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# Micropreparative fractionation of DNA fragments on metathesis-based monoliths: influence of stoichiometry on separation

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**Abstract**

Applying Grubbs' first generation benzylidene-type catalyst $Cl_2Ru(PCy_3)_2(CHPh)$ in ring opening metathesis polymerization (ROMP) of norborn-2-ene (NBE) and 1,4,5,8,8a-hexahydro-1,4,5,8, *exo, endo*-dimethanonapthalene (DMN-$H_6$), various monoliths were prepared within the confines of silanized borosilicate columns (100×3 mm I.D.) and investigated for the micropreparative separation of pBR322 DNA-Hae III restriction fragments ranging in size from 51 to 587 base pairs (bp), as a sample of double-stranded (ds) DNA. The approach to good resolution of dsDNA on monolithic columns entailed the modulation of the polymer morphology in terms of structure and porosity to suit such an analysis. Structural variations were achieved by changing the relative ratios of comonomers (NBE+DMN-$H_6$) at the expense of porogens, and by increasing the DMN-$H_6$ to NBE mass ratio. For dsDNA separations, eluents comprised 0.1 *M* aqueous triethylammonium acetate, pH 7.0, and acetonitrile. Alternatively, methanol was introduced in this study as a less polar gradient former. In terms of column evaluation, each column prepared was first tested in the separation of 5′-phosphorylated oligodeoxythymidylic acids [$p(dT)_{12-18}$], since good separation of oligodeoxynucleotides indicates the potential liability of the column tested for dsDNA analysis, and vice versa. It was noted that monoliths with combinations of 25:25:40:10, 28:28:35:9, and 30:30:32:8 (as weight% of NBE/DMN-$H_6$/2-propanol/toluene) showed good resolution of $p(dT)_{12-18}$. Moreover, they demonstrated good separation of the first 12 fragments (51–267 bp) of the pBR322 DNA-Hae III digest; however, reduced resolution in the separation of the last five highest molecular mass fragments (434–587 bp) was experienced. The best separation of these fragments was accomplished on a 25:25:40:10 NBE/DMN-$H_6$/2-propanol/toluene combination at a flow-rate of 2 ml/min, a temperature of 50 °C, and a gradient of 4–10% acetonitrile in 1 min, then 10–16% in 14 min. The total amount of pBR322 HaeIII digest that may be fractionated on these systems is 0.5–2.5 μg. 

*Keywords:* Stationary phases, LC; Monolithic stationary phases; DNA; Oligonucleotides

## 1. Introduction

Because of the importance of nucleic acids in all biochemical, biological, and biomedical sciences, the development and continuous improvement of technology for their fractionation, purification, quantitation, and structural analysis are of utmost impor-

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tance. By virtue of its high resolving capability, short cycle times, full automation, and preparative separation capability, high-performance liquid chromatography (HPLC) represents one of the most powerful tools for nucleic acid separation and analysis [1]. Among the various chromatographic techniques used for DNA separation, anion-exchange- and ion-pair reversed-phase HPLC are the most commonly applied modes, owing to their high efficiency, selectivity, and operable size range [2].

Stationary phases based on microparticles have been successfully used as separation media for HPLC of nucleic acids for more than three decades [3–7]. Nevertheless, the relatively large void volume between the packed particles as well as the slow diffusional mass transfer of solutes into and out of the stagnant mobile phase present in the pores of the stationary phase represent significant factors limiting the separation efficiency of conventional granular, porous packing materials. One means of enhancing mass transfer is the use of monolithic separation media [8–11] in which the chromatographic bed consists of a single piece of a rigid, porous polymer which has no interstitial volume but only internal porosity. Because of the absence of intraparticular volume, all of the mobile phase is forced to flow through the pores of the separation medium [12], resulting in enhanced convective mass transport [13].

In addition to improved chromatographic performance, monolithic separation media also possess distinctive advantages for preparative scale separations of nucleic acids [14]. Highly permeable chromatographic beds enable high percolation flow-rates at low column backpressure without loss in column efficiency, resulting in fast loading and elution times [15]. Moreover, very stable and uniform chromatographic beds can be manufactured reproducibly even in large-diameter preparative columns [16], which is very difficult to achieve with conventional, granular packing materials. Finally, the technology is readily scalable from laboratory devices to multigram loading levels required for manufacturing [17]. Monolithic columns of PS–DVB or of norborn-2-ene (NBE) and 1,4,5,8,8a-hexahydro-1,4,5,8, *exo, endo*-dimethanonapthalene (DMN-$H_6$), synthesized via ring-opening metathesis polymerization (ROMP) have been demonstrated to possess good separation capabilities for oligodeoxynucleotides and proteins [18–20]. Additionally, in particular when functionalized, they are applicable to the fast separation of various other compounds such as chiral drugs [21,22]. Single digit minute scale separations may be accomplished due to high flow-rates and diminished diffusion pathlength, where mass transport becomes the major lead in the separation [23–27]. It has been shown recently that protein and deoxyoligonucleotide separations on metathesis-based monoliths are reproducible and consistent within small relative deviations ($\sigma_{n-1}(R)$=1–10 and 9–23%, respectively) [18].

While variations in analysis conditions in terms of eluent composition, gradient slope, pH, temperature, and injection volume may generally help to improve separation performance, this may also be accomplished by structural variations or better, by a combination of both. In due consequence, morphology of a monolith and control over morphology is a key point for a better resolution. Though it is still hard to predict the optimum monolith composition for a particular separation problem, there are some general structural features that have already been elaborated for certain analytes [18]. Thus, besides good column permeability, low or no microporosity should be suited [20,28]. This would allow optimum mass transfer as well as good separations [29–32]. In the following, the synthetic approach to good resolution of dsDNA shall be outlined.

## 2. Experimental

### *2.1. Chemicals and reagents*

HPLC-grade water was purchased from Fluka (Buchs, Switzerland). HPLC-grade acetonitrile and methanol were purchased from Riedel-de Häen (Seelze, Germany) and Sigma (St Louis, MO). A stock solution of 1 mol/l triethylammonium acetate, pH 7.0, was prepared by mixing equimolar amounts of triethylamine and acetic acid (both from Fluka). The oligodeoxynucleotide ladder $p(dT)_{12\text{-}18}$ was from Pharmacia (Uppsala, Sweden), and the pBR322 DNA-Hae III digest was purchased from Sigma. Bicyclo[2.2.1]hept-2-ene (norborn-2-ene) and the initiator $Cl_2Ru(PCy_3)_2(CHC_6H_5)$ (Cy=cyclohexyl) were purchased from Fluka. The crosslinker DMN-

$H_6$ was prepared from freshly cracked dicyclopentadiene and pure norbornadiene (Fluka) according to the literature [33]. Toluene (Fluka) was dried over sodium/benzoketyl.

### 2.2. Instrumentation

A Model 616 pump and Model 600S controller (Waters, Milford, MA) equipped with a Model 200 UV–Vis detector (Linear Instruments, Fremont, CA) were used for HPLC analyses. All HPLC analyses were conducted applying SILK function to insure high sensitivity. All analyses were carried out at a wavelength of 260 nm. A Millennium 2000 Data System (Waters) was used for data acquisition and processing.

### 2.3. Synthesis of monoliths

Borosilicate glass columns (100×3 mm I.D., CP Analytika, Hagenbrunn, Austria) were etched with 2 *M* ethanolic KOH at 60 °C overnight. After repeated washing with water, columns were dried in vacuo. Silanization was performed at 60 °C overnight using a mixture of pyridine/toluene and bicyclo-[2.2.1]hept-2-ene-5-methyldichlorosilane (molar ratio 3:2:1). Glass columns were washed consecutively with acetone, water, and ethanol and dried under reduced pressure at ambient temperature.

Monoliths were prepared according to a protocol described previously [18]. A brief summary of the procedure is as follows: A pretreated HPLC column was placed in a test-tube and cooled to 0 °C. Two different solutions (A, B) were prepared by standard Schlenk techniques and cooled to −30 °C. Solution A consisted of NBE, DMN-H6, and 2-propanol; solution B consisted of the toluene and the initiator (**1**). Solution A and B were merged and mixed for a few seconds. The polymerization mixture was then filled into the test-tube in a way that the borosilicate column was completely filled and covered. The column was then kept at 0 °C for 30 min and a further 30 min at ambient temperature. The test-tube was broken, and the polymeric material at the outside of the HPLC column was removed. The column was provided with end fittings and attached to a HPLC system. A (1:1) mixture of ethylvinyl ether and toluene was pumped through the column to remove any initiator.

## 3. Results and discussion

### 3.1. Separation mode and analytes

The dissociated phosphodiester groups in oligodeoxynucleotides and dsDNA are capable of electrostatic interaction with the electrical double layer created by amphiphilic ions adsorbed onto the hydrophobic stationary phase in ion-pair reversed-phase chromatographic mode of HPLC. It was reported that elevated temperatures of 45–50 °C lead to a better resolution [6,34], while higher temperatures ensure the partial or complete denaturation of dsDNA, which can be used to detect sequence variation in DNA [35]. Thus, it was anticipated that hydrophobic monoliths of NBE/DMN-$H_6$ are suitable for fractionating dsDNA, since they were also applicable to efficient oligodeoxynucleotide separations [18]. However, dsDNA separation is more difficult to achieve due to the wide size range of fragments present and the small relative differences between them in terms of size, charge, and charge density. It was assumed that monoliths of higher surface area-to-pore size ratio would suite separation of dsDNA fragments by allowing different adsorption tendencies especially for the high molecular fragments (434–587 bp). Consequently, it is expected to reach better capacity and selectivity factors, and a better resolution for these fragments.

Both single-stranded, 5′-phosphorylated oligodeoxythymidylic acids $p(dT)_{12-18}$ as well as a pBR322 DNA-Hae III digest (51–587 bp) as a representative sample for dsDNA were used for column evaluation in this investigation. The fragments of the digest have been clearly separated by capillary electrophoresis [36], by ion-exchange [5] and by ion-pair reversed-phase HPLC in polystyrene/divinylbenzene (PS/DVB) [20], as well in conventional columns packed with $C_{18}$ alkylated PS/DVB [2,34]. However, to the best of our knowledge, there are no reports on semi-preparative scale separations of dsDNA fragments on monolithic systems. Preliminary analysis showed that monoliths with optimum separation of $p(dT)_{12-18}$ showed good

separation of the first 12 fragments but broad and overlapping peaks for the 434- to 587-bp fragments. To overcome this limitation, various polymer combinations were investigated to improve their separation capabilities for larger dsDNA fragments.

### *3.2. Design of the monolithic column packing*

The composition of a metathesis-based monolith is affected by the copolymer-to-porogens mass ratio, the type of porogens, the polymerization temperature, and the phosphine content as well [18,21,22]. For the present separation problem, and besides affecting the backpressure (Fig. 1), both the total amount of the NBE+DMN-$H_6$ and the NBE/DMN-$H_6$ mass ratio turned out to have a great effect on resolution. Among the many compositions, a 1:1 mass ratio of NBE/DMN-$H_6$ was previously reported to be the optimum for good separation of oligodeoxynucleotides. In due consequence, this stoichiometry was used quasi as a starting point and tested for $p(dT)_{12-18}$ and dsDNA fragments in order to develop the optimum conditions for the separation of the latter. An example of the separation of $p(dT)_{12-18}$ on a 1:1 NBE/DMN-$H_6$ monolith is illustrated in Fig. 2a. Good resolution of the oligomers was achieved at a flow-rate of 2.0 ml/min, applying a gradient of 11–19% acetonitrile in 10 min.

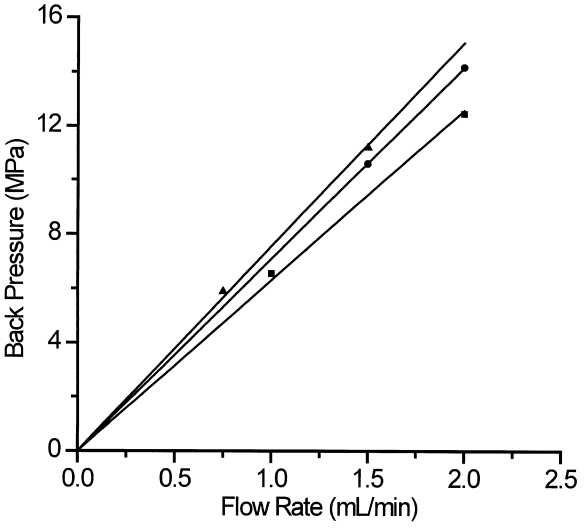


Fig. 1. Back pressure versus flow-rate at room temperature for a mobile phase consisting of 90% 0.1 *M* triethylammonium acetate (pH 7.0) and 10% acetonitrile. Monolith (■) 1a, (●) 2, (▲) 3a.

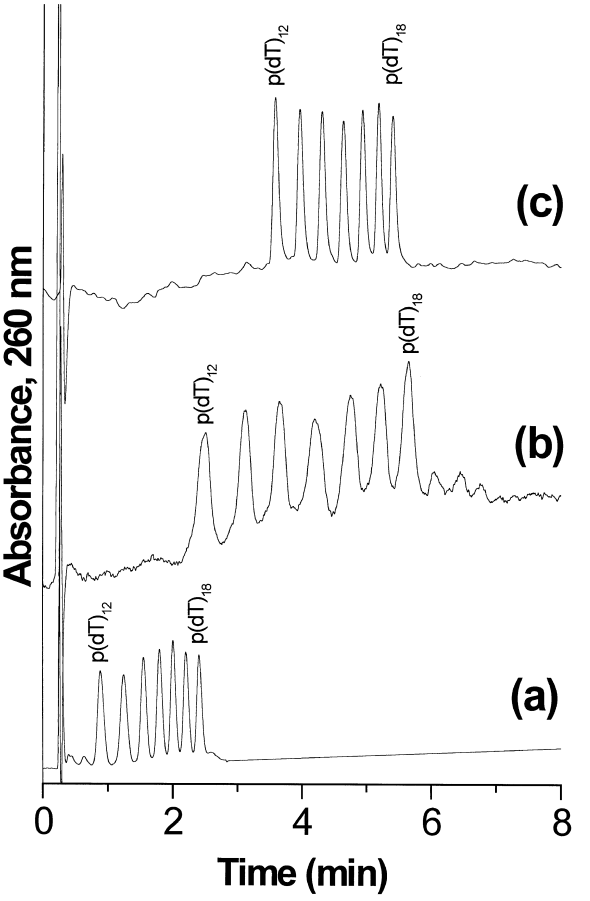


Fig. 2. Chromatograms of oligodeoxythymidylic acids on different monoliths, (a) 50%, (b) 56%, and (c) 60% NBE+DMN-$H_6$ (weight%). Column dimensions, 50×3 mm I.D.; mobile phase, 10–19% acetonitrile in 0.1 mol/l triethylammonium acetate in 10 min, flow-rate, 2 ml/min in (a) and (b), 1.5 ml/min in (c); temperature, room temperature; sample 1 μg $p(dT)_{12-18}$.

For the separation of pBR322 DNA-HaeIII fragments on the same monolith, a flow-rate of 2.0 ml/min applying a gradient of 4–10% acetonitrile within 1 min, followed by 10–16% in 14 min was chosen (Fig. 3a). The first 12 fragments (51–267 bp) were clearly separated. However, the higher molecular fragments (434–587 bp) were only partially separated (Fig. 3a). It was reported that longer fragments need either low flow-rate or shallower gradient to achieve good resolution [34]. Nevertheless, neither a shallower gradient slope at the same

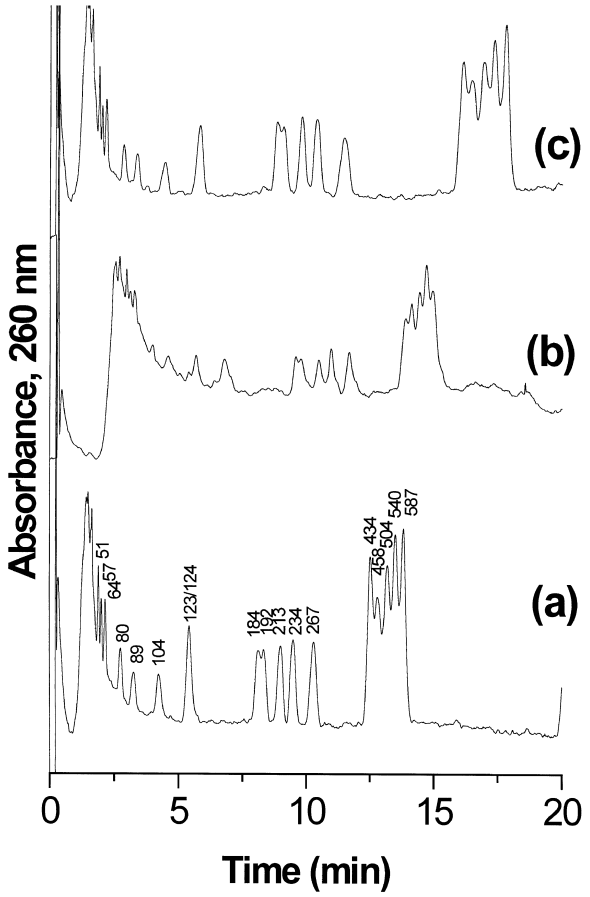


Fig. 3. Effect of gradient slope and flow-rate on the separation of dsDNA fragments separated on monolith consisting of 50% NBE+DMN-$H_6$. Mobile phase, A: 0.1 mol/l triethylammonium acetate, pH 7.0; B: 0.1 mol/l triethylammonium acetate, pH 7.0, 40% acetonitrile, gradient, (a and b) 10–24% B in 1 min, 24–40% B in 14 min, (c) 10–24% B in 1 min, 24–33% B in 9 min, 33–40% B in 10 min, flow rate, (a and c) 2.0 ml/min, (b) 1.5 ml/min; temperature, 50 °C; sample, 1.25 μg pBR322 DNA-Hae III digest.

flow-rate (16% change in acetonitrile in 20 min instead of 15 min, Fig. 3c,a) nor variation of the flow-rate (Fig. 3a,b) significantly influenced the resolution of the last five fragments of concern in the NBE/DMN-$H_6$ monolith.

The replacement of acetonitrile as gradient former by methanol resulted in faster elution of the DNA fragments as sharper peaks (chromatograms not shown). Consequently, methanol represents a stronger eluent than acetonitrile in ion-pair reversed-phase HPLC of dsDNA. Moreover, as can be deduced from the chromatogram of Fig. 4, the peak group of the 434- to 587-bp fragments was better resolved with the methanol gradient compared to the acetonitrile gradient (see Fig. 3).

### *3.3. Influence of morphology*

In order to further improve the chromatographic performance, we tried to enhance the specific surface area of the monolithic stationary phase. This may generally be accomplished by decreasing the pore size, which may be achieved by increasing the monomer to porogen mass ratio. For this purpose, three monoliths with a total (NBE+DMN-$H_6$) content of 50, 56, and 60%, respectively, were synthesized and employed in the separation of both $p(dT)_{12-18}$ and the pBR322 DNA-Hae III digest. A summary of the compositions of the monoliths is

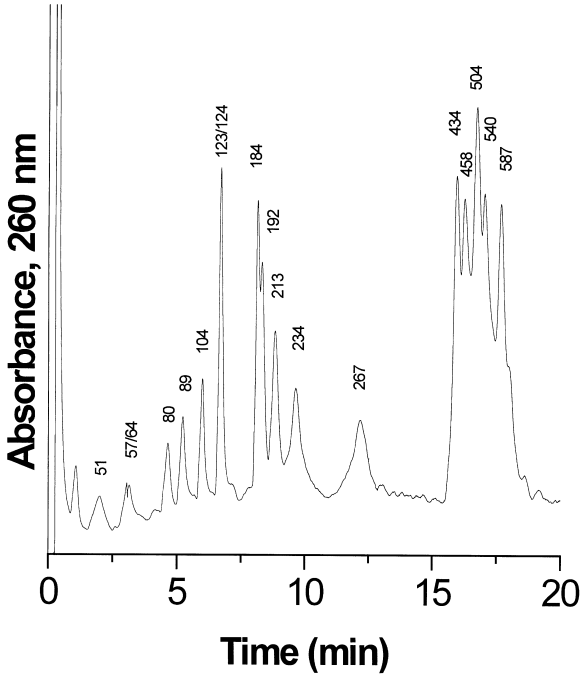


Fig. 4. Chromatogram of dsDNA fragments separated on a monolith consisting of 50% NBE+DMN-$H_6$. Mobile phase, A: 0.1 mol/l triethylammonium acetate, pH 7.0, 10% acetonitrile; B: 0.1 mol/l triethylammonium acetate, pH 7.0, 90% methanol, gradient 5% B isocratic for 2 min, 5–15% B in 10 min, and 15–20% B in 10 min, flow rate 1.5 ml/min; temperature 50 °C; sample, 2.5 μg pBR322 DNA-Hae III digest.

Table 1
Composition of polymerization mixtures for monoliths 1–3 and column back pressures at a flow of 1.5 and 2.0 ml/min, respectively

| Monolith | Composition[a] | Flow (ml/min)[b] | Backpressure (MPa) | $\sigma$ ($m^2/g$) | $\varepsilon_p$ (%) | $\varepsilon_z$ (%) | $\varepsilon_t$ (%) | $V_p$ (ml) |
|---|---|---|---|---|---|---|---|---|
| 1a | 25:25:40:10 | 2.0 | 12.7 | 97 | 59 | 22 | 81 | 0.42 |
| 1b | 24:26:40:10 | 2.0 | 10.0 | | | | | |
| 1c | 23:27:40:10 | 2.0 | 8.4 | | | | | |
| 2 | 28:28:35:9 | 2.0 | 14.3 | | | | | |
| 3a | 30:30:32:8 | 1.5 | 11.3 | 191 | 50 | 5 | 54 | 0.35 |
| 3b | 29:31:32:8 | 1.5 | 10.7 | | | | | |
| 3c | 28:32:32:8 | 1.5 | 10.4 | | | | | |

[a] NBE/DMN-$H_6$/2-propanol/toluene.
[b] Mobile phase, 0.1 mol/l triethylammonium acetate, pH 7.0, 10% acetonitrile.

given in Table 1. The chromatograms shown in Fig. 2a–c clearly demonstrate that a 60% ratio of monomers-to-porogens is the most suitable for efficient oligonucleotide separation, although the differences in resolving power between the different monoliths were rather subtle (Table 2). Nevertheless, the higher retentivity of the 60% monomers-to-porogens stationary phase is indicative of a higher specific surface area in the monolith. The pore size was known from previous experiments [18,21,22] and confirmed in a qualitative way by the backpressure experienced for each column under identical conditions (Table 1). It was noticed that the backpressure increased upon increasing the mass content of the monomers. The 60% NBE+DMN-$H_6$ could not be operated at 2.0 ml/min flow-rate because of exceeding the pressure limit of the HPLC instrument.

In the case of dsDNA, a different influence of monolith morphology on separation performance was observed. Table 3 collects the retention times and resolution values of the 17 detected fragments of the pBR322 DNA-HaeIII digest. Evaluation of the monoliths 1, 2, and 3 for pBR322 fragments revealed that the first 12 fragments were clearly separated on all three systems, whereas the last five fragments were still only partially separated. A comparison between these columns concerning the separation of the fragments reveals superior separation on the system with 50% monomers-to-porogens mass ratio. This suggests that the free access of the large dsDNA

Table 2
Retention times and resolution values for the separation of $p(dT)_{12-18}$ on various monoliths

| Fragment | Recipe[a] | | | | | |
|---|---|---|---|---|---|---|
| | 25:25:40:10 | | 28:28:35:9 | | 30:30:32:8 | |
| | $t_R$ | $R_s$ | $t_R$ | $R_s$ | $t_R$ | $R_s$ |
| $pdT_{12}$ | 0.89 | 1.44 | 2.51 | 1.14 | 3.58 | 1.59 |
| $pdT_{13}$ | 1.25 | 1.28 | 3.13 | 1.16 | 3.957 | 1.43 |
| $pdT_{14}$ | 1.55 | 1.18 | 3.65 | 1.10 | 4.30 | 1.42 |
| $pdT_{15}$ | 1.80 | 1.05 | 4.20 | 1.09 | 4.63 | 1.32 |
| $pdT_{16}$ | 2.01 | 1.01 | 4.75 | 1.03 | 4.93 | 1.15 |
| $pdT_{17}$ | 2.21 | 1.03 | 5.22 | 0.999 | 5.18 | 1.01 |
| $pdT_{18}$ | 2.41 | – | 5.65 | – | 5.399 | – |

[a] Given as NBE/DMN-$H_6$/2-propanol/toluene.

Table 3
Retention times and resolution values for restriction fragments from pBR322 DNA-HaeIII fragments on various monoliths

| Fragment | Recipe[a] | | | | | |
|---|---|---|---|---|---|---|
| | 25:25:40:10 | | 28:28:35:9 | | 30:30:32:8 | |
| | $t_R$ | $R_s$ | $t_R$ | $R_s$ | $t_R$ | $R_s$ |
| 51 | 1.90 | 0.04 | 5.02 | 0.33 | 4.25 | 0.44 |
| 57 | 1.91 | 0.84 | 5.25 | 0.39 | 4.47 | 0.43 |
| 64 | 2.16 | 1.11 | 5.49 | 0.89 | 4.73 | 0.80 |
| 80 | 2.75 | 0.68 | 6.21 | 0.46 | 5.46 | 0.34 |
| 89 | 3.26 | 1.27 | 6.61 | 0.61 | 5.78 | 0.64 |
| 104 | 4.23 | 1.69 | 7.16 | 0.76 | 6.35 | 0.49 |
| 123,124 | 5.42 | 4.41 | 7.84 | 1.57 | 6.87 | 1.17 |
| 184 | 8.12 | 0.01 | 9.40 | 0.05 | 8.21 | 0.47 |
| 192 | 8.34 | 1.24 | 9.44 | 0.93 | 8.59 | 0.43 |
| 213 | 9.00 | 0.9 | 9.81 | 0.63 | 8.82 | 0.46 |
| 234 | 9.50 | 1.35 | 10.07 | 0.72 | 9.15 | 1.45 |
| 267 | 10.30 | 3.73 | 10.47 | 2.73 | 10.21 | 0.49 |
| 434 | 10.52 | 0.47 | 11.77 | 0.32 | 10.44 | 0.14 |
| 458 | 12.80 | 0.63 | 11.87 | 0.54 | 10.49 | 0.14 |
| 504 | 13.19 | 0.52 | 12.06 | 0.48 | 10.61 | – |
| 540 | 13.50 | 0.57 | 12.23 | 0.39 | – | – |
| 587 | 13.82 | – | 12.40 | – | – | – |

[a] Given as NBE/DMN-$H_6$/2-propanol/toluene.

molecules into the smaller pores formed at higher monomers-to-porogen ratio is sterically hindered. Obviously, this was not noticed for the lower mass components such as the first 12 fragments of pBR322 DNA and the sample of $p(dT)_{12-18}$. Similar observations have been previously made for high molecular mass proteins [18].

### *3.4. Influence of glycerol as mobile phase additive*

Based on a recent report describing the influence of glycerol on dsDNA separations by capillary electrophoresis, we investigated as to what extent this method was applicable to HPLC separations of dsDNA on monolithic systems. According to these authors, organic modifiers possessing hydroxyl groups, including glycerol, methanol, and mannitol, enhanced the electrophoretic separation of dsDNA fragments due to their influence on conformation and the effective charge-to-mass ratio of the dsDNA fragments [37]. This improvement in separation was attributed to the interaction of these compounds with dsDNA. In fact, the chromatograms using methanol as gradient former showed some improvement in the peak shapes of the smaller dsDNA fragments, but it did not improve the separation of the large fragments of 434–587 bp. Based on the findings in capillary electrophoresis of dsDNA, glycerol was anticipated to be an appropriate additive to improve the separation especially of large dsDNS fragments.

Chromatograms of dsDNA separations under the same conditions as in Fig. 3a were performed with the addition of varying concentrations of glycerol as mobile phase additive. Upon addition of 1-, 2-, 3-, 4-, 5- and 10% glycerol to the mobile phase, better resolution and sharper peaks were observed in general, with an optimum at 4% glycerol (chromatograms not shown). Moreover, the addition of glycerol to the mobile phase enabled the application of gradient conditions that did not show the broad system peak appearing between 1.5 and 3 min in the blank gradient (Fig. 3a–c).

Consequently, a simple single step gradient of 4–24% acetonitrile in 15 min was applied at 4% glycerol (Fig. 5a). Good resolution was demonstrated with sharper peaks and the separation of all fragments was accomplished in less than 10 min, even the last five fragments were sufficiently, yet not

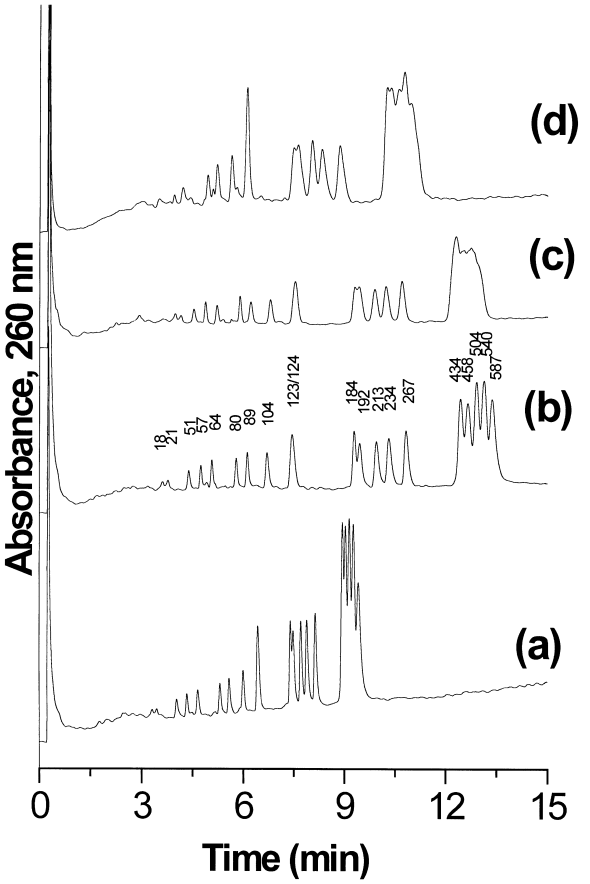


Fig. 5. Influence of glycerol as a mobile phase additive in the separation of dsDNA fragments. Mobile phase, A: 0.1 mol/l triethylammonium acetate, pH 7.0, (a and b) 4%, (c) 1%, and (d) 10% glycerol; B: 0.1 mol/l triethylammonium acetate, pH 7.0, 40% acetonitrile, (a and b) 4%, (c) 1%, and (d) 10% glycerol; gradient (a) 10–60% B in 15 min; (b, c, and d) 10–25% B in 5 min, 25–45% B in 12 min, flow-rate 2.0 ml/min; temperature, 50 °C; sample, 0.75 μg pBR322 DNA-Hae III digest.

baseline separated. A two-step gradient with shallower slope, namely 4–10% acetonitrile in 5 min then 10–18% B in 12 min, was attempted with 4% (v/v) glycerol, resulting in the separation shown in Fig. 5b. With this gradient, the resolution of the larger fragments, e.g. 184/192 and 434–587 bp was significantly improved. Moreover, the 18- and 21-bp fragments could be spotted applying this gradient where they could not be seen before due to gradient and solvent interference with the base line.

The same gradient was examined with 1 and 10%

glycerol as mobile phase additive (Fig. 5c,d) but it was noticed that 4% glycerol demonstrated the best resolution. With 10% glycerol, the resolution of the dsDNA fragments was considerably deteriorated and the peaks of the 18- to 64-bp fragments disappeared, while 1% glycerol was not enough to enhance the separation of the large DNA fragments. The enhancement in resolution with 4% glycerol, especially for the 18-, 21-, 184-, 192-, and 434- to 587-bp fragments could be due to establishing better differential interaction of the phosphodiester-groups with the amphiphiles adsorbed onto the polymer surface. This might be a consequence of the conformational change in these fragments induced by glycerol, which causes stretching of the DNA double helix [37]. Moreover, the replacement of solvating water molecules by the less polar glycerol molecules enhances intramolecular electrostatic repulsive forces between adjacent phosphate groups and enforces the chain stretching. A second important impact of glycerol on dsDNA is the reduction of the effective charge-to-mass ratio due to replacement of water with glycerol as solvating molecules on the nucleobases. These two factors augment the differential interaction between the surface potential at the stationary phase and the dsDNA active sites, resulting in height chromatographic selectivity especially for the large dsDNA fragments.

The amount of DNA material that could be loaded onto a 100×3 mm I.D. column without serious loss in separation efficiency was about 2.5 μg. Although this amount will suffice for most experiments involving nucleic acids, the process can be readily upscaled using monolithic columns of larger dimensions. Because of the ease of preparation and the high reproducibility of synthesis, column inner diameters of 7–10 mm can be readily reached, representing a 5- to 11-fold increase in sample throughput.

## 4. Conclusions

Hydrophobic monoliths prepared from norborn-2-ene (NBE) and 1,4,5,8,8a-hexahydro-1,4,5,8, *exo, endo*-dimethanonapthalene (DMN-$H_6$) are applicable to the micropreparative scale separation of dDNA. The monolith recipe of 25:25:40:10 (NBE/DMN-$H_6$/2-propanol/toluene) showed the best resolution of intermediate and high molecular mass nucleic acids. This polymer composition furnished a satisfactory bed for micropreparative separation and purification of dsDNA. Thus, amounts of up to 2.5 μg dsDNA may be separated on a 100×3 mm I.D. monolith in one single run in less than 15 min. Monolithic column efficiency is highly dependent on the morphology of the monolithic bed. While a small increase in the monomer-to-porogen mass ratio does not affect the resolution of low molecular mass analytes such as oligonucleotides, it seems to have a crucial influence on dsDNA separations. Thus, a 1:1 ratio of the co-monomers NBE/DMN-$H_6$ generally gave better separations. Instead of acetonitrile, methanol is an alternative gradient former for ion-pair reversed-phase HPLC that is more suitable for the separation of large nucleic acids. The chromatograms of pBR322 DNA-Hae III restriction fragments showed smoother base lines and sharper peaks of fragments of 80–267 bp with methanol. The use of 4% (v/v) glycerol was found to be very advantageous in separating dsDNA as it improves the resolution leading to sharper peaks and better selectivity.

## References

[1] M. Gilar, D.L. Smisek, A.S. Cohen, in: M. Gilar, D.L. Smisek, A.S. Cohen (Eds.), Nucleic Acids and Their Constituents, Elsevier, Amsterdam, 1998.
[2] C.G. Huber, J. Chromatogr. A 806 (1998) 3.
[3] C. Horváth, B.A. Preiss, S.R. Lipsky, Anal. Chem. 39 (1967) 1422.
[4] E. Westman, S. Eriksson, T. Laas, P.-A. Pernemalm, S.-E. Skold, Anal. Biochem. 166 (1987) 158.
[5] Y. Kato, M. Sasaki, T. Hashimoto, T. Murotsu, S. Fukushige, K. Matsubara, J. Chromatogr. 265 (1983) 342.
[6] C.G. Huber, P.J. Oefner, G.K. Bonn, Chromatographia 37 (1993) 653.
[7] J.J. Kirkland, F.A. Truszkowski, C.H. Dilks Jr., G.S. Engel, J. Chromatogr. A 890 (2000) 3.
[8] L.C. Hansen, R.E. Sievers, J. Chromatogr. 99 (1974) 123.
[9] S. Hjertén, Y.-M. Li, J.-L. Liao, J. Mohammad, K. Nakazato, G. Pettersson, Nature 356 (1992) 810.
[10] F. Svec, J.M.J. Fréchet, Anal. Chem. 64 (1992) 820.
[11] H. Minakuchi, K. Nakanishi, N. Soga, N. Ishizuka, N. Tanaka, Anal. Chem. 68 (1996) 3498.
[12] M. Petro, F. Svec, J.M.J. Fréchet, J. Chromatogr. A 752 (1996) 59.
[13] J.J. Meyers, A.I. Liapis, J. Chromatogr. A 852 (1999) 3.

[14] D. Josic, A. Strancar, Ind. Eng. Chem. Res. 38 (1999) 333.
[15] C. Viklund, F. Svec, J.M.J. Fréchet, K. Irgum, Chem. Mater. 8 (1996) 744.
[16] E.C. Peters, F. Svec, J.M.J. Fréchet, Chem. Mater. 9 (1997) 1898.
[17] R.R. Deshmukh, T.N. Warner, F. Hutchison, M. Murphy, W.E. Leitch II, P. De Leon, D.L. Cole, G.S. Srivatsa, Y.S. Sanghvi, J. Chromatogr. A 890 (2000) 179.
[18] B. Mayr, R. Tessadri, E. Post, M.R. Buchmeiser, Anal. Chem. 73 (2001) 4071.
[19] A. Premstaller, H. Oberacher, W. Walcher, A.M. Timperio, L. Zolla, J.-P. Chervet, N. Cavusoglu, A. van Dorsselaer, C.G. Huber, Anal. Chem. 73 (2001) 2390.
[20] A. Premstaller, H. Oberacher, C.G. Huber, Anal. Chem. 72 (2000) 4386.
[21] F. Sinner, M.R. Buchmeiser, Macromolecules 33 (2000) 5777.
[22] F. Sinner, M.R. Buchmeiser, Angew. Chem. 112 (2000) 1491.
[23] A.E. Rodrigues, B.J. Ahn, A. Zoulalian, AIChE J. 28 (1982) 541.
[24] A.E. Rodrigues, J.C. Lopes, Z.P. Lu, J.M. Loureiro, M.M. Dias, J. Chromatogr. 590 (1992) 93.
[25] A.E. Rodrigues, A.M.D. Ramos, J.M. Loureiro, M. Diaz, Z.P. Lu, Chem. Eng. Sci. 47 (1992) 4405.
[26] A.E. Rodrigues, J.M. Loureiro, C. Chenou, M.R. de la Vega, J. Chromatogr. A 664 (1995) 233.
[27] A.E. Rodrigues, J. Chromatogr. B 699 (1997) 47.
[28] H. Oberacher, C.G. Huber, Trends Anal. Chem. (2002) in press.
[29] E.C. Peters, F. Svec, J.M.J. Fréchet, Adv. Mater. 11 (1999) 1169.
[30] D. Josic, A. Buchacher, A. Jungbauer, J. Chromatogr. B 752 (2001) 191.
[31] M.B. Tennikov, N.V. Gazdina, T.B. Tennikova, F. Svec, J. Chromatogr. A 798 (1998) 55.
[32] C.D. Wood, A.I. Cooper, Macromolecules 34 (2001) 5.
[33] J.K. Stille, D.A. Frey, J. Am. Chem. Soc. 81 (1959) 4273.
[34] C.G. Huber, P.J. Oefner, G.K. Bonn, Anal. Chem. 67 (1995) 578.
[35] W. Xiao, P.J. Oefner, Hum. Mutat. 17 (2001) 439.
[36] A. Paulus, D. Hüsken, Electrophoresis 14 (1993) 27.
[37] D. Liang, L. Song, Z. Chen, B. Chu, J. Chromatogr. A 931 (2001) 163.