo}, Electroosmotic migration is expressed in $cm^2\ V^{-1}\ s^{-1}$ (10^{-4}).
[c] Separations performed in a 50 m*M* phosphate buffer, pH 6.5 containing an additive as indicated.
[d] PEG, poly(ethylene glycol).
[e] EG, ethylene glycol.
n/d, not determined

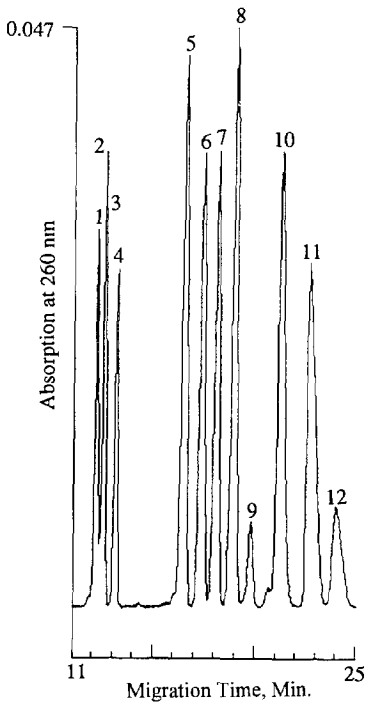

Fig. 13. Separation of deoxyribonucleotides by MEKC. Electrophoresis is carried out under the conditions given for Fig. 7, except that 40 m*M* SDS is added to the buffer. Peaks: 1 = dGMP; 2 = dAMP; 3 = dTMP; 4 = dCMP; 5 = dGDP; 6 = dADP; 7 = dTDP; 8 = dCDP; 9 = dGTP; 10 = dATP; 11 = dTTP; 12 = dCTP.

electroosmotic flow. Hydrophillic solutes elute in the void volume (at time t_o) because they are insoluble in the micelles. Hydrophobic and partially hydrophobic solutes partition between the dynamic micellar phase and the aqueous phase. Negatively charged micelles (SDS) and negatively charged solutes, such as the nucleotides studied in this work, are not strongly attracted to each other. Therefore, nucleotides are resolved by their differences in electrophoretic mobility, similar to CZE, see Fig. 6a. Even anionic solutes can be partially solubilized by negatively charged micelles [25]. Novotny et al. observed considerable improvement in the separation of ribonucleotides with the use of cationic surfactants over CZE alone or MEKC [7].

4. Conclusions

Ficoll forms a polymer phase in PS–CZE, but unlike MEKC, which needs a critical concentration (CMC), these beads of a spherical polymer yield a polymer phase even in a low concentration of the polymer [26]. We propose that a spherical polymer offers a second pseudo-phase for partitioning of charged solutes. Thus, nucleotides partition between the aqueous phase and this polymer phase due to

Table 3
Separation of nucleoside phosphates: Effect of different additives and electrical potentials in MEKC

Additive	M_r	Relative viscosity	15 000 V		20 000 V	
			Plates[a]	μ_{eo}^{b}	Plates[a]	μ_{eo}^{b}
None[c]		1.00	37 400	4.0	23 900	4.7
Ficoll 2.5%	400 000	1.20	n/d	n/d	53 800	4.2
Ficoll 5.0%	400 000	1.90	61 200	3.8	58 200	4.0
Ficoll 7.5%	400 000	2.60	n/d	n/d	87 100	3.2
PEG[d] 2.5%	200	1.00	n/d	n/d	44 000	4.0
Dextran 2.5%	70 000	1.40	50 900	3.8	63 900	4.2
EG[e] 2.5%	28	1.00	48 500	4.0	36 500	4.2

[a] Theoretical plate numbers per meter, derived from ddC and ddA.
[b] μ_{eo}, Electroosmotic migration is expressed in $cm^2\ V^{-1}\ s^{-1}$ (10^{-4}).
[c] Separations performed by MEKC using a 50 m*M* phosphate buffer, pH 6.5 containing 40 m*M* SDS and an additive as indicated.
[d] PEG, poly(ethylene glycol).
[e] EG, ethylene glycol.
n/d, not determined.

their hydrophobicity. Although nucleotides exhibit a negative charge against the electroosmotic flow, once partitioned with the polymer phase, their electrophoretic migration is considerably reduced (Fig. 11). In addition to the partitioning effect, Ficoll increases the solution viscosity, thus further influencing the separation. In excess of a critical concentration (>3%), Ficoll coils begin to influence the elution order (Fig. 10). For example, the purine nucleotides having stronger interaction with the Ficoll phase, emerge earlier than the pyrimidine nucleotides, and the difference increases in direct proportion to the number of their phosphate groups. Therefore, the abnormal elution sequence, i.e. dGTP and dATP emerging among the nucleoside diphosphates, is caused by nucleotide partitioning between the aqueous and the polymer phase of the Ficoll, see Fig. 9b. The spherical polymer offers a very powerful tool, the second pseudo-phase and also a low viscosity for the separation of charged solutes in PZ–CZE. Other spherical polymers need to be explored for CZE separations.

Acknowledgments

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References

[1] R.P. Singhal and J.P. Landes, J. Chromatogr., 458, (1988) 117–128.
[2] R.P. Singhal, P. Landes, N.P. Singhal, L.W. Bmwn, P.J. Anevski and J.A. Toce, Biochromatography, 4 (1989) 78–88.
[3] R.P. Singhal, D. Hughbanks and J. Xian, J. Chromatogr., 609 (1992) 147–161.
[4] J. Zhao, B. Todd and G.H. Fleet, J. Chromatogr. A, 673 (1994) 167–171.
[5] S. Cohen, A. Paulus and B.L. Kanger, Chromatographia, 24 (1987) 15–24.
[6] A.S. Cohen, S. Terabe, J.A. Smith and B.L. Kanger, Anal. Chem., 59 (1987) 1021–1027.
[7] J. Liu, F. Banks, Jr. and M. Novotny, J. Microcol. Sep., 1 (1989) 136–141.
[8] M. Huang, S. Liu, B.K. Murray and M.L. Lee, Anal. Biochem., 207 (1992) 231–239.
[9] T. Grune, G.A. Ross, H. Schmidt, W. Siems and D. Perrett, J. Chromatogr., 636 (1993) 105–111.
[10] A. Van Schepdael, K. Smets, F. Vandendriessche, A. van Aerschot, P. Herdewijn, E. Roets and Hoogmartens, J. Chromatogr. A, 687 (1994) 167–173.
[11] T. Tadey and W.C. Pundy, J. Chromatogr. B, 657 (1994) 365–372.
[12] J.A.A. Chambers, in J.A.A. Chambers and D. Rickwood (Editors), Biochemistry Lab Fax Series, Bios Scientific Publishers, Academic Press, Oxford, 1993, Ch. 1, p. 1.
[13] K.D. Lukacs and J.W. Jorgenson, J. High Resolut. Chromatogr. Commun., 8 (1985) 407–411.
[14] P.D. Grossman and D.S. Soane, J. Chromatogr., 559 (1991) 257–266.
[15] R.J. Sime, in Physical Chemistry: Methods, Techniques and Experiments, Saunders College Publications, Philadelphia, 1990, Part 3, p. 522.
[16] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya and T. Ando, Anal. Chem., 56 (1984) 113–116.
[17] S. Terabe, K. Ostuda and T. Ando, Anal. Chem., 57 (1985) 834–841.
[18] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya and T. Ando., Anal. Chem., 56 (1984) 111–113.
[19] S.F.Y. Li, Capillary Electrophoresis — Principles, Practice and Applications, Elsevier, Amsterdam, 1992, p. 14.
[20] Pharmacia (1983), in Ficoll-Paque Technique Note (Pharmacia Fine Chemicals), Tryckkontakt Press, Uppsala, Sweden, p. 4.
[21] S. Terabe, K. Otsuka and T. Ando, Anal. Chem., 57 (1985) 834–841.
[22] D.D. Burton, M.J. Sepaniak and M.P. Maskarinec, J. Chromatogr. Sci., 24 (1986) 347–351.
[23] H. Nishi, N. Tsumagari, T. Kakimoto and S. Terabe, J. Chromatogr., 465 (1989) 331–343.
[24] K. Otsuka, S. Terabe and T. Ando, J. Chromatogr., 332 (1985) 219–226.
[25] T. Kaneta, S. Tanaka, M. Taga and H. Yoshida, Anal. Chem., 64 (1992) 798–801.
[26] R.P. Singhal and J. Xian, Capillary electrophoresis: separation of nucleic acids, their components and sequencing of DNA fragments, in D.M. Radzik and S.M. Lunte (Editors), Application of Capillary Electrophoresis to Pharmaceutical and Biochemical Analysis, Pergamon Press, New York, 1996, in press.