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Review

Micropellicular stationary phases for high-performance liquid chromatography of double-stranded DNA

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Abstract

The central role of nucleic acids in biosciences has effectuated the rapid development of numerous techniques for their isolation, separation, characterization and quantitation. Advances in high-performance liquid chromatography, particularly the introduction of novel microparticulate sorbents, have greatly promoted the separation and quantitation of nucleic acids. Because of their favorable mass transfer properties, micropellicullar packing materials are advantageous for fast and high-resolution separations of double-stranded (ds) DNA molecules. With micropellicular packings, anion-exchange and ion-pair reversed-phase chromatography are the most popular chromatographic separation modes for dsDNA. The effective separation mechanisms in both chromatographic techniques are preferably described by nonstoichiometric models, that are founded on a better physicochemical background than traditional stoichiometric models. Column efficiency, retention characteristics, and size or sequence dependency of retention of dsDNA are greatly influenced by the chosen operational variables in both chromatographic modes. The applicability of HPLC with micropellicular stationary phases nucleic acids research includes preparative DNA fractionation, DNA restriction mapping, analysis of polymerase chain reaction products and purification of plasmid DNA. © 1998 Elsevier Science B.V.

Keywords: Reviews; Stationary phases, LC; DNA

Contents

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1. Introduction

Rapid developments in DNA technology, such as DNA cloning techniques, DNA restriction analysis, DNA sequencing, in situ hybridization, and polymerase chain reaction (PCR) have created the need for fast and automated analytical and preparative separation methods for nucleic acids. Characterization of genomic DNA by restriction mapping and hybridization is of high importance in recombinant DNA work and medical diagnostics [1]. Reverse transcription followed by PCR has provided a convenient and highly sensitive method for examining gene expression and for the direct identification of viral genomes [2]. The 'human genome project' to sequence the whole human genome [3], which is one of the largest projects in molecular biology, has also stimulated the search for faster and more reliable methods for DNA separations.

By virtue of its high-resolving capability, short analysis time, advanced instrumentation, and preparative separation capacity, high-performance liquid chromatography (HPLC) is a powerful technique for the separation and quantitation of nucleic acids. A comparison of HPLC with traditional gel electrophoretic and ultracentrifugal techniques reveals that the weakest aspects of the traditional techniques are actually the strengths of HPLC. This is because HPLC offers the opportunity for automatic sampling, fraction collection, data reduction, and reporting functions that greatly enhance quantitation and promote higher sample throughput and recovery. Five major chromatographic modes are utilized for separation of dsDNA: size-exclusion [4–7], anion-exchange [8–11], mixed-mode [12–14], ion-pair reversed-phase [15,16], and slalom chromatography [17–19]. Of these, anion-exchange chromatography is the most prominent technique so far. The various chromatographic separation techniques applicable to the separation of DNA fragments and numerous applications have been summarized in several informative reviews [20–24] and the size-dependent separation of DNA fragments with various chromatographic methods has been reviewed by Kasai [25]. Strege and Lagu compared different anion-exchange stationary phases for the separation of DNA restriction fragments and found that the performance of micropellicular stationary phases was superior to that of porous stationary phases [26]. Investigations in our laboratory demonstrated that short columns packed with micropellicular, alkylated polystyrene–divinylbenzene particles allowed the rapid and high-resolution separation of oligonucleotides and dsDNA fragments by means of ion-pair reversed-phase chromatography [27–30]. This review is intended to describe the strengths and weaknesses of micropellicular stationary phases for anion-exchange and ion-pair reversed-phase HPLC of double-stranded nucleic acids. The principles of separation, the most important operating variables, and selected examples of application are discussed.

2. Stationary phases and chromatographic modes for separation of dsDNA

2.1. Micropellicular stationary phases for HPLC of nucleic acids

Early attempts to utilize porous stationary phases, that have been optimized for the separation of small molecules, did not yield the expected speed and

resolution for HPLC of biopolymers because of the restricted access of large biomolecules to the small pores of the chromatographic support [31]. The subsequent development of advanced wide-pore silica packings with a nominal pore size of 300–4000 Å opened a broad field of biotechnological applications for HPLC, because the large pores provide the analytes with sufficient accessibility to the stationary phase. However, HPLC separations of biopolymers on macroporous supports suffer from additional limitations relative to small molecule separations, particularly slow mass transport within the chromatographic packing material resulting in broad peaks and low sample recovery [32]. Moreover, because of the low diffusivities of biopolymers, small flow-rates and shallow gradients have to be utilized with porous stationary phases which entail excessively long analysis times.

As a consequence, it has been a challenge to find ways of exposing the mobile phase and sample molecules rapidly and efficiently to the entire available surface area of the chromatographic packing. One promising approach to solve the mass transport problem so far is based on the application of stationary phases of the micropellicular configuration [33] which is characterized by a core of fluid-impervious support material (fused-silica particles or organic polymer microspheres) covered by a thin, retentive layer of stationary phase (Fig. 1). Due to the lack of pores providing an internal surface, the total surface area of micropellicular stationary phases is relatively low. The specific surface area of 5-μm nonporous silica particles is only 0.55 m^2/g compared to 300 m^2/g for porous, 10-μm/100 Å silica particles. To provide enough surface area for chromatographic retention, micropellicular particles of 1–3 μm diameter with a specific surface area of 2.7–0.9 m^2/g are therefore recommended [34]. Because the column back pressure increases with decreasing particle diameter, HPLC columns packed with particles smaller than 1 μm are difficult to operate within the pressure limit of conventional liquid chromatographic instrumentation. The effect of particle size on column efficiency and analysis time is well understood in HPLC [35]. Columns packed with small particles can, by virtue of favorable mass transfer properties, be used at flow velocities higher than the optimum flow velocity without serious loss in efficiency. Furthermore, steep gradient profiles can be used with micropellicular packings to rapidly elute the sample components. Therefore micropellicular stationary phases of 1–3 μm particle diameter offer many advantages such as fast mass transfer, high column efficiency, preservation of biological activity, the possibility of performing high-speed analyses, and fast column equilibration. The loading capacity of columns packed with micropellicular stationary phases for macromolecules is not much smaller than the loading capacity of totally porous sorbents [36,37]. Owing to the solid, fluid impervious core of the micropellicular packings, columns can be stable at high pressure and temperature unlike those packed with conventional porous materials [38].

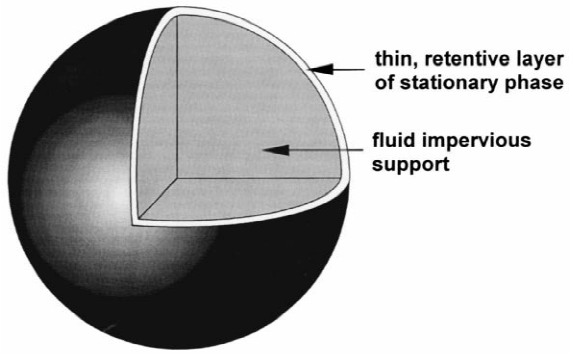

Fig. 1. Schematic illustration of the micropellicular stationary phase configuration (adapted from Ref. [33]).

Since most chromatographic DNA separations are carried out at neutral or alkaline pH, the considerable solubility of silica based packing materials at pH values above 8 can be a problem [39]. Moreover, silanophilic interactions between silanols and sample molecules can impair separation efficiency, peak symmetry, and sample recovery. Therefore, micropellicular stationary phases that are currently commercially available for the separation of DNA are exclusively based on organic polymers as the support material (Table 1). The greatest advantages of polymeric stationary phases are their excellent chemical stability in contact with acidic, alkaline and hydroorganic eluents and the homogeneity of the polymer surface without strong binding sites such as silanols on a silica surface [40]. A high crosslinking degree of the organic polymers guarantees high

Table 1
Commercially-available, micropellicular column packings for HPLC of dsDNA

Trade name	Support material, particle size	Stationary phase	Chromatographic mode	Supplier
Gen-Pak FAX	Methacrylate polymer, 2.5 μm	Diethylaminoethyl groups	HPAEC	Waters, Milford, MA, USA
TSKgel DEAE–NPR, TSKgel DNA–NPR	Hydrophilic polymer, 2.5 μm	Diethylaminoethyl groups	HPAEC	TosoHaas, Stuttgart, Germany
ProPac PA1, Nucleopac PA-100	PS–DVB, 10 μm or 13 μm	Quaternary ammonium groups	HPAEC	Dionex, Sunnyvale, CA, USA
Shodex IEC QA-620N	Methacrylate gel, 2.5 μm	Quaternary ammonium groups	HPAEC	Showa Denko, Tokyo, Japan
DNASep	PS–DVB, 2.5 μm	Octadecyl groups	IP–RP-HPLC	Transgenomic Inc., Santa Clara, CA, USA

mechanical stability and minimizes swelling in organic solvents so that packed columns can be operated even at high pressure [41].

The following two sections introduce the separation principles that have been realized until now with micropellicular stationary phases, namely anion-exchange and ion-pair reversed-phase chromatography. The principle of slalom chromatography was discovered independently in 1988 by Hirabayashi and Kasai [17] and Boyes et al. [18]. The separation is based on the fact that long DNA molecules (5–50 kbp) have to turn very fast during their passage through the narrow and tortuous openings between the closely packed spherical particles of a chromatographic column. The longer the DNA molecule, the more difficult it is to pass through the openings, and consequently, a separation according to size, where smaller DNA molecules elute first, is achieved. Important features of slalom chromatography are that DNA fragments do not interact with the column packing, that separation depends strongly on particle size and flow-rate, and that the DNA fragments are totally excluded from the pores, and therefore, pore size and pore volume are not important factors in determining the separation. From the latter it follows that slalom chromatography can be realized in principle also with micropellicular particles, although such an application of micropellicular particles has not been published yet in the literature. Details of the operating conditions and applications of slalom chromatography have been reviewed recently [25] and are, hence, not covered in this review.

2.2. Anion-exchange chromatography of dsDNA

The way in which nucleic acids interact with a chromatographic stationary phase strongly depends on the specific structure of the nucleic acid molecule. In this review however, we will focus just on double-stranded DNA molecules of different chain length. The double helical structure of DNA secludes the hydrophobic nucleobases inside the double helix whereas the two helical sugar-phosphate chains spiral down the outside of the double-stranded structure, presenting a polyanionic, highly hydrated and hydrophilic surface to the solvent. Double-stranded DNA fragments up to ca. 100 bp in length (dimensions 34×2 nm) can be treated as a straight rod with a certain degree of elasticity. Nevertheless, the longer the molecule becomes, the more it forms a random coil in free solution [25]. When a DNA solution flows through the narrow channels of a chromatographic packing, however, the random coil conformation of large DNA molecules may be stretched by shear forces [25], and therefore, it is mainly the stretched conformation of the DNA molecule which is undergoing interactions with the stationary phase.

In anion-exchange chromatography of DNA, the mobile phase is a buffer solution containing a strong electrolyte as eluting salt and a linear gradient of

increasing salt concentration is usually applied for elution. The traditional theories for ion-exchange chromatography are based on a stoichiometric ion-exchange process and the mass action law [42–44] but the newer, nonstoichiometric theories have been able to construct a more realistic physicochemical model for ion-exchange chromatography of biomacromolecules [45–47]. Ståhlberg et al. have elaborated a quantitative theory assuming that the interaction between a multiply-charged biomolecule and the surface of an ion-exchanger can be treated as nonspecific long-range electrostatic interaction between two charged surfaces in contact with a buffered salt solution [47,48]. The fixed-charged groups on the surface of the stationary phase create an electrostatic potential difference between the surface and the bulk solution that will attract solute molecules having their own surface potential of opposite sign. Therefore, the biomolecules are not regarded as bound to any discrete charged sites at the surface, but they are kept in the stationary phase domain by the electrostatic potential generated by the plurality of fixed charges in close proximity of the surface (Fig. 2) [45].

The magnitude of the surface potential of a stationary phase or a biomolecule is determined by the charge density at the surface. The charge density depends on the total density of fixed-charged groups, i.e. anion-exchange functional groups of the stationary phase or phosphodiester groups of the dsDNA, the concentration of eluent counterions, and their association constants to the fixed-charged groups. The overall strength of interaction between a biomolecule and the stationary phase is then determined by the magnitude of the interacting surface potentials and the extent of the interacting surface area [47]. Since the charge density of dsDNA is constant (two negative charges per base pair), its surface potential does not depend on size, whereas the magnitude of the contact area increases with increasing length of the DNA molecule. Thus, anion-exchange separation of dsDNA is primarily based on length. The pre-

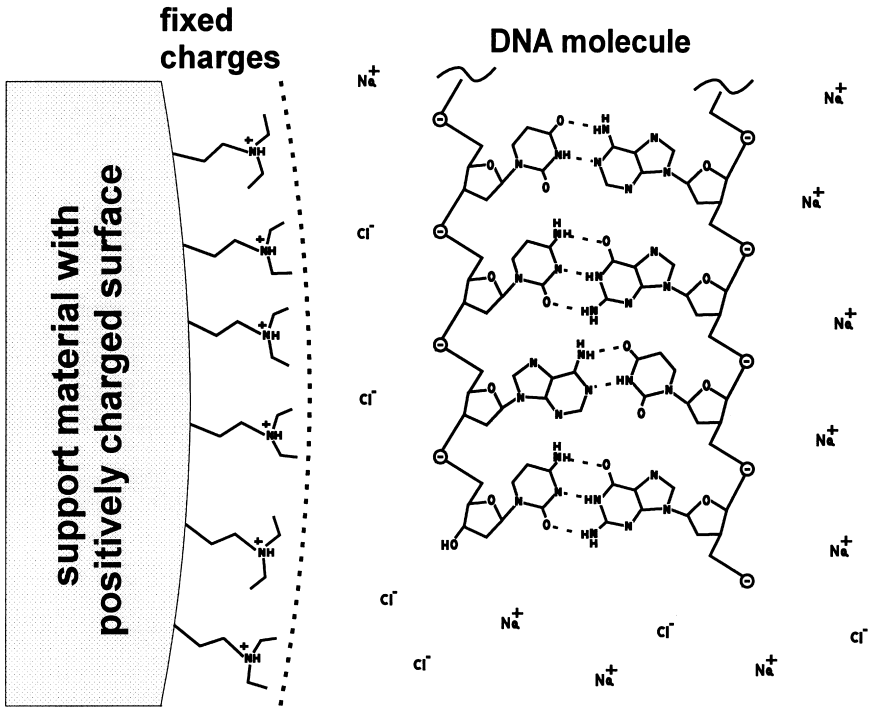

Fig. 2. Schematic illustration of retention of dsDNA by electrostatic interactions between the surface potentials created by the positively-charged DEAE groups of an anion-exchange stationary phase and the negatively-charged phosphodiester groups of dsDNA.

dominant factors determining retention of dsDNA in anion-exchange chromatography can be summarized as follows: ion-exchange capacity of the stationary phase, ionic strength, temperature and dielectric constant of the eluent, association tendency of the eluting salt with the fixed charges of the stationary phase and the DNA, respectively, and length of the DNA molecule (see equation 10 in [47]). Virtually all anion-exchange functional groups are based on amino groups, where the degree of alkylation of the nitrogen determines the pH range over which the functional group is positively charged. Secondary and tertiary amino groups, diethylaminoethyl (DEAE) groups for instance, serve as weak anion-exchange functionalities. The maximum surface charge density of DEAE bonded stationary phases is reached at pH values below 4. With increasing pH the charge density decreases and at pH 9 it is about 50% of the maximum value [49]. Therefore, the practical operating pH with weak anion-exchange stationary phases for HPLC of dsDNA lies between 6 and 9. Quaternary ammonium groups such as trimethylammonium (TMA) groups, on the other hand, ensure a constant charge density independent of the eluent pH.

While the predominant attractive force in anion-exchange chromatography is electrostatic, other forces and mechanisms may play a role in determining the final separation. These forces include dispersive forces, dipole–dipole attraction, hydrogen bonding, and solvophobic repulsion. In many cases, these additional forces are undesirable because they are difficult to control and because the retention behavior of nucleic acids becomes unpredictable (see also Section 5.1). Therefore, micropellicular polymeric support materials for anion-exchange chromatography of DNA are synthesized directly from hydrophilic monomers (methacrylates, vinylalcohol, for instance) in many cases. Alternatively, the surface of hydrophobic polymeric support materials is hydrophilized by derivatization with polar or ionic groups (hydroxyl groups [37], sulfonic acid groups [24]). These polar surfaces will strongly attract water molecules from the mobile phase so that interactive forces other than electrostatic are efficiently suppressed. Table 1 lists commercially available micropellicular stationary phases for anion-exchange chromatography of DNA fragments. Most stationary phases are synthesized by chemically bonding DEAE or TMA groups to the surface of hydrophilic polymer particles (Gen-Pak FAX, TSKgel DEAE–NPR, Shodex IEC QY-620N). An exception is the resin used in ProPac PA1 and NucleoPac PA-100 columns, where the stationary phase is formed by physically adsorbing 0.2-μm strong anion-exchange microbeads onto micropellicular, sulfonated PS–DVB particles.

Fig. 3 compares the separation of DNA restriction fragments from the pBR322 plasmid obtained upon digestion with *Hae*III by anion-exchange chromatography on a micropellicular DEAE stationary phase (TSKgel DEAE–NPR, [50], Fig. 3a) and a macroporous quaternary ammonium anion-exchanger (Mono Q, [11], Fig. 3b). Separation was accomplished in both instances with combinations of linear sodium chloride gradients in 20 m*M* Tris–HCl buffer. It can be seen that the chromatographic pattern is very similar with both stationary phases. The most substantial difference between the two chromatograms is their time scale. The separation on Mono Q was finished within 8 h, which makes the method impractical for routine work. A considerable reduction in analysis time to less than 15 min, as well as a significant enhancement in resolution, is achieved by the use of the micropellicular TSKgel DEAE–NPR stationary phase. The elution order of the fragments was ascertained by preparative fractionation followed by strictly size-dependent separation of the fractions by polyacrylamide gradient electrophoresis [11]. Although DNA fragments are eluted primarily according to chain length, the fragments 123, 192, 504, and 458 are retained longer than expected indicating that retention mechanisms other than ion-exchange are effective (see also Section 5.1).

2.3. Ion-pair reversed-phase chromatography of dsDNA

Ion-pair reversed-phase chromatography is a well-established technique for separating mono- and oligonucleotides, and a variety of silica- and polymer-based columns, mostly based on porous or macroporous support materials, are commercially available [51–53]. For the chromatographic analysis of dsDNA, however, ion-pair reversed-phase chromatography was not as popular as anion-exchange

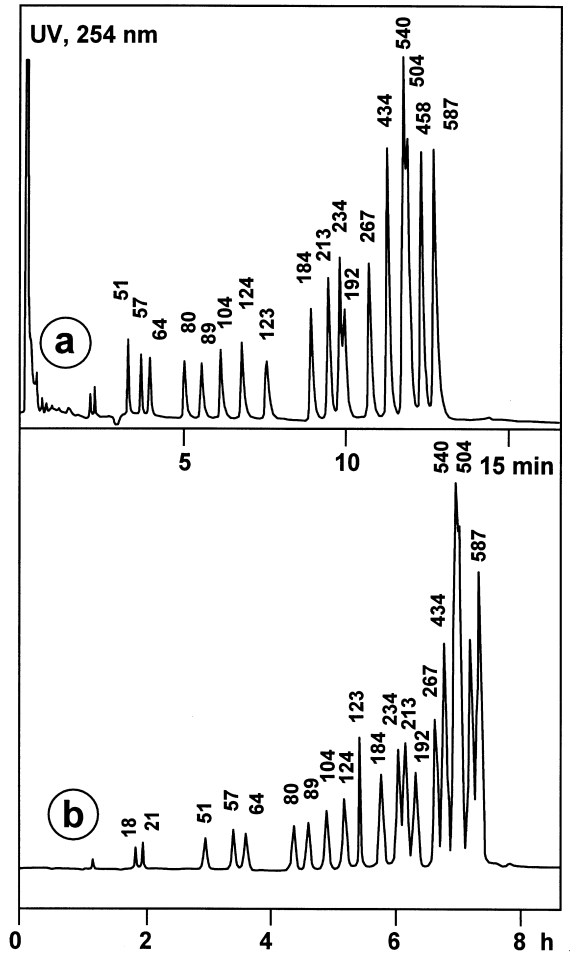

Fig. 3. Separation of a pBR322 DNA–*Hae*III digest on a micropellicular (a) and a macroporous (b) anion-exchange stationary phase. Conditions in (a): column, TSKgel DEAE–NPR (2.5 nm, 35×4.6-mm I.D.); mobile phase, (A) 20 m*M* Tris–HCl, pH 9.0, (B) 1 *M* sodium chloride in 20 m*M* Tris–HCl, pH 9.0; linear gradient, 25–45% B in 0.1 min, 45–50% B in 2.9 min, 50–100% B in 57 min; flow-rate, 1.5 ml/min; temperature, 25°C; sample, 4.8 μg of pBR322 DNA–*Hae*III digest (reproduced from Ref. [50] with permission). Conditions in (b): column, Mono Q (10 μm, 700 Å pore size); mobile phase, (A) 20 m*M* Tris–HCl, pH 8.3, (B) 1 *M* sodium chloride in 20 m*M* Tris–HCl, pH 8.3; linear gradient, 40–65% B in 2.2 h, 65–75% B in 5.8 h; flow-rate, 0.15 ml/min; temperature, ambient temperature; sample, 20 μg of pBR322 DNA–*Hae*III digest (reproduced from Ref. [11] with permission).

chromatography in the past, mainly because of the lack of commercially-available stationary phases that allow fast and efficient separations. This situation has been changed by the commercial availability of alkylated PS–DVB microbeads [54] (Table 1) and several reports have demonstrated the applicability of this stationary phase for DNA separations by ion-pair reversed-phase chromatography [30,55–58].

In ion-pair reversed-phase chromatography, the phase system comprises a hydrophobic stationary phase and a hydroorganic eluent containing an amphiphilic ion and a small, hydrophilic counterion. Although a number of alternative names such as soap chromatography [59], ion-interaction chromatography [60], dynamic ion-exchange chromatography [61], solvophobic ion chromatography [62] exist, ion-pair reversed-phase chromatography is the most commonly used name for the technique. Stoichiometric models [63] suggest that solute ions form stoichiometric complexes either in the mobile phase (ion-pair model [62]) or at the stationary phase (dynamic ion-exchange model [61]) but again, non-stoichiometric models are founded on a better understanding of the underlying physicochemical principles [64,65].

According to the electrostatic retention model developed by Ståhlberg [65,66], the positively- or negatively-charged amphiphilic ions are adsorbed at the interface between the nonpolar stationary phase and the hydroorganic mobile phase, resulting in the formation of an electric double layer. The excess of either positive or negative charges at the surface entails a surface potential ψ_0 between the bulk mobile phase and the hydrophobic stationary phase (Fig. 4). Electroneutrality requires that the surface potential has to be neutralized by charge of opposite sign, either as excess of counterions or as depletion of coions on the solution side of the interface. The plane-of-closest-approach of hydrated species, such as dsDNA, to the surface of the stationary phase is called the outer Helmholtz plane (OHP) [67]. Fig. 4 presents an idealized view of the electrostatic interactions of a dsDNA molecule with the surface potential generated by triethylammonium ions at the surface of a hydrophobic support material. In ion-pair reversed-phase chromatography, the magnitude of the surface potential at the stationary phase is determined by several factors such as hydrophobicity of the column packing, charge, hydrophobicity and concentration of the pairing ion, ionic strength, temperature and dielectric constant of the mobile

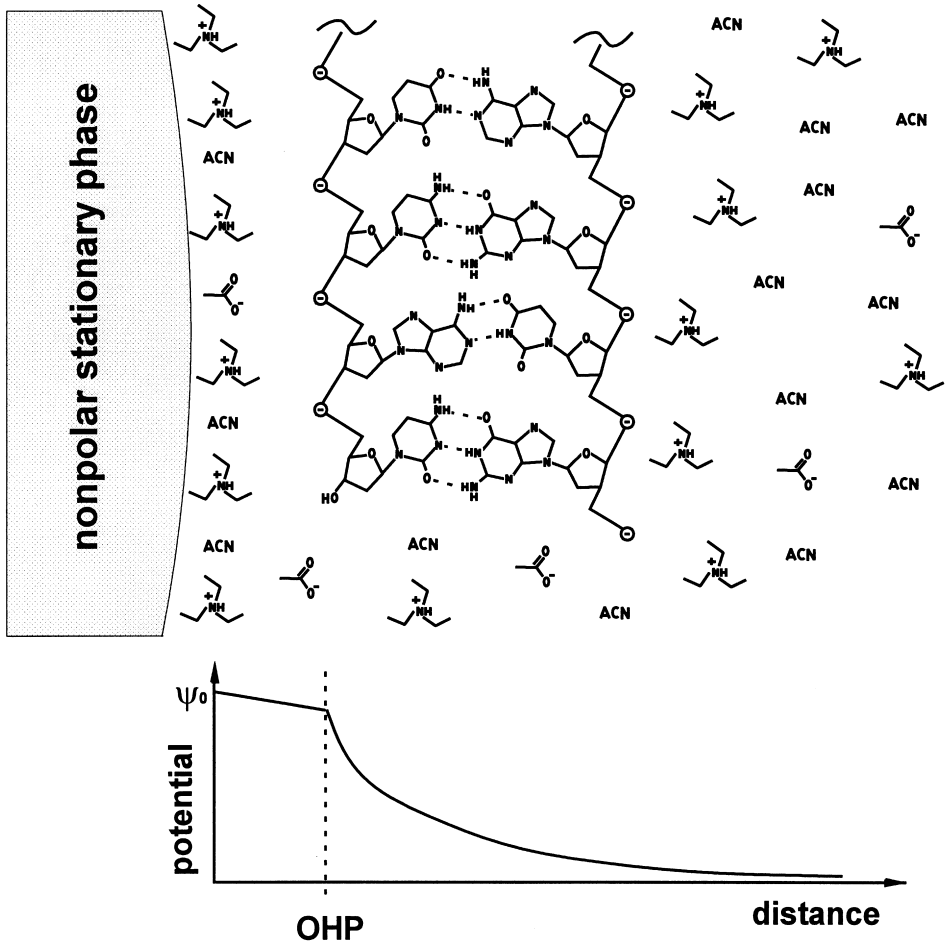

Fig. 4. Schematic illustration of electrostatic interactions between an electric double layer created by selective adsorption of amphiphilic ions and dsDNA. ACN, acetonitrile molecule; OHP, outer Helmholtz plane, plane-of-closest-approach of hydrated solute molecules.

phase, and concentration of organic modifier (see equation 10 in [65]). Retention is governed by the magnitude of the individual surface potentials and the contact area involved in electrostatic interaction. Double-stranded DNA molecules are therefore retained according to chain length in ion-pair chromatography. In principle, additional solvophobic interactions between hydrophobic solutes and the hydrophobic surface of the stationary phase are possible in ion-pair reversed-phase chromatography, but this kind of interaction is of minor importance in the case of dsDNA, nondenaturing conditions assumed, because of its highly hydrophilic outer surface.

From the models discussed above it can be concluded that there is no fundamental difference in the electrostatic interactions responsible for retention of dsDNA in ion-exchange and ion-pair reversed-phase chromatography. An important difference between the two techniques is the way the electrical potential is created at the surface of the stationary phase: fixed charges bound to the chromatographic support in ion-exchange chromatography, and adsorption of amphiphilic ions from the eluent onto the surface of a hydrophobic stationary phase in ion-pair reversed-phase chromatography. Another difference between ion-exchange chromatography and ion-pair reversed-phase chromatography is the condition used to accomplish elution of the adsorbed DNA: in the former the surface potential is lowered by a gradient of increasing ionic strength, in the latter it is the

desorption of amphiphilic ions from the hydrophobic surface of the stationary phase due to an increase in the concentration of organic solvent that lowers the surface potential.

The comparison of a micropellicular and a porous stationary phase for the separation of dsDNA fragments by ion-pair reversed-phase chromatograph is depicted in Fig. 5. The micropellicular stationary phase was octadecylated PS–DVB (Fig. 5a), the porous stationary phase PepRPC (C_2/C_{18}) [15] (Fig. 5b). Triethylammonium acetate (TEAA) at a concentration of 0.1 *M* and 0.04 *M*, respectively, was used as ion-pair reagent and linear gradients of acetonitrile were applied to elute the DNA at neutral pH. The time necessary to complete the separation was 5 min with the micropellicular stationary phase and 4 h with the porous stationary phase, which proves once more the superiority of micropellicular phases in enabling rapid and high-resolution separations. Subsequent polyacrylamide gel electrophoretic analysis of fractionated DNA fragments revealed that, in contrast to anion-exchange chromatography, all fragments were eluted strictly according to size [15,30].

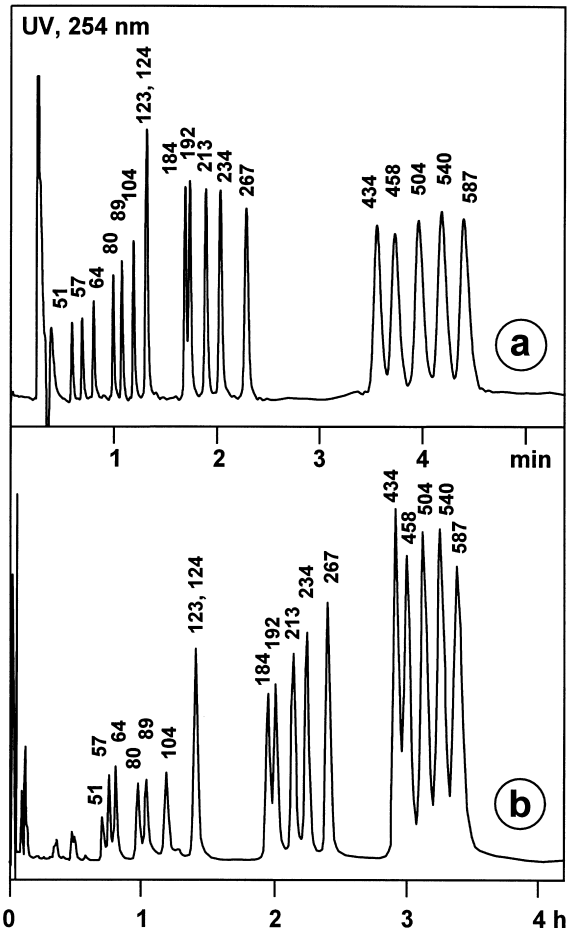

Fig. 5. Separation of a pBR322 DNA–*Hae*III digest by ion-pair reversed-phase chromatography on a micropellicular (a) and a porous (b) stationary phase. Conditions in (a): column, PS–DVB-C_{18} (2.1 μm, 30×4.6-mm I.D.); mobile phase, (A) 0.1 *M* TEAA, pH 7.0, (B) 0.1 *M* TEAA, 25% acetonitrile, pH 7.0; linear gradient, 35–55% B in 1 min, 55–65% B in 5 min; flow-rate, 1.2 ml/min; temperature, 50°C; sample, 0.5 μg pBR322 DNA–*Hae*III digest. Conditions in (b): column, PepRPC (C_2/C_{18}) HP 5/5 (5 μm, 100 Å pore size, 50×5-mm I.D.); mobile phase, 40 m*M* TEAA, pH 6.5; linear gradient, 8.5–10.5% acetonitrile in 40 min, 10.5–12% in 120 min, 12–12.3% in 70 min; flow-rate, 0.5 ml/min; temperature, ambient temperature; sample, 10 μg pBR322 DNA–*Hae*III digest (reproduced from Ref. [15] with permission).

3. Operational variables in anion-exchange chromatography of dsDNA

3.1. Columns and column equilibration

Nakatani et al. investigated the influence of particle diameter on the separation of a 1-kbp ladder with a TSKgel DNA–NPR column packed with 3.5-μm and 2.5-μm micropellicular particles [68]. With the 2.5-μm particles, 17 out of 23 fragments were baseline resolved, 4 fragments were partly resolved, and 2 fragments coeluted. With the 3.5-μm particles, 13 fragments were baseline resolved, 8 were partly resolved and 2 fragments coeluted. Generally, peaks were sharper and less asymmetrical on the 2.5-μm stationary phase and retention times of DNA fragments were between 7 and 9% shorter with the 3.5-μm stationary phase under identical gradient conditions. Several studies have suggested that the resolution of macromolecules is rather insensitive to column length under conditions of gradient elution [69,70]. Hence, short columns of 30 to 50 mm length are frequently used for the chromatographic separation of biomolecules. Kato et al. have shown, however, that better separations of DNA fragments ranging in size from 75 to 12 216 bp were achieved with two 35×4.6-mm I.D. columns

connected in series compared to the separation with one single column [50].

Strege and Lagu compared the three micropellicular anion-exchange packings Gen-Pak FAX, TSKgel DEAE–NPR, and ProPac PA1 (Table 1) through the separation of a pBR322 DNA–*Hae*III digest [26]. The mobile phase was 25 m*M* phosphate buffer, pH 7.0, and sodium chloride was used as the gradient former. The separation patterns obtained with these three packings were almost identical but the time required to obtain comparable resolution was shortest with the 35×4.6-mm I.D. TSKgel DEAE–NPR column (20 min), followed by the 100×4.6-mm I.D. Gen-Pak FAX column (25 min), and the 250×4-mm ProPac PA1 column (35 min). Another study examined TSKgel DEAE–NPR, Gen-Pak FAX, and NucleoPac PA100 with the pBR322 DNA–*Hae*III digest and concluded that there is little advantage of one column over the others [24]. The columns packed with the 2.5 μm particles exhibited substantial back pressure (10–15 MPa at 0.5 ml/min, 100×4.6-mm I.D., 20°C) which is, nevertheless, well within the normal operating range of analytical HPLC equipment. The column packed with the 10-μm particles exhibited a comparatively low back pressure (0.5–0.8 MPa at 1 ml/min, 250×4 mm I.D., 24°C) which makes this stationary phase suitable for scale-up and preparative chromatography of milligram quantities of DNA fragments.

The total time required for a chromatographic run includes the time for separation of the sample components of interest, the time for column regeneration, and the time for reequilibrating the column to initial conditions. Regeneration of the column is usually achieved by flushing the column with an eluent of high ionic strength to elute strongly adsorbed compounds. Columns packed with micropellicular stationary phase have a very small intraparticulate void volume and can, therefore, be equilibrated very fast after gradient elution to starting conditions [38]. With a new column or after changing the eluent, a 10–20-min flush with mobile phase at 1 ml/min was sufficient for equilibration of micropellicular anion-exchange columns in our experience. Between gradient runs, 1.5–3-min equilibration was adequate to obtain reproducible separations with a 35×4.6-mm I.D. TSKgel DEAE–NPR column. Stowers et al. describe a rather time-consuming protocol for equilibration of the Gen-Pak FAX column [71]. The column had to be equilibrated in between runs for at least 30 min at 0.5 ml/min, and the equilibration time had to be kept constant in order to avoid memory peaks. Moreover, daily cleaning of the column was recommended to preserve constant performance.

3.2. Mobile phase composition and pH

The most widely employed buffer is tris(hydroxymethyl)aminomethane (Tris) at concentrations between 5 and 50 m*M*, adjusted to a pH between 7.5 and 8.5 with hydrochloric acid or boric acid [11,50,71,72]. Alternative buffer compounds used include phosphate [73,26], acetate [21], ethanolamine [11], piperazine [11], 2-(*N*-cyclohexylamino)ethanesulfonic acid [74], and 2-(*N*-morpholino)ethanesulfonic acid [74]. An investigation with a macroporous, silica-based column (Nucleogen DEAE) showed, that superior resolution of DNA fragments was possible with phosphate buffer [9]. With TSKgel DEAE–NPR and Gen-Pak FAX, however, no significant difference between Tris buffer and phosphate buffer was discernible [26,50,75]. On weak anion-exchangers, retention times increased upon decreasing the pH from 9 to 7 because of the higher charge density of the stationary phase. Concomitantly, column efficiency deteriorated notably at lower pH, and thus, the usual operating pH with DEAE stationary phases lies between 8 and 9.

Sodium chloride, potassium chloride, and sodium perchlorate are the most commonly used gradient formers [50,72,68]. Sodium sulfate failed to elute the DNA from a Mono Q column even at a concentration of 2 *M* [11]. Poor resolution was obtained with sodium chlorate and ammonium sulfate with a Nucleogen DEAE column [9]. The effect of sodium chloride and sodium perchlorate as gradient former was studied by Nakatani et al. [68]. Similar retention times and slightly smaller, but still acceptable efficiency was observed with a gradient of 0.17–0.26 *M* sodium perchlorate in 20 m*M* Tris–HCl buffer, pH 9.0, compared to a gradient of 0.5–0.75 *M* sodium chloride in the same buffer. Variation of the cation (lithium, sodium, potassium, and cesium) while keeping chloride as the anion showed, that with smaller cations the DNA eluted at lower ionic

strength, an effect simply reflecting the different activities of chloride in the presence of different cations [10].

The double helical structure of DNA does not denature in buffer in the presence of moderate concentrations of chaotropic additives at room temperature, but urea and/or formamide are sometimes added to the eluent to suppress intermolecular interactions between nucleic acids and nonelectrostatic interactions between the nucleic acids and the stationary phase resulting in superior resolution and prevention of cross-contamination [21]. Di- or multivalent metal ions present as impurities or released from the wetted parts of the HPLC apparatus into the eluent are known to facilitate intramolecular interactions between nucleic acids. Maa et al. demonstrated, that DNA fragments from a pGEM-3Z DNA–*Taq*I digest eluted as a single, broad peak in the presence of 1 m*M* magnesium chloride in the starting eluent and proved that all three fragments of the digest coeluted. The addition of 0.5–1 m*M* EDTA to the eluent may complex the metal ions and thereby eliminate the observed carry-over phenomenon if it is due to binding of the smaller fragments to the larger ones [37].

The carry-over phenomenon was also found with the Gen-Pak FAX column after gel–electrophoretic analysis of eluted DNA fragments. Fig. 6a shows the separation of 1 μg λDNA–*Eco*R1 digest with a gradient of 0.55–0.7 *M* sodium chloride in 25 m*M* phosphate, pH 7, on a Gen-Pak FAX column. The gel electrophoretic analysis of collected fractions showed that two of the fragments were distributed among two peaks (5540 bp, 5930 bp), and that the 21 800 bp fragment was split into two peaks [26]. A possible explanation for the carry-over phenomenon is a solvophobic interaction between the DNA and the stationary phase because it could be completely suppressed in the presence of 10% isopropanol in the eluent (Fig. 6b). Another phenomenon most probably related to sovophobic interactions between the DNA and the stationary phase was observed with the ProPac PA1 column, where a gradient of 0.57–0.68 *M* sodium chloride in 25 m*M* sodium phosphate, pH 7.0, eluted all DNA fragments with a shoulder on the leading side of the peaks. The shoulders were completely eliminated upon the addition of 10% isopropanol to the eluent [26].

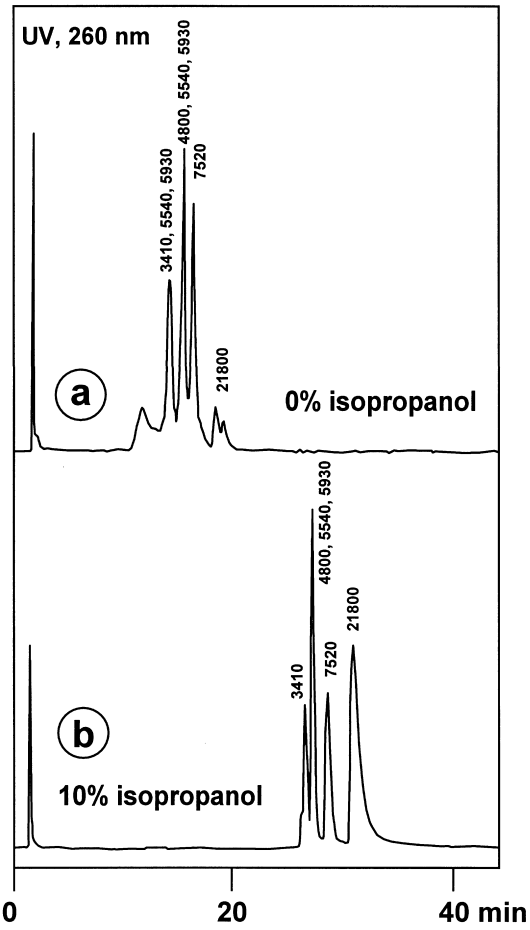

Fig. 6. Influence of organic modifier on the separation of DNA fragments on a Gen-Pak FAX column. Column, Gen-Pak FAX (2.5 μm, 100×4.6-mm I.D.); mobile phase, 25 m*M* sodium phosphate, pH 7.0, linear gradient, 0.55–0.70 *M* sodium chloride in 40 min in (a), 25 m*M* sodium phosphate, pH 7.0, 10% isopropanol; linear gradient, 0.45– 0.60 *M* sodium chloride in 40 min in (b); flow-rate: 0.5 ml/min; temperature, ambient temperature; sample, 1 μg λDNA–*Eco*RI digest (reproduced from Ref. [26] with permission).

3.3. Flow-rate and gradient slope

The optimum resolution of DNA fragments ranging in size from 564 to 23 130 bp on the Gen-Pak FAX column was achieved at flow-rates higher than 0.8 ml/min [26]. The effect of flow-rate on resolution of small and large DNA fragments on the TSKgel DEAE–NPR column is depicted in Fig. 7a. With fragments shorter than 3000 bp, highest res-

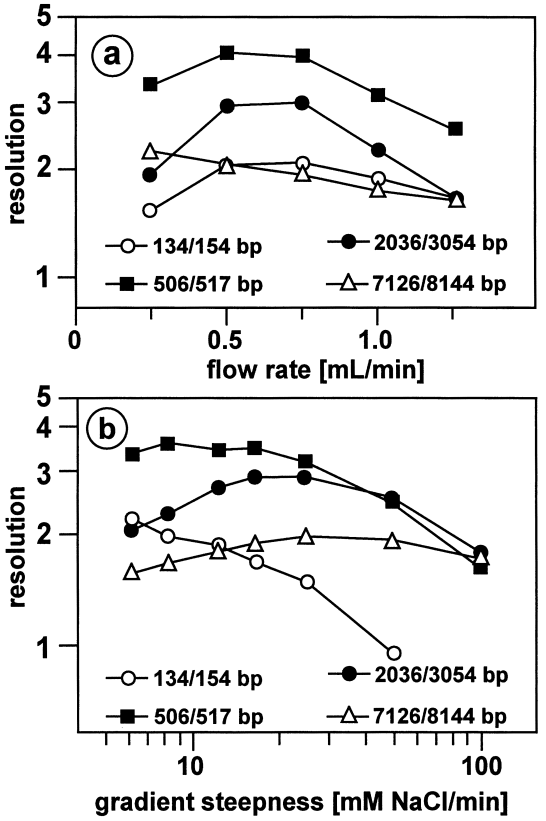

Fig. 7. Dependence of resolution on flow-rate (a) and gradient steepness (b) in anion-exchange HPLC on a TSKgel DNA–NPR column. ○, 134/154 bp; ■, 506/517 bp; ●, 2036/3054 bp; △ 7126/8144 bp (reproduced from Ref. [76] with permission).

olution was observed at flow-rates of 0.5–0.75 ml/min. Large DNA fragments were better resolved at flow-rates smaller than 0.5 ml/min [76]. Fig. 7b shows the dependence of resolution on gradient steepness. Resolution increases with decreasing gradient steepness and goes through a maximum, after which the resolution decreases again. As can be seen from Fig. 7b, the optimum gradient steepness shifts to higher values for large DNA fragments.

Two chromatograms showing the separation of 22 DNA fragments of a 1-kbp ladder with fragments ranging in size from 75 to 12 216 bp eluted with a convex gradient of sodium chloride on the Gen-Pak FAX column (Fig. 8a) and with a linear gradient of sodium chloride on the TSKgel DEAE–NPR column (Fig. 8b) are compared in Fig. 8. The concentration range of gradient former exploitable for the elution

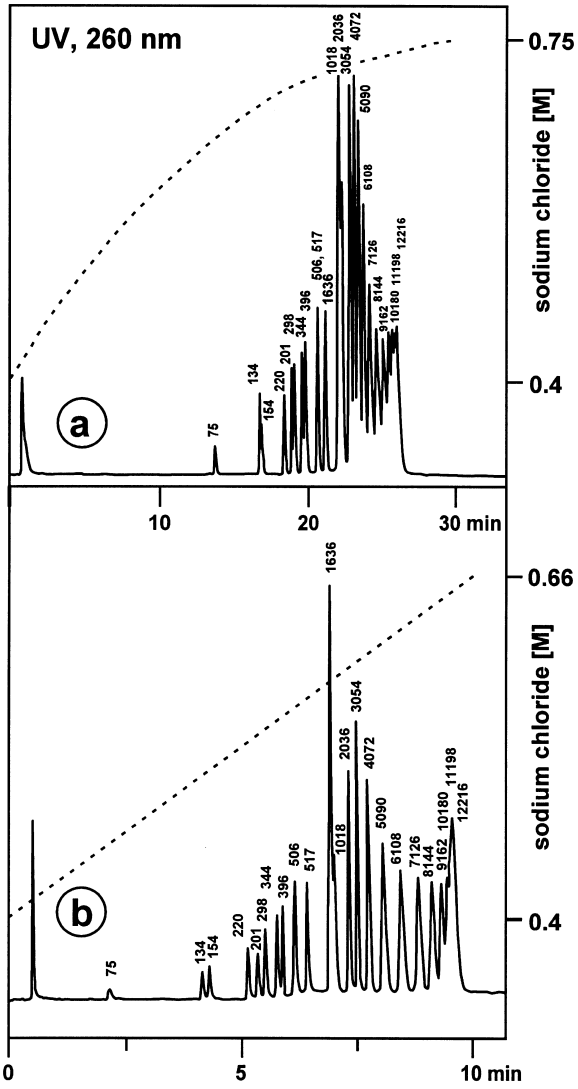

Fig. 8. Separation of 1-kbp ladder on Gen-Pak FAX (a) and TSKgel DEAE–NPR (b) column. Chromatographic conditions in (a): Column, Gen-Pak FAX (2.5 μm, 100×4.6-mm I.D.); mobile phase, 25 m*M* sodium phosphate, pH 7.0, convex gradient, 0.40–0.75 *M* sodium chloride in 30 min; flow-rate, 0.5 ml/min; sample, 50 μg of 1-kbp ladder (reproduced from Ref. [75] with permission); Chromatographic conditions in (b): column, TSKgel DEAE–NPR (2.5 μm, 75×4.6-mm I.D.); mobile phase, 20 m*M* Tris–HCl, pH 9.0; linear gradient, 0.50–0.75 *M* sodium chloride in 15 min; flow-rate, 1 ml/min; temperature, 25°C; sample, 2-μg of 1-kbp ladder (reproduced from Ref. [68] with permission).

of small and large fragments is broader with the linear gradient. Particularly the later eluting, large DNA fragments, that encounter a steeper linear

gradient (Fig. 8b), are better resolved on the TSKgel DEAE–NPR column.

3.4. Temperature

Several authors report that there is little effect of temperature on the quality of DNA separations in anion-exchange chromatography [50,21,23]. Müller found, however, that an increase in temperature from ambient to 60°C improved the resolution of DNA fragments on a strong-base Mono Q anion-exchange column [10]. Generally, retention times increased at higher column temperature most probably due to the partial desolvation of the DNA and better accessibility of the stationary phase at higher temperature. The increase in retention is exemplified in Fig. 9 by the chromatograms of λDNA–*Hin*dIII fragments separated at 25, 45, and 60°C on the Gen-Pak FAX column. Resolution decreased significantly at 45 and 60°C as can be seen by the coelution of the 4371-bp fragment with the 2028-bp and 2322-bp fragments at these temperatures [26]. At 60°C, the 23 130 bp fragment was split into two peaks, which may be due to partial denaturation. A similar temperature dependence of resolution was found with the TSKgel DEAE–NPR column [76]. It appears that temperatures between ambient and 35°C are optimal for the separation of dsDNA on micropellicular weak-base anion-exchangers.

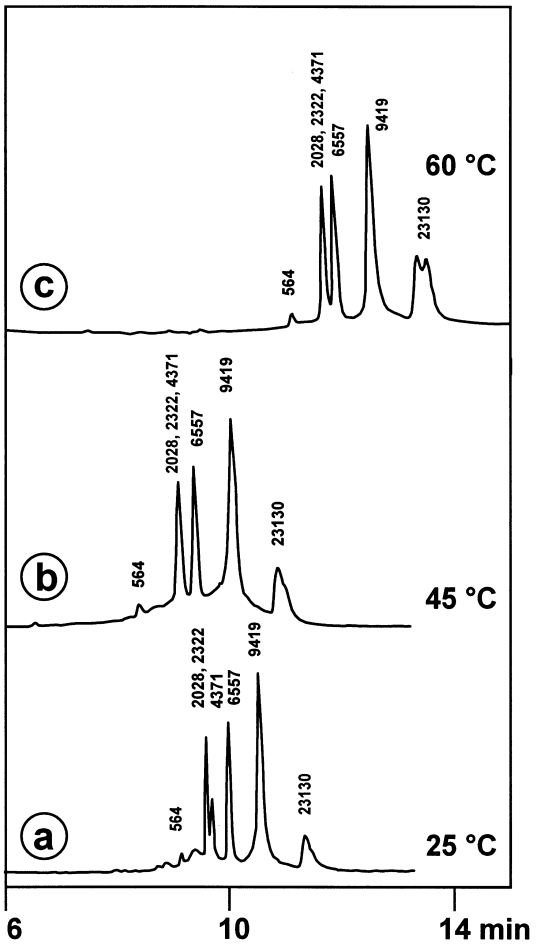

Fig. 9. Effect of temperature in anion-exchange chromatography of DNA fragments. Column, Gen-Pak FAX (2.5 μm, 100×4.6-mm I.D.); mobile phase, 25 m*M* sodium phosphate, pH 7.0; linear gradient, 0.40–0.75 *M* sodium chloride in 10 min; flow-rate, 0.8 ml/min; temperature, 25°C (a), 45°C (b), 60°C (c); sample, 1 μg λ DNA–*Hin*dIII digest (reproduced from Ref. [26] with permission).

4. Operational variables in ion-pair reversed-phase chromatography of dsDNA

4.1. Columns and column equilibration

Alkylation of micropellicular PS–DVB particles has been shown to be a prime prerequisite to accomplish high-resolution separations of nucleic acids [30]. On unmodified PS–DVB particles, double-stranded DNA restriction fragments larger than 100 bp could not be resolved sufficiently. An improvement in resolving power was obtained after coating the particles with polyvinylalcohol, but satisfactory resolution over a broad size range was achieved only with octadecylated PS–DVB particles. The high efficiency of columns packed with the alkylated particles is believed to be a consequence of the effective shielding of the aromatic rings of the PS–DVB support material, which may otherwise unwantedly interact with the DNA.

Sufficient equilibration of a newly packed PS–DVB–C_{18} column is an important determinant for obtaining high-performance separations. The time course of equilibration with 0.1 *M* TEAA at a flow-rate of 1 ml/min is summarized in Fig. 10. It is evident, that retention times remained constant already after 20 min of equilibration. Separation efficiency, on the other hand, increased notably

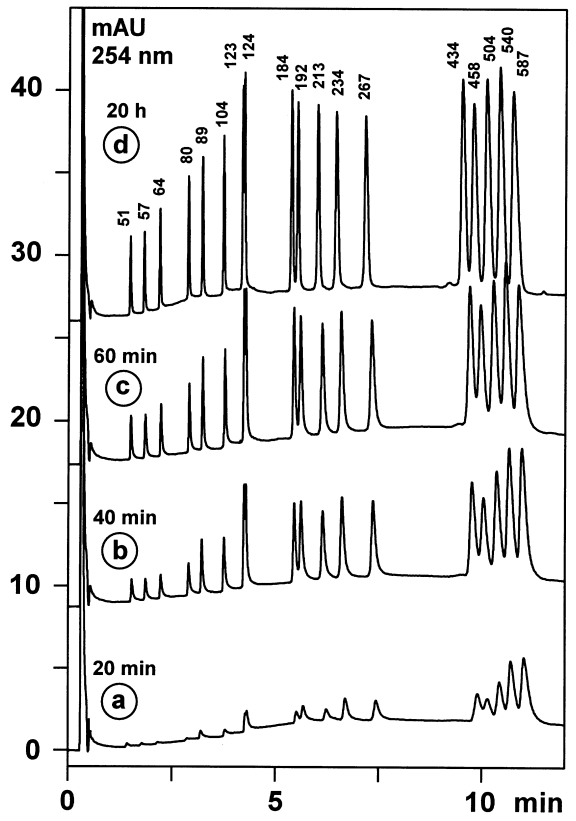

Fig. 10. Equilibration of a newly-packed column for ion-pair reversed-phase HPLC. Column, PS–DVB–C_{18} (2.1 μm, 50×4.6-mm I.D.); mobile phase, (A) 0.1 *M* TEAA, pH 7.0, (B) 0.1 *M* TEAA, 25% acetonitrile, pH 7.0; linear gradient, 35–55% B in 4 min, 55–67% B in 8 min; flow-rate, 1 ml/min; temperature, 50°C; sample, 1.25 μg pBR322 DNA–*Hae*III digest injected after 20 min (a), 40 min (b), 60 min (c), and 20 h (d) of equilibration time (reproduced from Ref. [56] with permission).

between 20 and 60 min of equilibration, with a further increase being observed after 20 h of equilibration [56]. Nonetheless, once a newly-packed column has been adequately equilibrated, reequilibration between chromatographic analyses is accomplished very quickly in less than 3 min (see also Section 6.3).

4.2. Mobile phase composition

Triethylammonium acetate (TEAA) is the ion-pair reagent of choice for ion-pair chromatography of dsDNA. A 1 *M* stock solution is prepared by dissolving equimolar amounts of triethylamine and acetic acid in water and final adjustment of the pH to 7–8 with triethylamine or acetic acid. Acetonitrile is used almost exclusively as the gradient former because of its low viscosity and good UV transmissivity. Eluents are prepared by dilution of the 1 *M* TEAA stock solution with water–acetonitrile mixtures usually without further addition of a buffering component. The influence of TEAA concentration on retention and resolution of DNA fragments is shown in Fig. 11. Between 25 and 75 m*M* TEAA, retention times generally increased with increasing

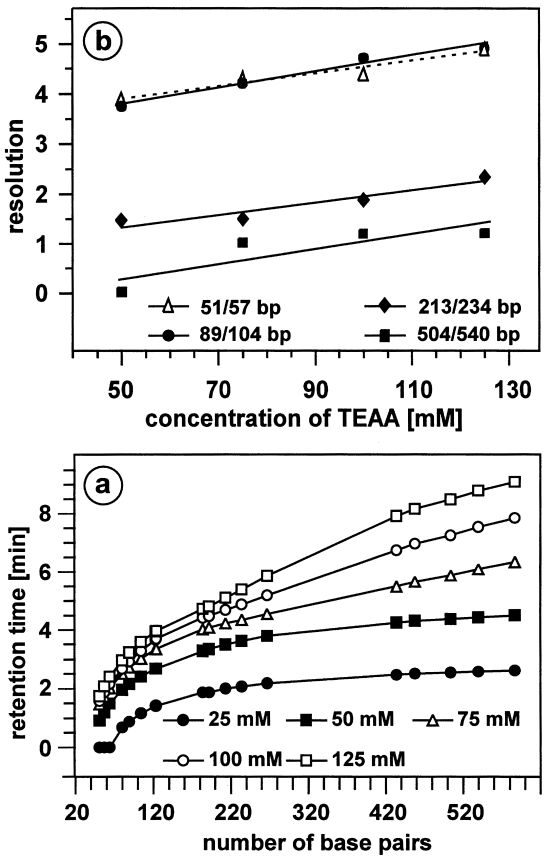

Fig. 11. Influence of ion-pair reagent concentration on retention times (a) and resolution (b) of DNA fragments. Column, PS–DVB–C_{18} (2.1 μm, 30×4.6-mm I.D); mobile phase, (A) 0.025–0.125 *M* TEAA, pH 7.0, (B) 0.025–0.125 *M* TEAA, 25% acetonitrile, pH 7.0; linear gradient, 30–55% B in 4 min, 55–65% B in 6 min; flow-rate 1 ml/min; temperature, 50°C; sample, 0.5 μg pBR322 DNA–*Hae*III digest; line symbols in (a), ●, 25 m*M*; ■, 50 m*M*; △, 75 m*M*; ○, 100 m*M*; □, 125 m*M* TEAA; line symbols in (b), △, 51/57 bp, ●, 89/104 bp, ♦, 213/234 bp, ■, 504/540 bp.

TEAA concentration because of the higher surface potential of the stationary phase. Above a concentration of 75 m*M* TEAA, the retention times of DNA fragments smaller than 200 bp remained almost constant whereas the retention of large fragments was still increasing (Fig. 11a). The increase in the concentration of TEAA from 25 to 125 m*M* in the eluent resulted in a linear enhancement in resolution (Fig. 11b) [30]. Concentrations higher than 125 m*M* TEAA are not practical because of the high viscosity of the TEAA solution.

Eriksson et al. [15] investigated the influence of TEAA and tetrabutylammonium bromide on the separation of DNA fragments. They found that a higher concentration of acetonitrile together with a lower concentration of the tetrabutylammonium salt was needed to elute the DNA from a PepRPC (C_2/C_{18}) column and that resolution was equivalent with TEAA and tetrabutylammonium bromide. Ion-pair reagents other than TEAA have also been tried for the separation of dsDNA fragments on PS–DVB-C_{18}, but only TEAA allowed the separation of dsDNA fragments with high resolution. The use of eluents containing trialkylammonium salts with alkyl groups longer than ethyl resulted in longer retention of DNA accompanied by serious deterioration of separation efficiency and peak shape [77]. Tetraalkylammonium salts were not applicable to the separation of dsDNA fragments.

4.3. Flow-rate and gradient slope

The flow-rate of the mobile phase exerts a significant impact on resolution (Fig. 12). For DNA fragments smaller than 200 bp, higher resolution was observed at higher flow-rates due to minimization of peak dispersion by longitudinal diffusion. But the longer the DNA molecules grow, the more restricting becomes their resistance to mass transfer between the stationary and mobile phase, which causes broader peaks at higher flow-rates [30]. Shallower gradients allow higher resolution of DNA fragments in ion-pair chromatography. To study the influence of gradient steepness on separation efficiency a pBR322 DNA–*Hae*III digest was chromatographed with a gradient of 8–20% acetonitrile in 30 min (corresponding to a slope of 0.4% acetonitrile per min) and with another gradient of 9.8–18.2% acetonitrile in 25

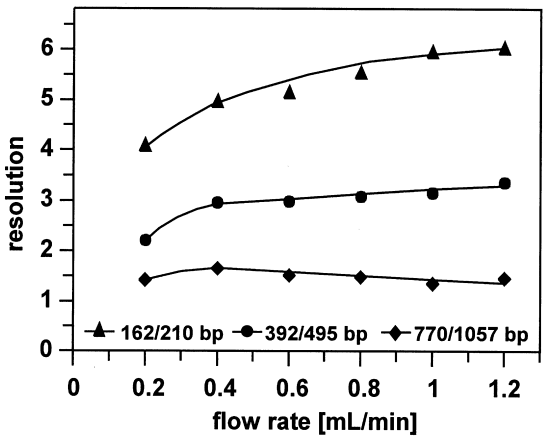

Fig. 12. Influence of flow-rate on resolution of DNA fragments. Column, PS–DVB-C_{18} (2.1 μm, 30×4.6-mm I.D.); mobile phase, (A) 0.1 *M* TEAA, pH 7.0, (B) 0.1 *M* TEAA, 25% acetonitrile, pH 7.0; linear gradient, 30–55% B in 4 min, 55–75% B in 12 min; flow-rate, 0.2–1.2 ml/min; temperature, 50°C; sample, 0.65 μg ΦX174 DNA–*Hin*dII digest; line symbols, ▲, 162/210 bp; ●, 392/495 bp; ♦, 710/1057 bp (reproduced from Ref. [30] with permission).

min (corresponding to a slope of 0.34% acetonitrile per min). The time-window between elution of the 51 bp and 587 bp fragment expanded from 5.81 min to 22.69 min with the first gradient, and from 1.13 min to 20.82 min with the second gradient. Table 2 lists the resolution between selected pairs of DNA fragments. Resolution values for small as well as large DNA fragments were between 9.6 and 21.7% higher with the shallower gradient, which clearly demonstrated, that shallower gradients that begin at a higher concentration of acetonitrile allow better resolution in a shorter period of time.

4.4. Temperature

Fig. 13 illustrates the impact of column temperature on the separation of DNA fragments [30]. It is evident that retention times and resolution increased continually with increasing temperature due to the same reasons valid for anion-exchange chromatography (see Section 3.4). At temperatures above 50°C, some of the peaks became broad and distorted due to partial or complete denaturation of the DNA double helix (see Section 5.2). No carry-over phenomena like those observed at lower temperatures in anion-

Table 2
Resolution of DNA fragments at different gradient slopes

Fragment pair	Resolution	
	8–30% ACN in 30 min 0.4% ACN per min	9.8–18.2% ACN in 25 min 0.34% ACN per min
123/124	0.955	1.122
184/192	1.995	2.187
234/267	4.971	6.051
540/587	1.307	1.454

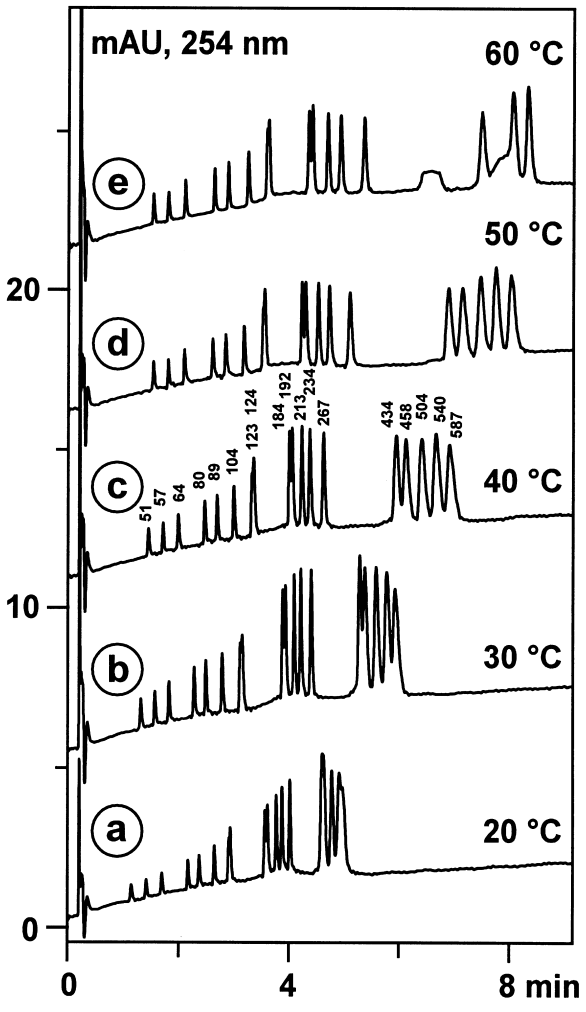

Fig. 13. Separation of pBR322 DNA–*Hae*III fragments at different column temperatures. Column, PS–DVB-C_{18} (2.1 μm, 30× 4.6-mm I.D.); mobile phase, (A) 0.1 *M* TEAA, pH 7.0, (B) 0.1 *M* TEAA, 25% acetonitrile, pH 7.0; linear gradient, 30–55% B in 4 min, 55–65% B in 6 min; flow-rate, 1 ml/min; temperature, 20–70°C; sample, 0.5 μg pBR322 DNA–*Hae*III digest (reproduced from Ref. [30] with permission).

exchange chromatography were noticed in ion-pair reversed-phase chromatography.

5. Dependence of chromatographic retention on fragment size and base sequence

Various biochemical and molecular biological techniques yield more or less complex mixtures of nucleic acids which subsequently have to be separated, identified, and quantitated. Chromatographic separation of dsDNA according to size is highly desirable because the size, usually expressed as the number of base pairs, is a basic information necessary to identify a DNA molecule. But besides size, there are other important molecular properties and structural features such as shape, conformation, curvature, melting behavior, single-stranded segments, and sequence variations, that are responsible for the biological function of a DNA molecule and that are directly related to its specific base sequence. Therefore, base sequence is another helpful separation criterion for the fractionation of nucleic acids. Ideally, in order to provide as much analytical information as possible about a nucleic acid sample, two-dimensional separation according either to size or to base sequence should be realizable in one single separation system by simply changing the separation conditions such as eluent composition or column temperature.

5.1. Anion-exchange chromatography

The separation principle of anion-exchange chromatography implies that elution order is determined only by size. However, anion-exchange separation of dsDNA fragments on a variety of different stationary phases has suffered from inversions in retention time

as a function of chain length, preventing its use for accurate-size fragment identification [8,75,50,37,9,11,26]. Retardation of some *Hae*III restriction fragments from pBR322 has been reported on DEAE- or quaternary ammonium-based HPLC columns such as Nucleogen DEAE 400, Mono Q, Gen-Pak FAX or TSKgel DEAE–NPR. The size calibration graph constructed from the retention of DNA fragments of a pBR322 DNA–*Hae*III digest on TSKgel DEAE–NPR shows clearly, that some of the fragments, especially the 123-, 192-, 267-, 458-bp fragments, are retained more strongly than expected from their size (Fig. 14a). Although this was not verified for all of them, the retardation of these fragments was attributed to their high AT content [10].

In fact, more detailed analysis revealed that retardation is not always proportional to the high percentage of AT in a fragment, indicating that another feature is responsible for the observed retardation [78]. Fack et al. remarked that the delayed retention is related to a curved geometry of DNA fragments [79]. Since curved geometries are mainly, but not exclusively the result of poly A runs, curved fragments exhibit inherently high AT content [79]. Fig. 15 shows that two DNA fragments of identical chain length can be almost baseline separated by the Gen-Pak FAX column. Two adjacent bending motives are present in the later-eluting 316-bp fragment and DNA modelling predicted that this fragment is curved. This finding corroborated the hypothesis that sequence-directed conformation particularities play an important role in determining the chromatographic retention of DNA fragments in anion-exchange chromatography. The increase in retention was attributed to the dipole character caused by local compression of charges in the curved fragments resulting in stronger electrostatic interaction with the stationary phase [79].

As suggested by Bloch, a more length-relevant separation of DNA fragments is feasible when a gradient of tetraethylammonium chloride is applied instead of sodium chloride [74]. The result is depicted in Fig. 14b where the calibration points are much closer to a straight line. The local reduction in surface potential through the preferential binding of positively-charged tetraethylammonium ions to AT rich sequences [80,81] is believed to be responsible

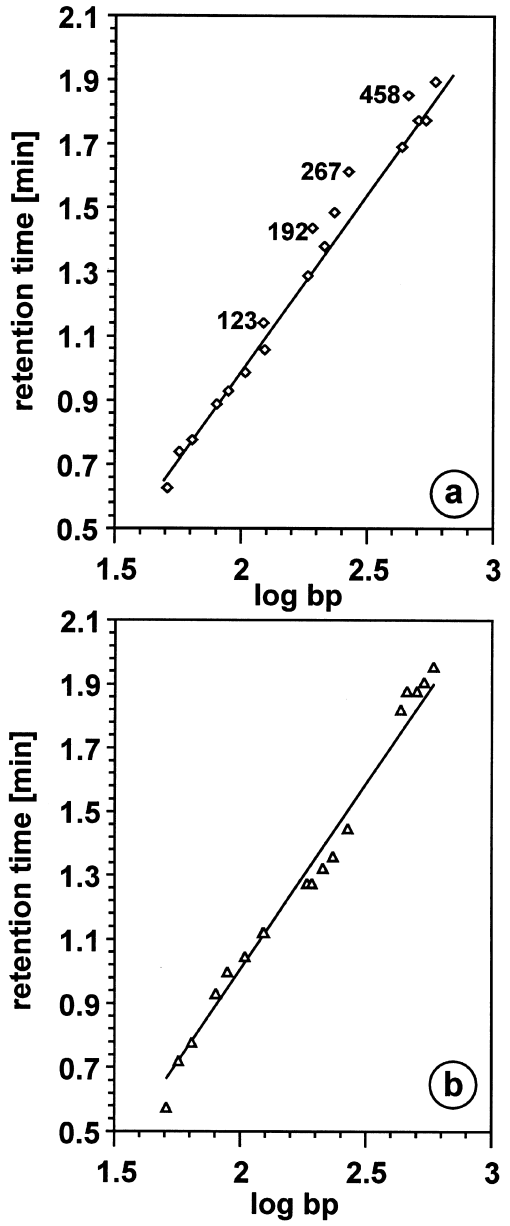

Fig. 14. Dependence of retention time on fragment length in anion-exchange chromatography with sodium chloride (a) and tetramethylammonium chloride (b) as gradient formers. Column, TSKgel DEAE–NPR (2.5 μm, 35×4.6 mm I.D.); mobile phase, 10 m*M* CHES, pH 9.0; linear gradient, 0.516–0.554 *M* sodium chloride in 0.3 min, 0.554–0.6 *M* in 1 min, 0.6 *M* for 0.3 min, 0.6–0.7 *M* in 0.1 min, 0.7 *M* for 0.4 min in (a), 10 m*M* MES, pH 8.0, linear gradient, 0.884–1.045 *M* tetramethylammonium chloride in 1 min, 1.045–1.115 in 2 min in (b); flow-rate, 1 ml/min; temperature, 30.4°C (a), 29.7°C (b); sample, pBR322 DNA–*Hae*III digest; (data from Ref. [74]).

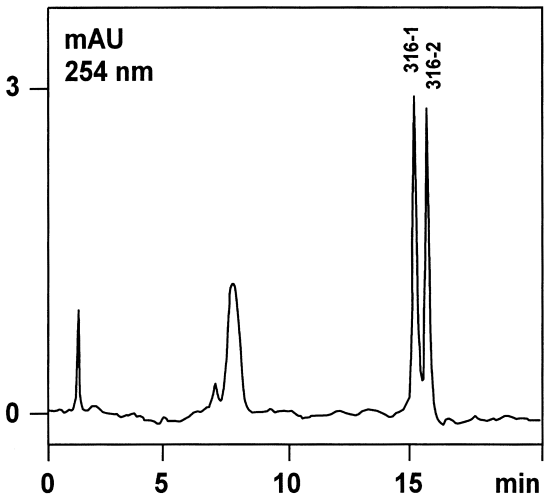

Fig. 15. Separation of two fragments of identical length by anion-exchange chromatography. Column, Gen-Pak FAX (2.5 μm, 100×4.6-mm I.D.); mobile phase, 50 m*M* Tris–HCl, 1 m*M* EDTA, pH 7.5; convex gradient, 0.22–0.58 *M* sodium chloride in 45 min; flow-rate, 0.5 ml/min (reproduced from Ref. [79] with permission).

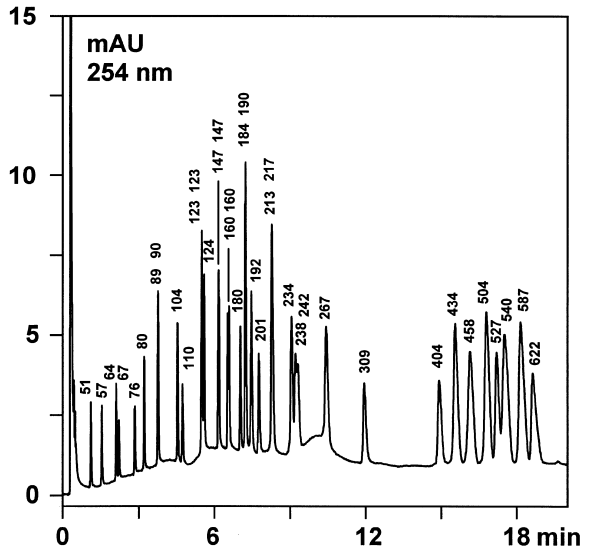

Fig. 16. High-resolution separation of dsDNA fragments by ion-pair reversed-phase chromatography. Column, PS–DVB-C_{18} (2.1 μm, 50×4.6-mm I.D.); mobile phase, (A) 0.1 *M* TEAA, pH 7.0, (B) 0.1 *M* TEAA, 25% acetonitrile, pH 7.0; linear gradient, 37–55% B in 6 min 55–65% B in 14 min; flow-rate, 1 ml/min; temperature, 50°C; sample, 0.75 μg pBR322 DNA–*Hae*III digest and 0.4 μg pBR322 *Msp*I digest (reproduced from Ref. [56] with permission).

for the lower tendency of AT-rich DNA to bind tightly to the anion-exchanger, resulting in improved size dependence of retention. This study showed that the replacement of sodium chloride by tetraethylammonium chloride as gradient former is an easy and simple way of making an anion-exchange chromatographic separation size dependent.

5.2. Ion-pair reversed-phase chromatography

No retention inversions could be detected by ion-pair chromatography on PS–DVB-C_{18} at temperatures between 20 and 50°C. However, a closer examination the high-resolution separation of 37 DNA restriction fragments depicted in Fig. 16 reveals some slight details of fragment retention [56]. Fragments 89 bp and 90 bp coelute whereas fragments 123 bp and 124 bp with the same difference in length are nearly baseline separated. No correlation between this observed anomalous retention behavior and base composition could be detected. This finding is corroborated by the coelution of both 123-bp fragments with significantly different base compositions (33.3 and 43.9% AT, respectively), and the separation of fragments of 123 and 124 bp with very similar base compositions (33.3 and 34.7% AT, respectively). In most cases, fragments of identical size coelute (123 and 147 bp) with the only exception of the two 160-bp fragments, which are partially separated despite of the same length and almost identical base composition (38.1 and 39.4% AT, respectively). From this observations it is concluded that although the high separation efficiency of the alkylated PS–DVB stationary phase allows the detection of minute influences of DNA sequence and ensuing secondary structure on retention behavior, the separation is size dependent to a very high degree. The strict size dependence of retention is also illustrated by the size calibration graph obtained by plotting the retention times of 57 DNA fragments, ranging in size from 51 to 2176 bp, against the logarithm of fragment length (Fig. 17). The correlation between retention and size was almost linear up to a size of 500 bp and allowed the calculation of fragment size from retention data with an accuracy of better than 3.2% [56]. Moreover, unlike in anion-exchange chromatography, no specific influence of

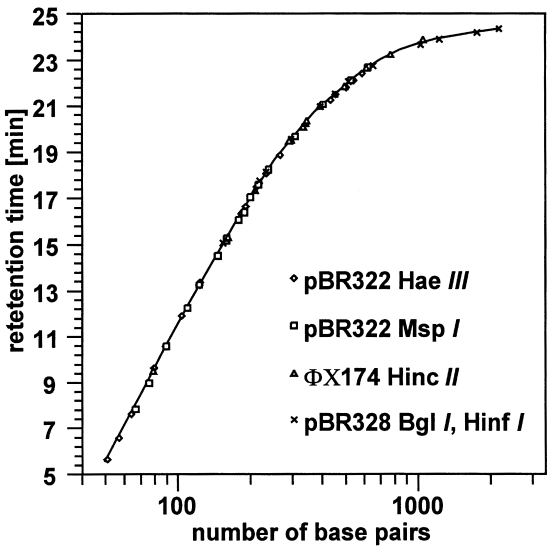

Fig. 17. Dependence of retention time on fragment length in ion-pair reversed-phase chromatography. Column, PS–DVB-C_{18} (2.1 μm, 50×4.6-mm I.D.); mobile phase, (A) 0.1 *M* TEAA, pH 7.0, 8% acetonitrile, (B) 0.1 *M* TEAA, 20% acetonitrile, pH 7.0; linear gradient, 0–100% B in 30 min; flow-rate, 1 ml/min; temperature, 50°C; sample, 57 restriction fragments ranging from 51 to 2176 bp in length.

AT content on retention behavior was detected at temperatures between 20 and 50°C.

In ion-pair chromatography at temperatures above 50°C, DNA fragments are subject to sequence-dependent separation due to partial denaturation of AT-rich segments [82]. As with increasing GC content the stability of the helical structure of DNA molecules increases, AT-rich DNA fragments melt at lower temperatures than GC-rich DNA fragments of comparable length. The temperature at which denaturation begins is determined by the total number and the extent of repetitive AT tracts that can form melting domains of 30–300 bp resulting in partially-denatured DNA fragments. Compared to stretched, helical fragments of the same length, retention times of partially-denatured DNA fragments are smaller because of the perturbation of the stretched structure upon formation of single strands which causes a reduction of the contact area available for chromatographic interaction. Reduced retention as an indicator for partial denaturation can be readily recognized from plots of retention time versus temperature, as shown in Fig. 18. First, a linear increase in retention is observed in the temperature range, where the double-stranded structure of DNA is stable. However, upon further increasing the temperature, the retention times of some DNA fragments decrease significantly due to partial denaturation.

It is concluded that in ion-pair reversed-phase chromatography, the control of column temperature allows the realization of two different separation dimensions, one is size, one is base sequence: in the temperature range between 20 and 50°C, retention of DNA fragments is strictly size dependent, whereas at temperatures above 50°C, retention is influenced by sequence-dependent denaturation of DNA fragments. The ion-pair reversed-phase chromatographic sepa-

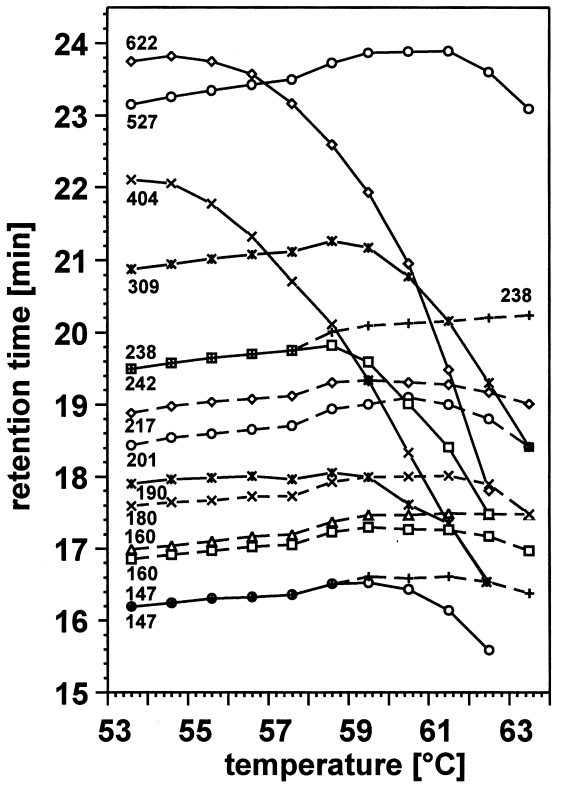

Fig. 18. Effect of temperature-dependent partial denaturation on retention of DNA fragments. Column, PS–DVB-C_{18} (2.3 μm, 50×4.6 mm I.D.); mobile phase, (A) 0.1 *M* TEAA, pH 7.0, 7% acetonitrile, (B) 0.1 *M* TEAA, 19% acetonitrile, pH 7.0; linear gradient, 0–100% B in 30 min; flow-rate, 0.75 ml/min; temperature, 54.6–63.5°C; sample, 0.41 μg pBR322 DNA–*Msp*I digest (reproduced from Ref. [82] with permission).

ration of two fragments of identical length from the pBR322 DNA–*Msp*I digest is illustrated in Fig. 19. At 54.6°C, retention depends on size only and hence, the two 147-bp fragments coelute (Fig. 19a). At 61.5°C, however, partial denaturation in one of the two fragments reduces its retention and permits baseline separation of the two fragments (Fig. 19b). Inspection of the pBR322 sequence reveals that the earlier eluting, partially denatured fragment (coordinates 1666–1812) contains two tracts of 6 consecutive AT base pairs and one tract of 4 consecutive base pairs, whereas the later eluting, helical fragment (coordinates 2683–2829) contains only one tract of 4 consecutive AT base pairs. The agreement between observed retention behavior and base sequence suggests that the interpretation or prediction of retention data under partially denaturing conditions is straightforward in ion-pair reversed-phase chromatography in contrast to anion-exchange chromatography, where very little is known about the actual mechanism that causes delayed retention. Moreover, since the physicochemical background of partial denaturation is well known and theories are elaborated to calculate the melting process [83], retention data can be used to obtain information about structural features of DNA fragments.

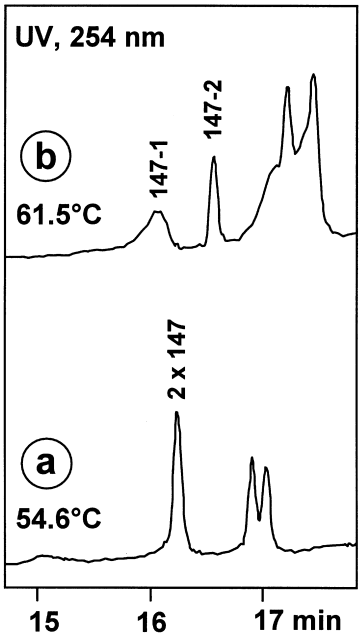

Fig. 19. Separation of DNA fragments of identical length but different sequence under partially denaturing conditions. Column, PS–DVB-C_{18} (2.1 μm, 50×4.6-mm I.D.); mobile phase, (A) 0.1 *M* TEAA, pH 7.0, 7% acetonitrile, (B) 0.1 *M* TEAA, 19% acetonitrile pH 7.0; linear gradient 0–100% B in 30 min; flow-rate, 0.75 ml/min; temperature, 54.6 (a) and 61.5 °C (b); sample, 0.41 μg of pBR322 DNA–*Msp*I digest.

6. Examples of application

6.1. Preparative DNA fractionation

The amount of dsDNA which is available in molecular biological or biochemical experiments is usually much less than 1 μg. Therefore, the loading capacity of analytical columns (30−250 mm length, 2–4.6 mm I.D.) is adequate for most applications. Ten μg of a λDNA–*Hin*dIII digest could be loaded onto a 35×4.6-mm I.D. TSKgel DEAE–NPR column without serious loss in separation efficiency [50]. Recovery of DNA fragments depended on buffer pH and was 78.4% at pH 7.0 and 93.6% at pH 9.0 [76]. Fifty μg of a 1-kbp ladder could be separated on a 100×4.6 mm I.D. Gen-Pak FAX column (Fig. 8a, [75]) and recovery was more than 95% with phosphate buffer, pH 7.0. Waterborg and Robertson were able to purify up to 750 μg of digested plasmid DNA by solute-displacement ion-exchange HPLC [84]. They showed that at extreme column overload solutes with different adsorption characteristics compete for the limited binding sites at the stationary phase resulting in solute–solute displacement effects and separation of large amounts of dsDNA.

Preparative fractionation of nucleic acids by anion-exchange chromatography requires elution with gradients of increasing salt concentration and, therefore, requires time-consuming removal of the salt from the collected nucleic acid fractions by precipitation, dialysis, ultrafiltration, size exclusion chromatography, or solid-phase extraction. In ion-pair reversed-phase chromatography, on the other hand, the mobile phase is composed exclusively of volatile compounds (TEAA, water, acetonitrile), and the isolation of pure DNA fragments is possible in yields above 95% simply by evaporation of the eluent [30]. Fig. 20a depicts the micropreparative fractionation of 4 μg of a pB322 DNA–*Hae*III digest in less than 15 min. The eluted peaks were collected and some of the fractions were rechromato-

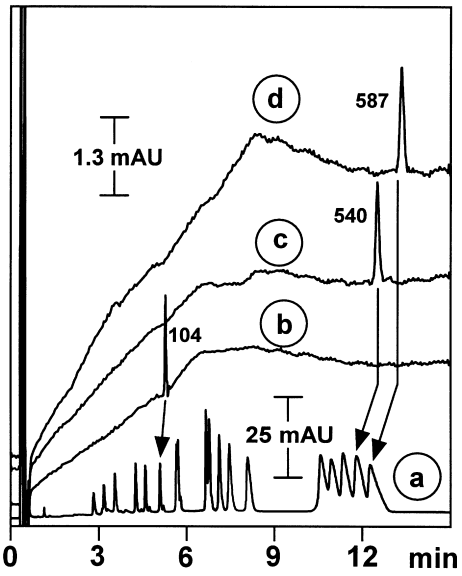

Fig. 20. Micropreparative fractionation of 4 μg of a pBR322 DNA–*Hae*III (a) digest and rechromatography (b–d) of some of the isolated fragments. Column, PS–DVB-C_{18} (2.3 μm, 50×4.6 mm I.D.); mobile phase, (A) 0.1 *M* TEAA, pH 7.0, (B) 0.1 *M* TEAA, 25% acetonitrile, pH 7.0; linear gradient, 30–55% B in 6 min, 55–65% B in 9 min; flow-rate, 1 ml/min; temperature, 50°C; sample, 4 μg pBR322 DNA–*Hae*III digest (a) and isolated fragments 104 (b), 540 (c) and 587 (d) (reproduced from Ref. [56] with permission).

graphed under identical separation conditions. As shown in the chromatograms of Fig. 20b–d, no cross contamination of eluted fractions could be detected [56].

6.2. DNA restriction analysis

Separation and purification of restriction fragments is a key element in a number of molecular biological techniques, including cloning, DNA sequencing, genome fingerprinting, and DNA hybridization. DNA restriction fragments are products of digestion of larger DNA molecules with restriction endonucleases that cleave phosphodiester bonds at specific nucleotide sequences [85]. The set of fragments generated by this enzymatic cleavage may range in size from a few base pairs to several thousands of base pairs. Anion-exchange HPLC [50,86] and ion-pair reversed-phase HPLC [56] are fast and reliable alternatives to traditional slab gel electrophoresis for the separation of DNA restriction fragments. Several chromatograms in this paper have confirmed, that anion-exchange chromatography is optimally suited for the separation of DNA fragments ranging in size from 18 to 23 000 bp (Figs. 3, 6, 8, 9 and 15). The difference in fragment length that can be resolved in anion-exchange chromatography is ca. 8–10% of the total length for fragments smaller than 1000 bp. The length difference necessary to allow separation increases with increasing chain length, especially for DNA molecules longer than 100 bp.

The size range of separated DNA fragments investigated so far in ion-pair chromatography is 20–2200 bp [56]. Ion-pair reversed-phase chromatography provides higher resolving power than anion-exchange chromatography for DNA fragments smaller than 1000 bp, since DNA fragments can be separated if their overall length differs by 2–8% (Figs. 5,16,19).

6.3. Analysis of PCR products

The simplicity of PCR has lead to its wide use in biochemistry, molecular biology and clinical diagnostics [87]. Following the PCR process, the amplified DNA has to be separated from other reaction components (DNA polymerase, oligonucleotide primers, nucleotides, buffer additives) to allow its identification and quantitation. HPLC appears to be an ideally suited technique for fast and automated separation of amplified PCR products [88,89,55,57]. Katz et al. studied the suitability of anion-exchange chromatography for the rapid separation and quantitation of PCR products [88]. They amplified a 500-bp segment of bacteriophage λDNA and separated the PCR reaction mixture without further sample pretreatment with a 12-min sodium chloride gradient in Tris buffer, pH 9.1, on a TSKgel DEAE–NPR column. The relative standard deviation of retention times and peak areas for 24 measurements was less than 5% and 10%, respectively [88]. The baseline level of the UV detector was found to increase with time, indicating accumulation of poorly eluted compounds, and the use of an appropriate guard column and the occasional cleaning of the column with 20% aqueous acetonitrile was therefore recommended. Fig. 21 shows the anion-exchange chromatographic analysis of a PCR amplified 409 bp segment from the human androgen receptor. The PCR reaction was spiked with restriction fragments from a ΦX174

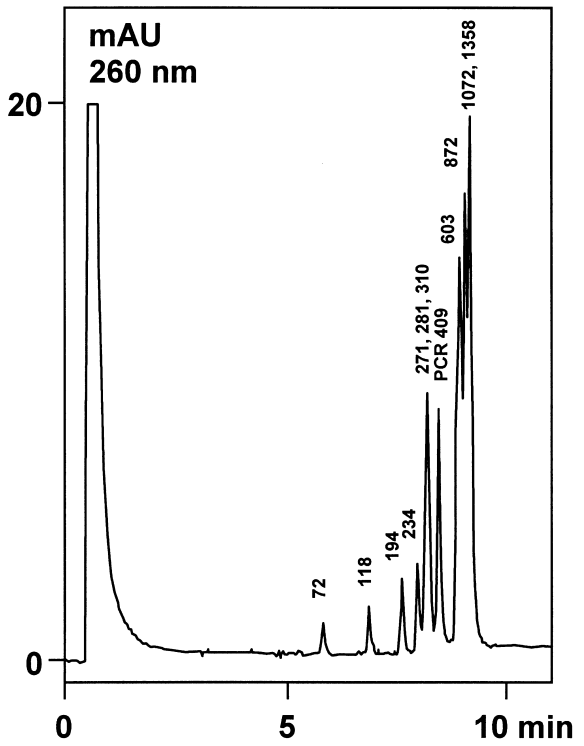

Fig. 21. Anion-exchange analysis of a PCR amplified 409-bp fragment of the androgen receptor spiked with a ΦX174 DNA–*Hae*III digest. Column, TSKgel DEAE–NPR (2.5 μm, 35×4.6 mm I.D.); mobile phase, 25 m*M* Tris–borate, pH 8.6, 0.25 m*M* EDTA; linear gradient, 0.45–0.75 *M* sodium chloride in 15 min; flow-rate, 1 ml/min; temperature, 25°C; sample 409 bp PCR product and 1 μg ΦX174 DNA–*Hae*III digest (reproduced from Ref. [90] with permission).

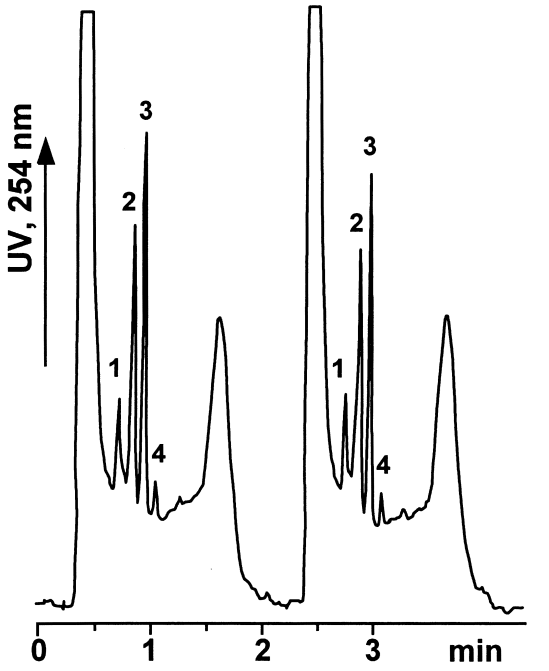

Fig. 22. Fast separation and column regeneration for routine analysis of PCR products. Column, PS–DVB-C_{18} (2.1 μm, 50× 4.6 mm I.D.); mobile phase, (A) 0.1 *M* TEAA, pH 7.0, (B) 0.1 *M* TEAA, 25% acetonitrile, pH 7.0; linear gradient, 45–65% B in 1 min, 0.1 min at 90% B for elution of strongly retained compounds, 0.9 min at 45% B for equilibration; flow-rate, 3 ml/min; temperature, 50°C; sample, 1, unspecific PCR product, 2, 120-bp PCR product, 3, 132-bp PCR product, 4, 167-bp PCR product (reproduced from Ref. [91] with permission).

DNA–*Hae*III digest in order to confirm the expected length of the amplified PCR fragment by its elution position between standard fragments of known length [90].

In ion-pair chromatography, the relatively low viscosity of acetonitrile–water mixtures at elevated temperature permits the application of high flow-rates and very steep gradient profiles. Fig. 22 depicts two successive chromatograms of repetitive injections of four PCR products ranging from 100 to 167 bp in size [91]. The separation was completed within 1 min at 50°C and a flow-rate of 3 ml/min by a linear gradient from 11.25 to 16.25% acetonitrile in 0.1 *M* TEAA. Thereafter, column regeneration was achieved by a 0.1-min flush with 22.5% acetonitrile followed by 0.9-min equilibration with starting eluent. The application of such a gradient program enabled the injection of a PCR sample every 2 min. The reproducibility of retention times and peak areas with manual injection was 2.7% and 8%, respectively. Through the use of an automatic sampling system the reproducibility could be effectively reduced to 1.91% and 2.53%, respectively. In routine analysis of PCR products, column performance and baseline stability can be impaired by components of the PCR reaction that are strongly adsorbed and accumulated on the stationary phase such as high molecular weight DNA, DNA polymerase, gelatin, or mineral oil. It is therefore recommended to wash the column after ca. 100 analyses for 30 min with 100% methanol, which elutes the strongly-adsorbed compounds and completely regenerates the original baseline stability and column performance [55].

6.4. Fluorescent-dye-labelled DNA fragments

PCR is also one of the most efficient techniques for measuring the viral load of infected samples. Fig. 23 demonstrates that fluorescent dye labelled HIV–PCR products could be separated by ion-pair chromatography with high separation efficiency and detection sensitivity. For quantitation of viral genomes in clinical samples, known amounts of synthetic RNA (wild-type RNA with 15-bp deletion and wild-type RNA with 12 bp insertion, respectively) were added as internal standards before extraction [92]. Retention of labelled DNA was influenced by the structure of the DNA and the type of fluorophore added to the DNA molecule. Whereas retention times of single-stranded oligonucleotides increased by more than 100% (1.9 to 4 min), the effect was less pronounced (5 to 5.8 min, 16%) in the case of a double-stranded 127-bp PCR product after labelling with 5-carboxyfluorescein [93]. The detection limit of fluorescence detection using a single 5-carboxyfluorescein label attached to one of the PCR primers was found to be approximately 1 fmole or 30–70 times higher than that of UV absorbance detection.

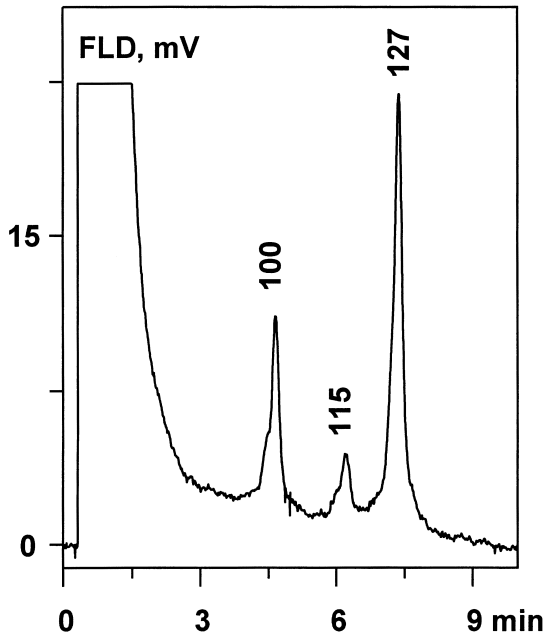

Fig. 23. Ion-pair chromatographic analysis of 5-carboxyfluorescein-labelled HIV PCR-products. Column, PS–DVB-C_{18} (2.1 μm, 50×4.6-mm I.D.); mobile phase, (A) 0.1 *M* TEAA, pH 7.0, (B) 0.1 *M* TEAA, 25% acetonitrile, pH 7.0; linear gradient, 45–55% B in 10 min, 5; flow-rate, 1 ml/min; temperature, 50°C; detection, fluorescence 488/520 nm; sample, 115-bp segment of HIV amplifed by PCR, 100-bp and 127-bp internal standards [92].

6.5. Detection of partial denaturation in DNA fragments

Fig. 24 depicts a detailed study of temperature-dependent separation of DNA restriction fragments between 48.8 and 58.6°C [82]. A strictly size-dependent separation is obtained at 48.8°C. After raising the temperature to 53.6°C, retention of fragment 458 bp is decreased due to partial denaturation in relation to fragment 434 bp so that these two fragments are only partly resolved. At a temperature of 56.6°C, the 458-bp fragment as well as the 587-bp fragment elute before the 434-bp fragment and the 46-bp fragment is completely denatured. A further increase in temperature to 58.6°C reveals the 587 bp fragment eluting between the 267-bp and 434-bp peaks and the 458-bp fragment eluting between the 234-bp and 267-bp fragments. All the other peaks remain unchanged in their relative elution positions between 48.8 and 58.6°C. Fig. 24 also shows that, in a temperature interval of 10°C, partially-denatured DNA fragments eluted as relatively sharp peaks, suggesting that complete strand dissociation does not occur and that the secondary structure of partially-melted DNA is relatively uniform at a given temperature.

Oefner and Underhill utilized the decreased retention of partially denatured DNA to detect mutations by denaturing HPLC [94]. The mutant sample is first hybridized with wild-type DNA to form a mixture of two homo- and two heteroduplices which are subsequently analyzed by denaturing HPLC at temperatures around 60°C. Because of partial denaturation in the locality of the base mismatch, heteroduplices elute earlier than homoduplices indicating that a mutation is present in the analyzed DNA sequence. Denaturing HPLC allowed the detection of single mismatches in DNA fragments as large as 1000 bp.

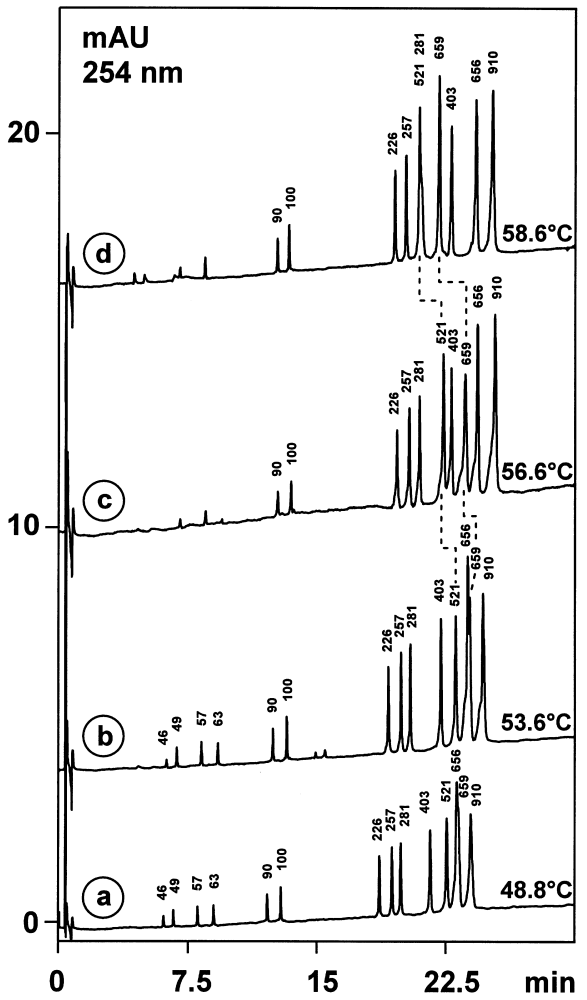

Fig. 24. Separation of the pBR322 DNA–*Alu*I digest at temperatures above 48.8°C. Column, PS–DVB-C_{18} (2.1 μm, 50×4.6 mm I.D.); mobile phase, (A) 0.1 *M* TEAA, pH 7.0, 7% acetonitrile, (B) 0.1 *M* TEAA, 19% acetonitrile, pH 7.0; linear gradient, 0–100% B in 30 min; flow-rate, 0.75 ml/min; temperature, 48.8 (a), 53.6 (b), 56.6 (c), and 58.6 °C (d); sample, 5 μg pBR322 DNA–*Alu*I digest (reproduced from Ref. [82] with permission).

6.6. Purification of plasmid DNA

Plasmids are used as cloning vectors for recombinant DNA studies, as templates for RNA transcription, as substrates to determine enzyme activity, or for biophysical studies on superhelical structures. Most protocols used for isolation and purification of plasmid DNA are based on cesium chloride density-gradient centrifugation [95], size-exclusion chromatography [96], or anion-exchange chromatography [73,97]. Most desirable is a separation procedure that yields plasmids in their native supercoiled form. Therefore, after removal of proteins, small RNA and high-molecular-mass cellular RNA and DNA, plasmid DNA is further purified by separation of the supercoiled from the nicked form. Maa et al. synthesized a micropellicular DEAE anion-exchanger based on highly crosslinked PS–DVB particles to separate different forms of the pGEM-3Z plasmid [37]. Fig. 25 shows that supercoiled plasmid DNA

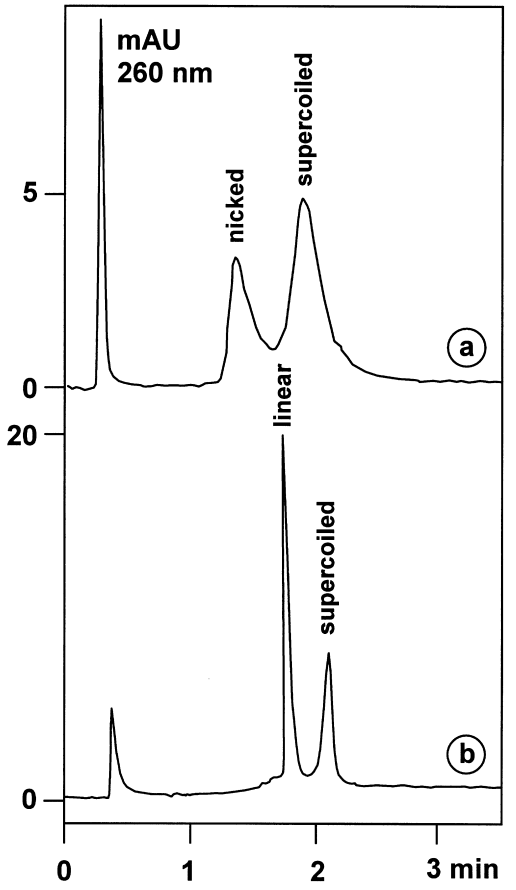

Fig. 25. Anion-exchange HPLC of linear, supercoiled and nicked plasmid DNA. Column, micropellicular PS–DVB–DEAE (3.3 μm, 30×4.6 mm I.D.), mobile phase, 20 m*M* Tris–HCl, pH 8.0, 1 m*M* EDTA, linear gradient, 0.40–0.55 *M* sodium chloride in 5 min (a), 0.45–0.5 *M* sodium chloride in 5 min (b); flow-rate, 2 ml/min; temperature, 25°C; sample, 0.25 μg pGEM-3Z plasmid DNA and 0.25 μg of *Hin*dII digested pGEM-3Z plasmid DNA (reproduced from Ref. [37] with permission).

elutes after the nicked and linear form within less than 3 min. This chromatographic separation followed by ethanol precipitation provides purified plasmid DNA within a few hours, as opposed to conventional cesium chloride density centrifugation which can take several days.

7. Conclusions

It is concluded that anion-exchange chromatography and ion-pair reversed-phase chromatography using micropellicular stationary phases are complementary techniques for the fast and high-resolution separation of double-stranded nucleic acids. The lack of internal pore structure is responsible for high efficiency, short analysis times, and high sample recovery. With both separation modes, the choice and optimization of several chromatographic parameters allows one to adjust the desired resolution, speed of analysis, and detection limits for dsDNA molecules. Hundreds of samples can be injected without sample pretreatment and columns can be regenerated by simple cleaning procedures. Owing to the small specific surface area of micropellicular packings, sample loads up to only a few micrograms are possible for micropreparative purposes, but this is sufficient for most molecular biological applications.

Table 3
Characteristics of anion-exchange and ion-pair reversed-phase HPLC with micropellicular packings

	HPAEC	IP–RP–HPLC
Typical stationary phase	2.5-μm DEAE-bonded micropellicular, hydrophilic polymer beads	2.5-μm micropellicular PS–DVB-C_{18} beads
Typical eluent	20 m*M* Tris–HCl, pH 7–9	0.1 *M* TEAA, pH 7
Gradient formers	0.2–1 *M* Sodium chloride or 0.5–1.5 *M* tetramethylammonium chloride	2.5–20% acetonitrile
	very steep gradients applicable for fast elution	
Flow-rates	0.5–3 ml/min (with 50×4.6-mm columns)	
Pressure limit	≥40 MPa	
Analysis times	Less than 1 min to 30 min	
Optimum temperature	20–35°C	50°C
Resolution of fragments ≤1000 bp	8–10% difference in length	2–8% difference in length
Resolution of fragments ≥2200 bp	500–1000 bp difference in length	Not yet investigated
Size-dependent retention	With tetraalkylammonium salts as gradient formers	At 20–50°C
Sequence-dependent separation	With sodium chloride as gradient former, retention depends primarily on size but also on base sequence	At temperatures above 50°C due to partial denaturation
UV detection limit, 260 nm	20 fmol 404 bp PCR product (20 μl sample size) [30]	
Fluorescence detection limit, Ex 490 nm, Em 520 nm	1 fmol 127 bp 5-carboxyfluorescein-labelled PCR product (20 μl sample size) [55]	
Analytical loading capacity	ca. 1 μg [30,50]	
Micropreparative loading capacity	ca.10 μg [50]	ca. 5 μg [56]
Sample recovery	95–100%	
Recovery of dsDNA in pure form by evaporation of eluent	Usually not possible because of high salt content	Possible with volatile ion-pair reagents (TEAA)

Compared to ion-pair reversed-phase chromatography, anion-exchange HPLC offers higher sample loading capacity (e.g. 10 versus 5 μg) at the cost of more laborious post-separation sample workup. The resolving power of ion-pair chromatography for DNA fragments up to 1000 bp in length is higher than that of anion-exchange chromatography, but the latter can better separate large DNA fragments of 1000 bp to more than 20 000 bp in length. Size-dependent or sequence-dependent separation can be achieved through appropriate choice of separation conditions. Variation of temperature (20–50°C for size-dependent separation, >50°C for sequence-dependent separation) allows a two-dimensional separation according to molecular size or base sequence in ion-pair reversed-phase chromatography. Table 3 summarizes some of the characteristics of anion-exchange and ion-pair reversed-phase chromatography for the separation of dsDNA.

8. Abbreviations

ACN	Acetonitrile
bp	Base pairs
C_{18}	Octadecyl-
CHES	2-(*N*-cyclohexylamino)ethanesulfonic acid
DEAE	Diethylaminoethyl-
dsDNA	Double-stranded deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FLD	Fluorescence detection
HIV	Human immunodeficiency virus
HPAEC	Anion-exchange HPLC
HPLC	High-performance liquid chromatography
IP–RP-HPLC	Ion-pair reversed-phase HPLC
MES	2-(N-morpholino)ethanesulfonic acid
kbp	Kilobase pairs
PCR	Polymerase chain reaction
PS–DVB	Polystyrene–divinylbenzene
TEAA	Triethylammonium acetate
TMA	Trimethylammonium
Tris	Tris(hydroxymethyl)aminomethane

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