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High-Performance Liquid Chromatography−Electrospray Ionization Mass Spectrometry of Single- and Double-Stranded Nucleic Acids Using Monolithic Capillary Columns

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Monolithic capillary columns were prepared by copolymerization of styrene and divinylbenzene inside a 200-μm i.d. fused silica capillary using a mixture of tetrahydrofuran and decanol as porogen. With gradients of acetonitrile in 100 mM triethylammonium acetate, the synthesized columns allowed the rapid and highly efficient separation of single-stranded oligodeoxynucleotides and double-stranded DNA fragments by ion-pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC). Compared with capillary columns packed with micropellicular, octadecylated poly-(styrene/divinylbenzene) particles, an improvement in column performance of approximately 40% was obtained, enabling the analysis of an 18-mer oligodeoxynucleotide with a column efficiency of more than 190 000 plates per meter. The chromatographic separation system was on-line-coupled to electrospray ionization mass spectrometry (ESI-MS). To improve the mass spectrometric detectabilities, 25 mM triethylammonium bicarbonate was utilized as an ion-pair reagent at the cost of only little reduction in separation performance and acetonitrile was added postcolumn as the sheath liquid through the triaxial electrospray probe. High-quality mass spectra of femtomole amounts of 3-mer to 80-mer oligodeoxynucleotides were recorded showing very little cation adduction. Double-stranded DNA fragments ranging in size from 51 to 587 base pairs were separated and detected by IP-RP-HPLC-ESI-MS. Accurate mass determination by deconvolution of the mass spectra was feasible for DNA fragments up to the 267-mer with a molecular mass of 165 019, whereas the spectra of longer fragments were too complex for deconvolution because of incomplete separation due to overloading of the column. Finally, on-line IP-RP-HPLC tandem MS was applied to the sequencing of short oligodeoxynucleotides.

For more than 30 years, columns packed with microparticulate sorbents have been successfully applied as separation media in high-performance liquid chromatography (HPLC).[1] Despite many advantages, HPLC columns packed with microparticulate, porous stationary phases have some limitations, such as the relatively large void volume between the packed particles and the slow diffusional mass transfer of solutes into and out of the stagnant mobile phase present in the pores of the separation medium.[2,3] One approach to alleviate the problem of restricted mass transfer and intraparticular void volume is the concept of monolithic chromatographic beds, where the separation medium consists of a continuous rod of a rigid, porous polymer which has no interstitial volume but only internal porosity consisting of micropores and macropores.[4−8] Because of the absence of intraparticular volume, all of the mobile phase is forced to flow through the pores of the separation medium.[9] According to theory, mass transport is enhanced by such convection[10,11] and has a positive effect on chromatographic efficiency.[12] Monolithic chromatographic beds are usually prepared by polymerization of suitable monomers and porogens in a stainless steel or fused silica tube which acts as a mold.[6,13,14] The porous structure is achieved as a result of the phase separation which occurs during the polymerization of a monomer or monomer mixture containing appropriate amounts of both a cross-linking monomer and a porogenic solvent or a mixture of porogenic solvents.[15,16]

Miniaturized chromatographic separation systems applying capillary columns of 10−500-μm inner diameter are frequently the method of choice for the separation and characterization of biopolymer mixtures, when the amount of available sample is limited. The concept of monolithic stationary phases is especially

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favorable for the fabrication of capillary columns, because the immobilization of the monolith at the capillary wall eliminates the necessity of preparing a tiny retaining frit,[17] which is one of the more tedious and difficult-to-control steps during the manufacture of packed-bed capillary columns.[18] Moreover, the possibility of direct on-line conjugation of capillary HPLC to mass spectrometry makes available highly valuable information about the structure and identity of the separated compounds.[19] Electrospray ionization mass spectrometry (ESI-MS), by virtue of the multiple charging of biopolymers and the very soft ionization process, has become one of the most important mass spectrometric techniques for the analysis of nucleic acids.[20] Nevertheless, the success of ESI-MS for the characterization of nucleic acids largely depends on the purity of the sample that is introduced into the mass spectrometer.[21] The major difficulties arise due to the tendency of nucleic acids to form quite stable adducts with cations, resulting in mass spectra of poor quality.[22,23] The on-line sample preparation of nucleic acids by chromatographic separation prior to ESI-MS is very attractive because it not only removes cations from nucleic acid samples but also fractionates nucleic acids in mixtures that are too complex for direct-infusion ESI-MS.

Although continuous-bed columns have proved to be efficient for the separation of biopolymers such as peptides[24,25] and proteins,[6,13,26] the concept of continuous beds has not successfully been transferred to the separation of nucleic acids by ion-pair reversed-phase HPLC (IP-RP-HPLC).[27] We have utilized 200-μm i.d. capillary columns packed with micropellicular polymer beads for the chromatographic separation and mass spectrometric characterization of nucleic acids up to 40 nucleotides by on-line IP-RP-HPLC-ESI-MS.[28] Therefore, our goal in this study was to investigate the applicability of monolithic chromatographic beds to high-resolution separations of nucleic acids by IP-RP-HPLC and to extend the size range of the separated and detected nucleic acids. It is shown that both single- and double-stranded nucleic acids in a size range of 3 nucleotides (nt) to 600 base pairs (bp) can be separated with high efficiency and identified after separation by ESI-MS.

EXPERIMENTAL SECTION

Chemicals and Oligodeoxynucleotide Samples. Acetonitrile (HPLC gradient grade), divinylbenzene (synthesis grade), methanol (HPLC gradient grade), styrene (synthesis grade), and tetrahydrofuran (analytical reagent grade) were obtained from Merck (Darmstadt, Germany). Styrene and divinylbenzene were distilled before use. Acetic acid (analytical reagent grade), azobisisobutyronitrile (synthesis grade), decanol (synthesis grade), and triethylamine (p.a.) were purchased from Fluka (Buchs, Switzerland). A 1.0 M stock solution of triethylammonium acetate (TEAA) was prepared by dissolving equimolar amounts of triethylamine and acetic acid in water. A 0.50 M stock solution of triethylammonium bicarbonate (TEAB) was prepared by passing carbon dioxide gas (AGA, Vienna, Austria) through a 0.50 M aqueous solution of triethylamine at 5 °C until pH 8.4−8.9 was reached. For preparation of all aqueous solutions, high-purity water (Epure, Barnstead Co., Newton, MA) was used. The standards of phosphorylated and nonphosphorylated oligodeoxynucleotides ($p(dT)_{12-18}$, $p(dT)_{12-18}$, $p(dT)_{19-24}$, $p(dT)_{25-30}$) were purchased as sodium salts from Pharmacia (Uppsala, Sweden) or Sigma-Aldrich (St. Louis, MO). The synthetic oligodeoxynucleotides $(dT)_{24}$ (M_r 7 238.71), a 5′-dimethoxytritylated 5-mer (DMTr-ATGCG, M_r 1805.42), and an 80-mer (CCCCAGTGCT GCAATGATAC CGCGAGACCC ACGCTCACCG GCTCCAGATT TATCAGCAAT AAACCAGCCA GCCGGAAGGG, M_r 24 527.17) were ordered from Microsynth (Balgach, Switzerland) and used without further purification. The size standard of double-stranded DNA restriction fragments (pBR322 DNA-*Hae* III digest) was purchased from Sigma Aldrich.

Preparation of Monolithic and Packed-Bed Capillary Columns.[29] Polyimide-coated fused silica capillary tubing of 350-μm o.d. and 200-μm i.d. was obtained from Polymicro Technologies (Phoenix, AZ). A 1-m piece of fused silica capillary tubing was silanized with 3-(trimethoxysilyl)propyl methacrylate according to the procedure published in ref 30[30] in order to ensure immobilization of the monolith at the capillary wall. Then, a 300-mm piece of the silanized capillary was filled, using a plastic syringe, with a mixture comprising 50 μL of styrene, 50 μL of divinylbenzene, 130 μL of decanol, 20 μL of tetrahydrofuran, and 10 mg/mL of azobisisobutyronitrile. The mixture was polymerized at 70 °C for 24 h. After polymerization, the capillary was extensively flushed with acetonitrile at a flow rate of 5.0 μL/min and finally cut into 60-mm-long pieces. Octadecylated PS-DVB particles (PS-DVB−C18) were synthesized as published in the literature.[31] The PS-DVB-C18 stationary phase has been commercialized as DNASep by Transgenomic Inc. (Santa Clara, CA). Packed-bed capillary columns were prepared according to the procedure described in ref 18.[18]

High-Performance Liquid Chromatography. The HPLC system consisted of a low-pressure gradient micro pump (model Rheos 2000, Flux Instruments, Karlskoga, Sweden) controlled by a personal computer, a vacuum degasser (Knauer, Berlin, Germany), a column thermostat made from 3.3-mm o.d. copper tubing which was heated by means of a circulating water bath (model K 20 KP, Lauda, Lauda-Königshofen, Germany), a microinjector (model C4-1004, Valco Instruments Co. Inc., Houston, TX) with a 200- or 500-nL internal sample loop, a variable wavelength detector

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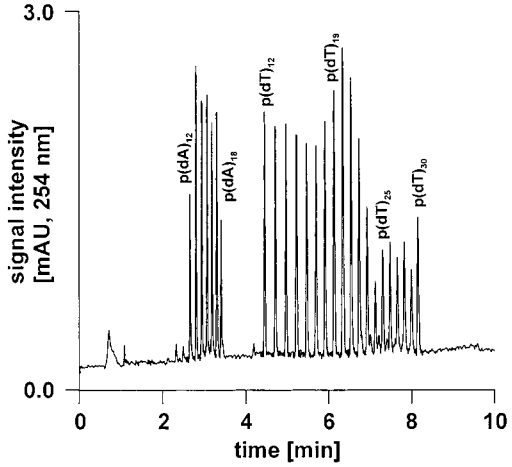

Figure 1. High-resolution capillary IP-RP-HPLC separation of phosphorylated oligodeoxynucleotide ladders in a monolithic capillary column. Column, continuous PS-DVB, 60 × 0.20 mm i.d.; mobile phase, (A) 100 mM TEAA, pH 6.97, (B) 100 mM TEAA, pH 6.97, 20% acetonitrile; linear gradient, 15−45% B in 3.5 min, 45−55% B in 2.5 min, 55−65% B in 4.0 min; flow-rate, 2.5 μL/min; temperature, 50 °C; detection, UV, 254 nm; sample, $p(dA)_{12-18}$, $p(dT)_{12-30}$, 40−98 fmol of each oligodeoxynucleotide.

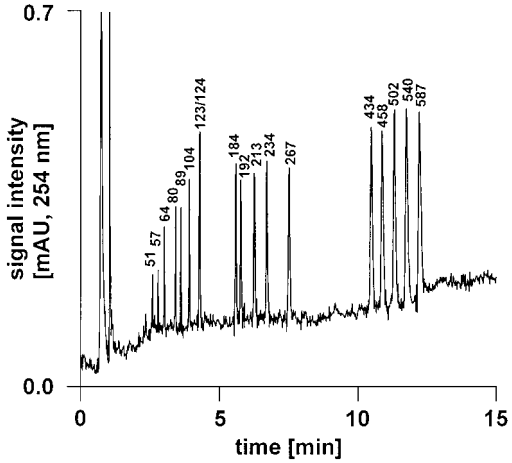

Figure 2. High-resolution capillary IP-RP-HPLC separation of a mixture of double-stranded DNA fragments in a monolithic capillary column. Column, continuous PS-DVB, 60 × 0.20 mm i.d.; mobile phase, (A) 100 mM TEAA, pH 7.00, (B) 100 mM TEAA, pH 7.00, 20% acetonitrile; linear gradient, 35−75% B in 3.0 min, 75−95% B in 12.0 min; flow-rate, 2.2 μL/min; temperature, 50 °C; detection, UV, 254 nm; sample, pBR322 DNA-*Hae* III digest, 1.81 fmol of each fragment.

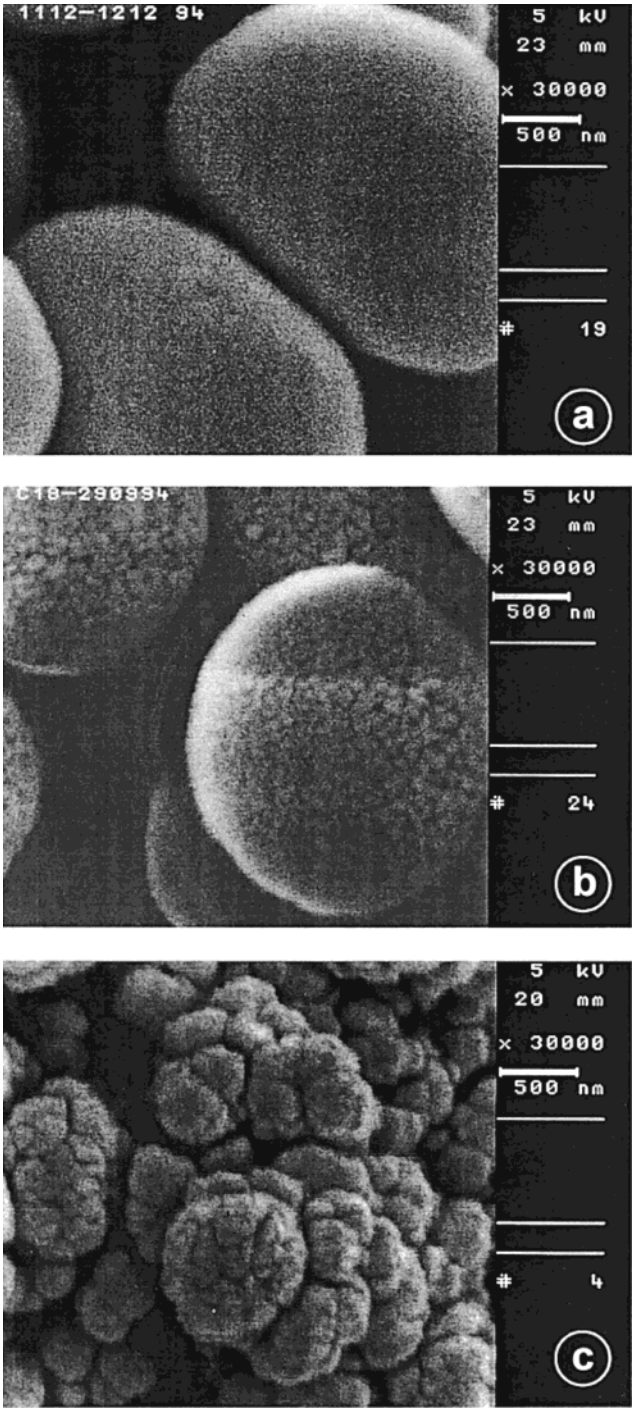

Figure 3. Scanning electron micrographs of (a) underivatized PS-DVB particles, (b) octadecylated PS-DVB particles, and (c) an underivatized PS-DVB monolith.

(model UltiMate UV detector, LC Packings, Amsterdam, Netherlands) with a Z-shaped capillary detector cell ULT-UZ-N-10, 3nL cell, LC Packings), and a PC-based data system (Chromeleon 4.30, Dionex-Softron, Germering, Germany).

Electrospray Ionization Mass Spectrometry and Coupling with Capillary Liquid Chromatography. ESI-MS was performed on a Finnigan MAT LCQ quadrupole ion trap mass spectrometer (Finnigan MAT, San Jose, CA, used in Figures 4−7) or a Finnigan MAT TSQ 7000 triple quadrupole mass spectrometer (used in Figure 3) equipped with an electrospray ion source. The capillary column was directly connected to the spray capillary (fused silica, 105-μm o.d., 40-μm i.d., Polymicro Technologies) by means of a microtight union (Upchurch Scientific, Oak Harbor, WA). A syringe pump equipped with a 250-μL glass syringe (Unimetrics, Shorewood, IL) was used for continuous-infusion experiments and for pumping sheath liquid. For analysis with pneumatically assisted ESI, an electrospray voltage of 3.2−3.7 kV and a nitrogen sheath gas flow of 20−30 arbitrary units (LCQ) or 28−33 psi (TSQ) were employed. The temperature of the heated capillary was set to 200 °C. Total ion chromatograms and mass spectra were recorded on a personal computer with the LCQ Navigator software version 1.2 or on a DEC-Alpha 3000 workstation with the ICIS software version 8.3.0 (Finnigan). Mass calibration and coarse tuning was performed in the positive-ion mode by direct infusion of a solution

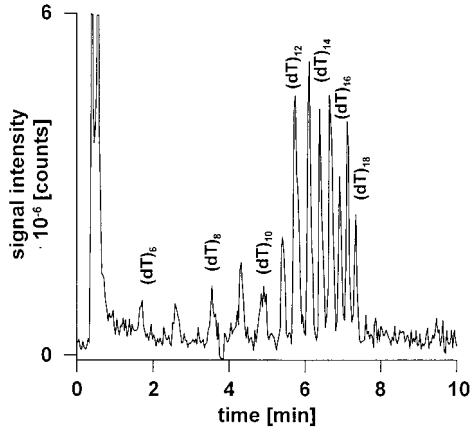

Figure 4. Separation and mass analysis of a series of oligodeoxythymidylic acids. Column, continuous PS-DVB, 60 × 0.20 mm i.d.; mobile phase, (A) 10 mM TEAA, pH 7.00, (B) 10 mM TEAA, pH 7.00, 20% acetonitrile; linear gradient, 20−60% B in 10.0 min; flow-rate, 3.2 μL/min; temperature, 50 °C; scan, 800−2000 amu in 2 s; electrospray voltage, 3.8 kV; sheath gas, 34 psi N_2; sheath liquid, acetonitrile; flow rate, 3.0 μL/min; sample, $(dT)_{6-18}$, 50 ng.

of caffeine (Sigma, St. Louis, MO), methionyl-arginyl-phenylalanyl-alanine (Finnigan), and Ultramark 1621 (Finnigan). Fine tuning for ESI-MS of oligodeoxynucleotides in the negative-ion mode was performed by infusion of 3.0 μL/min of a 20 pmol/μL solution of $(dT)_{24}$ in 25 mM aqueous TEAB containing 20% acetonitrile (v/v). A sheath flow of 3.0 μL/min acetonitrile was added through the triaxial electrospray probe. For all direct infusion experiments, cations present in the oligodeoxynucleotide samples were removed by on-line cation exchange using a 20 × 0.50 mm i.d. cation-exchange microcolumn packed with 38−75-μm Dowex 50 WX8 particles (BioRad, Richmond, CA).[23] For IP-RP-HPLC-ESI-MS analysis, oligodeoxynucleotides and DNA fragments were injected without prior cation removal.

RESULTS AND DISCUSSION

Performance of the Monolithic Capillary Columns for Oligodeoxynucleotide- and dsDNA Separations. Since slow mass transfer kinetics is often the limiting factor for speed and efficiency in biopolymer separations, the enhancement of intraparticular mass transfer is particularly important for the rapid chromatographic separation of large molecules having low diffusivities such as nucleic acids.[32] One way to circumvent intraparticular diffusion is the complete elimination of the support pores resulting in stationary phases of the micropellicular configuration.[27,33,34] The main advantage of such sorbents rests with the rapid mass transfer, because the only remaining particle-based diffusion limitations are in a thin layer at the surface of nonporous particles. This allows the rapid separation of oligodeoxynucleotides and DNA fragments within a fraction of the time that is required with conventional porous packing materials.[27,35] Consequently, to maintain the separation speed and performance upon the transition from a packed to a monolithic chromatographic bed, the synthesis of a monolith has to be tuned such that its morphology resembles that of a chromatographic bed formed by nonporous particles with relatively large channels for convective flow and without any micropores. Therefore, we used decanol and tetrahydrofuran as porogens with relatively poor solvency for poly(styrene/divinylbenzene) (PS-DVB), resulting in the formation of large channels in the monolithic bed.

Following polymerization, extensive washing with acetonitrile, and equilibration with 100 mM TEAA-5.0% acetonitrile solution,

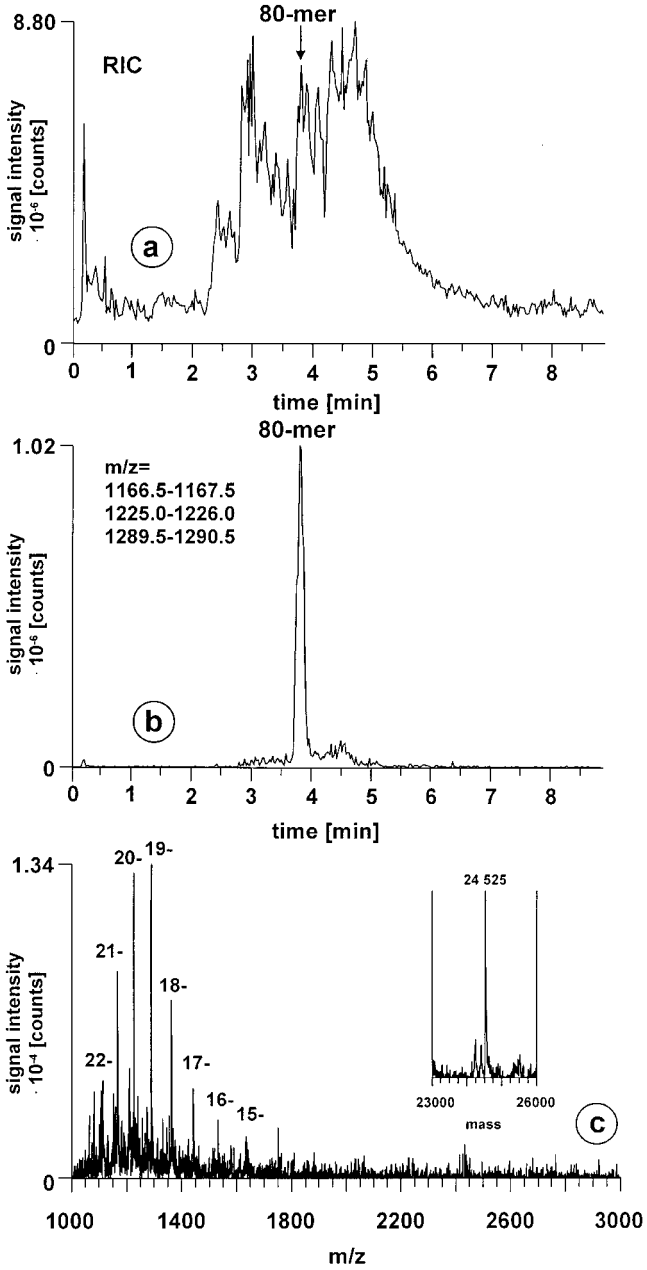

Figure 5. Quality control of a synthetic 80-mer oligodeoxynucleotide. Column, continuous PS-DVB, 60 × 0.20 mm i.d.; mobile phase, (A) 25 mM TEAB, pH 8.40, (B) 25 mM TEAB, pH 8.40, 20% acetonitrile; linear gradient, 20−100% B in 15 min; flow-rate, 3.0 μL/min; temperature, 50 °C; scan, 1000−3000 amu; electrospray voltage, 3.2 kV; sheath gas, 30 units; sheath liquid, acetonitrile; flow rate, 3.0 μL/min; sample, 5.0 pmol raw product.

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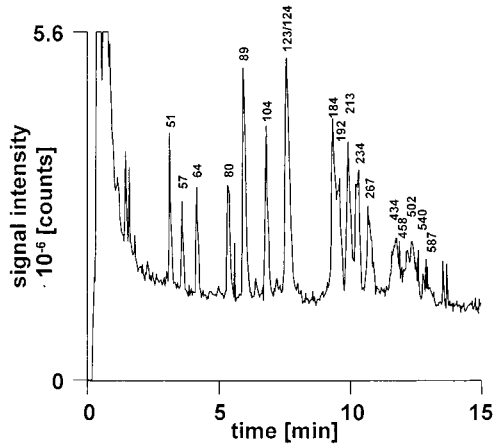

Figure 6. Separation and mass analysis of double-stranded DNA fragments from a restriction digest of the pBR322 plasmid. Column, continuous PS-DVB, 60 × 0.20 mm i.d.; mobile phase, (A) 25 mM TEAB, pH 8.40, (B) 25 mM TEAB, pH 8.40, 20% acetonitrile; linear gradient, 15−30% B in 3.0 min, followed by 30−50% B in 12 min; flow rate, 2.8 μL/min; temperature, 40 °C; scan, 1000−3000 amu; electrospray voltage, 3.2 kV; sheath gas, 32 units; sheath liquid, acetonitrile; flow rate, 3 μL/min; sample, pBR322 DNA-*Hae* III digest, 180 fmol of each fragment.

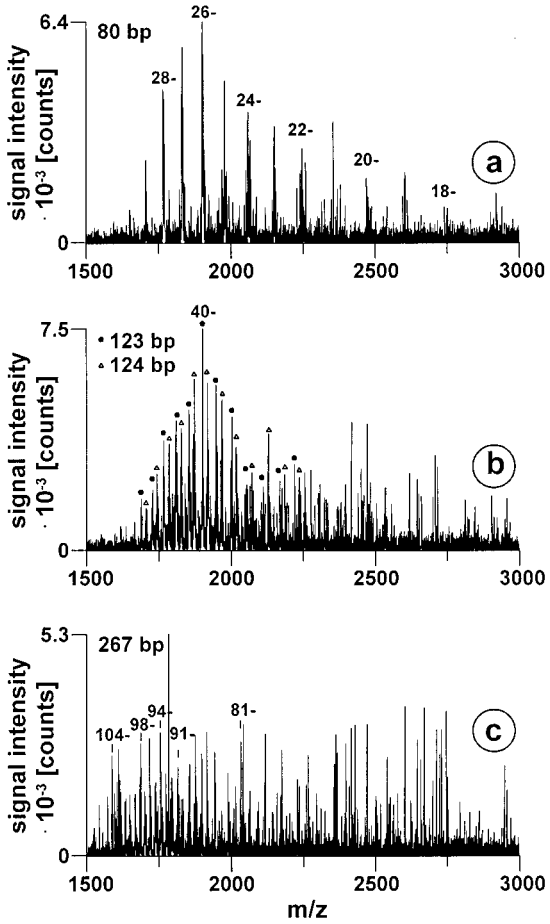

Figure 7. Extracted mass spectra of the 80 bp (a), 123/124 bp (b), and 267 bp (c) fragments of the pBR322 DNA-*Hae* III digest. Conditions as in Figure 6.

the performance of three different 60 × 0.20 mm i.d. monolithic capillary columns was compared with that of three columns packed with octadecylated, 2.3-μm micropellicular PS-DVB particles of the same dimensions. The permeabilities of the monolithic columns and the packed columns were similar, resulting in back pressures between 18 and 20 MPa at a flow rate of 2.6 μL/min and a 50 °C column temperature, which indicates that the dimension of the channels for convective flow in both chromatographic beds is of approximately the same size. The relative standard deviations of the peak widths at half-height, both among various batches of packed capillary columns and monolithic capillary columns, were better than 10%, which demonstrates that column preparation was reproducible and allowed the comparison of the chromatographic performance of both column types. The chromatographic performance was evaluated by gradient separation of a mixture of $(dT)_{12-18}$ with a gradient of 5.0−12.0% acetonitrile in 100 mM TEAA in 10 min. Three injections of the standard onto each of the three columns gave average peak widths at half-height for $(dT)_{18}$ of 2.28 ± 0.22 s (sample size $N = 9$, standard deviation sd = 0.29 s, level of significance $P = 95\%$) for the monolithic columns and 3.84 ± 0.16 s ($N = 9$, sd = 0.20 s, $P = 95\%$) for the packed-bed capillary columns. These values demonstrate that the chromatographic performance of monolithic columns for oligodeoxynucleotide separations is approximately 40% better than that of packed-bed columns. The chromatographic efficiency of the monolithic columns was determined by isocratic elution of $(dT)_{18}$ with an eluent containing 7.8% acetonitrile in 100 mM TEAA at a flow rate of 2.4 μL/min. At a column temperature of 50 °C, the number of theoretical plates exceeded 11 500 plates for a 60-mm column, corresponding to 191 000 theoretical plates per meter.

Table 1. Comparison of the Resolution Values for Oligodexynucleotides and Double Stranded DNA using Packed and Monolithic Capillary Columns

	resolution	
compounds	with packed column	with monolithic column
$p(dT)_{12}/p(dT)_{13}$	3.05	5.38
$p(dT)_{29}/p(dT)_{30}$	1.04	2.38
51/57 bp	3.88	5.15
540/587 bp	1.11	2.70

Figure 1 illustrates the high-resolution separation of phosphorylated oligodeoxyadenylic- and oligothymidylic acids ranging in size from 12 to 30 nt. Gradient elution with 3.0−9.0% acetonitrile in 3.5 min, followed by 9.0−11.0% acetonitrile in 2.5 min, and finally 11.0−13.0% acetonitrile in 4.0 min in 100 mM TEAA resulted in peak widths at half-height of 1.3 s for $p(dA)_{12}$ to 2.4 s for $p(dT)_{30}$, which allowed the baseline resolution of the whole series up to the 30-mer within 8.2 min. The resolution of homologous oligodeoxynucleotides obtained with the monolithic column clearly surpasses that of a capillary column packed with PS-DVB-C18 beads (Table 1, compare also Figure 1 in ref 28[28]).

IP-RP-HPLC has been shown to be efficient not only for the rapid separation of single-stranded oligodeoxynucleotides but also for the fractionation of double-stranded DNA fragments up to chain lengths of 2000 bp.[36] The applicability of the monolithic PS-DVB stationary phase to the IP-RP-HPLC separation of double-stranded DNA was tested by injection of a pBR322 DNA-*Hae* III digest, which was separated in 12.5 min using a gradient of 7.0−15.0% acetonitrile in 3 min, followed by 15.0−19.0% acetonitrile in 12

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min in 100 mM TEAA at a flow rate of 2.2 μL/min. Again, the chromatogram of the mixture depicted in Figure 2 with fragments ranging from 51 to 587 bp, as well as the resolution values given in Table 1, demonstrate that the separation performance of monolithic columns is superior to that of packed-bed columns with respect to their separation capability for nucleic acids (compare also Figure 1 in ref 36[36]). In this context, one distinctive difference between the octadecylated PS-DVB stationary phase and the PS-DVB monolith deserves discussion. While derivatization with octadecyl groups has been shown to be essential to obtain high chromatographic efficiency with PS-DVB particles,[31,37] monolithic stationary phases exhibit superior efficiency already without derivatization. One possible explanation for this different behavior is the formation of the polymer in two different chemical environments. The PS-DVB particles were polymerized in aqueous suspension, where poor solvation of the hydrophobic polymer by the hydrophilic solvent resulted in a relatively flat surface, as revealed by the scanning electron micrograph depicted in Figure 3a. The particle surface became rugulose after derivatization with octadecyl groups offering a contact area greater than that of a smooth spherical particle (Figure 3b). The formation of the monolithic bed, on the other hand, took place in an entirely organic environment. During polymerization, small primary particles of approximately 0.5 μm coagulated to form the porous monolith, resulting in a surface structure which came close to that of the octadecylated PS-DVB particles (Figure 3c).

On-line Separation and Mass Determination of Synthetic Oligodeoxynucleotides. For many of the analytical problems encountered with oligodeoxynucleotides, chromatographic separation in combination with UV detection is not sufficient to get a conclusive answer. The on-line conjugation of chromatographic separation to mass spectrometry, however, offers a potent tool for the characterization and identification of oligodeoxynucleotides on the basis of accurate mass determinations and fragmentation patterns. For example, the HPLC-UV analysis of a $(dT)_{12-18}$ standard that was left overnight at room temperature showed a number of small peaks eluting before the seven major peaks (chromatogram not shown). We supposed that the small peaks were phosphorylated or nonphosphorylated hydrolysis products of $(dT)_{12-18}$, but this assumption was not definitive until the separation system was on-line-coupled to ESI-MS, which revealed that they were nonphosphorylated hydrolyzates ranging from the 6-mer to the 11-mer (Figure 4). Application of a gradient from 4 to 12.0% acetonitrile in 10 mM TEAA enabled the separation of all oligothymidylic acids from the 6-mer to the 18-mer. As suggested in a recent report, acetonitrile was added postcolumn as sheath liquid to enhance the mass spectrometric detectability of the separated oligodeoxynucleotides.[38] This example demonstrates that, by using on-line IP-RP-HPLC-ESI-MS, the unequivocal identification of low femtomole amounts of oligodeoxynucleotides is feasible on the basis of their molecular masses (Table 2). With a gradient of 4.0−12.0% acetonitrile in 50 mM TEAA in 10 min, oligothymidylic acids as small as the 3-mer were eluted as sharp and symmetric peaks (chromatogram not shown), whereas mononucleotides could not be chromatographed, even with a neat aqueous eluent, due to the lack of retention. From the crystal structure of the trinucleotide $(A)_3$ it can be inferred that a 3-mer oligodeoxynucleotide has an almost globular structure with a diameter of approximately 1.0 nm.[39] Because penetration of analytes into micropores of commensurate size would cause considerable band broadening, the capability of the monolithic stationary phase to efficiently separate such small molecules is a good indicator for the absence of micropores.

Table 2. Measured and Theoretical Masses of $(dT)_{6-18}$

oligodeoxy-nucleotide	retentiontime (min)	molecular mass		rel deviation (%)
		measured	theoretical	
$(dT)_6$	1.77	1763.09	1763.21	0.006
$(dT)_7$	2.63	2066.96	2067.40	0.021
$(dT)_8$	3.59	2371.90	2371.59	−0.013
$(dT)_9$	4.35	2675.28	2675.79	0.019
$(dT)_{10}$	4.94	2978.95	2979.98	0.035
$(dT)_{11}$	5.44	3284.43	3284.18	−0.008
$(dT)_{12}$	5.76	3589.29	3588.37	−0.026
$(dT)_{13}$	6.13	3892.78	3892.57	−0.006
$(dT)_{14}$	6.39	4197.47	4196.76	−0.017
$(dT)_{15}$	6.66	4501.81	4500.96	−0.019
$(dT)_{16}$	6.92	4806.26	4805.15	−0.023
$(dT)_{17}$	7.12	5109.19	5109.35	0.003
$(dT)_{18}$	7.35	5413.35	5413.54	0.004

Refined chemistry has significantly improved the efficiency of automated solid-phase synthesis of long oligodeoxynucleotide sequences. However, assuming a coupling efficiency of 98−99% per synthesis cycle, the maximum yield of an 80-mer oligodeoxynucleotide will be only 20−45%, and contamination of the target sequence with a number of failure sequences or partially deprotected sequences is generally observed.[28,40] Figure 5a illustrates the analysis of 5.0 pmol of a crude 80-mer oligodeoxynucleotide. The high number of partly resolved peaks eluting between 2 and 6 min made identification and quantitation of the target sequence from the reconstructed ion chromatogram impossible. However, extraction of a selected ion chromatogram at m/z 1167.0, 1225.5, and 1290.0 clearly identified the target sequence eluting at 3.8 min (Figure 5b). Averaging and deconvolution of four mass spectra between 3.7 and 3.8 min yielded a molecular mass of 24 525.0 (Figure 5c), which correlates well with a theoretical mass of 24 527.17 (0.009% relative deviation). Moreover, the deconvoluted mass spectrum (inset in Figure 5c) did not show notable cation adduction, which verifies that IP-RP-HPLC is an efficient method for the desalting of oligodeoxynucleotides. Comparison of the mass spectrum extracted from the chromatogram (Figure 5c) with that of an 80-mer obtained by direct-infusion ESI-MS (compare Figure 3 in ref 23[23]) clearly corroborates the high value of on-line coupling of chromatographic separation to mass spectrometry, because the chemical background in the mass spectrum is greatly reduced upon chromatographic separation, and exact mass measurement is possible using IP-RP-HPLC-ESI-MS with only one fiftieth of the amount of sample that is consumed during direct-infusion ESI-MS.

On-line Separation and Mass Determination of dsDNA Fragments. The potential to obtain high-quality ESI mass spectra

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Table 3. Molecular Masses of Double-Stranded DNA Fragments from the pBR322 DNA-Hae III Digest

fragment	position[a]	molecular mass measured[b]	molecular mass theoretical	rel deviation (%)
51	942−992	31 565 ± 24 (4)	31 559.57	0.018
57	993−1049	35 252 ± 54 (6)	35 263.04	−0.032
64	534−597	39 573 ± 84 (7)	39 592.83	−0.026
80	3410−3489	49 494 ± 43 (10)	49 475.35	0.038
89	832−920	55 058 ± 41 (14)	55 038.97	0.034
104	298−401	64 391 ± 56 (22)	64 312.99	0.12
123	175−297	76 059 ± 49 (15)	76 045.76	0.017
124	402−525	76 731 ± 44 (17)	76 675.05	0.073
184	1263−1446	113 802 ± 140 (15)	113 747.36	0.048
192	4344−174	118 722 ± 123 (17)	118 668.82	0.045
213	1050−1262	131 733 ± 148 (18)	131 674.02	0.045
234	598−831	144 708 ± 127 (25)	144 646.56	0.042
267	3490−3756	165 091 ± 230 (12)	165 019.11	0.044
434	2518−2951	n. d.[c]	268 240.41	n. d.
458	2952−3409	n. d.	283 002.81	n. d.
502	1447−1948	n. d.	310 240.12	n. d.
540	1949−2488	n. d.	333 738.33	n. d.
587	3757−4343	n. d.	362 707.09	n. d.

[a] Position relative to the *Eco* RI restriction site in pBR322. [b] Molecular mass given as average ± standard deviation (number of charge states used to calculate the average molecular mass). [c] Not determined.

of large, double-stranded DNA is essentially determined by the amount of salt as well as the number of different compounds present in the sample mixture.[21,41] Recently, Muddiman et. al. published the mass spectrum of a 500-bp polymerase chain reaction product, which had been purified by ethanol precipitation followed by microdialysis.[42] Although the amount of DNA that was analyzed in the ion cyclotron resonance mass spectrometer was in the low femtomole range, much more material was required for purification before mass measurement. Hence, there is an urgent need for rapid on-line separation and purification protocols requiring only minute sample amounts. HPLC has been successfully applied for the desalting and separation of single-stranded oligodeoxynucleotides ranging in size up to 75 nt;[28,43] nevertheless, the applicability of HPLC to efficiently remove cation adducts in large nucleic acid samples has not been elucidated as yet. Figure 6 illustrates the chromatogram of DNA fragments from 486 ng (180 fmol) of a pBR322 DNA-*Hae* III restriction digest with detection by ESI-MS. For this separation, the gradient was ramped from 3 to 6.0% acetonitrile in 3.0 min, followed by 6.0−10.0% acetonitrile in 12 min at a flow rate of 2.8 μL/min and a column temperature of 40 °C. It can be seen that the fragments from 51 to 123 bp were completely resolved in the chromatogram, whereas the separation of the longer fragments was incomplete due to overloading of the column.[18] Mass spectra were extracted from the reconstructed ion chromatogram by averaging 4−8 scans and three examples for fragments ranging in size from 80 to 267 bp are illustrated in Figure 7. Whereas relatively few charge states (23− to 29−) were found in the mass spectrum of an 80 bp fragment (Figure 7a), the number of observed signals rapidly

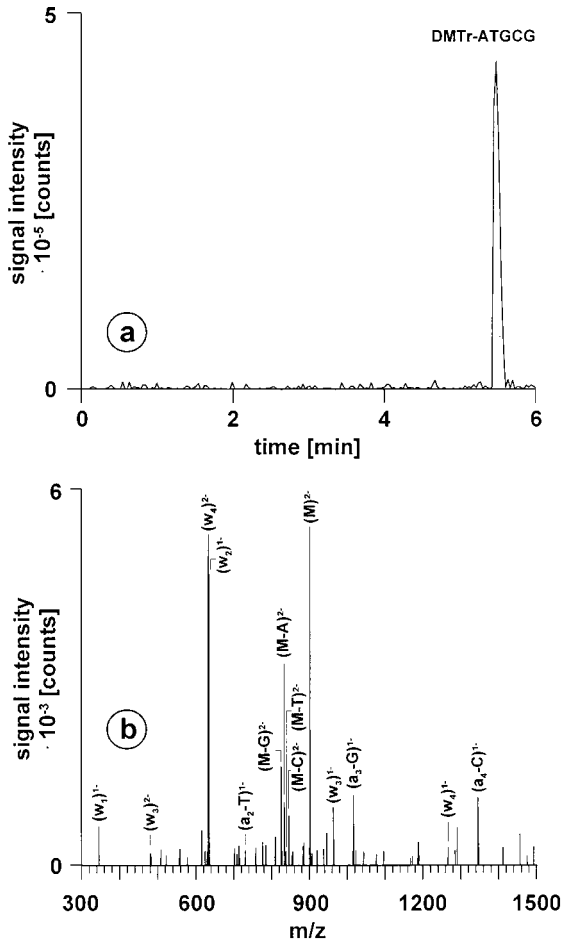

Figure 8. IP-RP-HPLC-MS/MS sequencing of a 5-mer oligodeoxynucleotide. Column, continuous PS-DVB, 60 × 0.20 mm i.d.; mobile phase, (A) 25 mM TEAB, pH 8.40, (B) 25 mM TEAB, pH 8.40, 20% acetonitrile; linear gradient, 10−100% B in 5.0 min; flow-rate, 3.0 μL/min; temperature, 50 °C; daughter ions of *m*/*z* 901.5, 4.0 amu isolation width, 19% relative collision energy; scan, 250−1810 amu; electrospray voltage, 3.2 kV; sheath gas, 30 units; sheath liquid, acetonitrile; flow rate, 3.0 μL/min; sample, 25 pmol raw product.

increased with the size of the DNA fragments (Figure 7b and c). The appearance of all charge state signals with sharp and defined peak shapes indicates that cation adducts have been efficiently removed by IP-RP-HPLC.

The molecular mass of the DNA fragments was calculated by a three-step procedure. First, a rough molecular mass was obtained by automatic deconvolution of the raw spectrum using the Bioworks software application. For the fragments from 51 to 267 bp, this deconvolution step readily yielded definite mass information, and even the mass spectrum of the coeluting 123- and 124-bp fragments was easily deconvoluted into two separate mass peaks. For the longer DNA fragments (434−587 bp), signals for the individual charge states could be only identified using the knowledge of the theoretical molecular mass of the investigated fragments from their DNA sequence. Subsequently, the charge states of all *m*/*z* signals in the mass spectrum having an abundance more than five times the signal-to-noise ratio were calculated. Finally, the *m*/*z* values and the corresponding integer charges state were used to calculate a molecular mass. Statistical treatment of the molecular masses of the individual charge states gave the average molecular mass and its standard deviation. The

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Table 4. Fragment Ions for Sequencing of a 5-mer Oligodeoxynucleotide

ion assignment	m/z
$(M)^{2-}$	901.37
$(M-A)^{2-}$	833.65
$(M-T)^{2-}$	838.57
$(M-G)^{2-}$	826.13
$(M-C)^{2-}$	845.89
$(w_1)^{1-}$	345.87
$(w_2)^{1-}$	635.06
$(w_3)^{1-}$	964.18
$(w_4)^{1-}$	1267.01
$(w_3)^{2-}$	481.45
$(w_4)^{2-}$	633.53
$(a_2-T)^{1-}$	714.16
$(a_3-G)^{1-}$	1016.07
$(a_4-C)^{1-}$	1345.98

results of these calculations are summarized in Table 3, which shows that the masses of the double-stranded DNA fragments ranging in size up to 267 bp were measured with an accuracy of better than 0.08%. As mentioned above, the masses of the longer fragments could not be calculated, mainly because too many species were coeluting, resulting in spectra of high complexity. To tackle this problem, the ESI-MS detectability has to be further improved to allow the injection of smaller amounts of sample which do not overload the column.

IP-RP-HPLC-ESI-MS/MS Sequencing of Oligodeoxynucleotides. In addition to information regarding the molecular mass, tandem mass spectrometry (MS/MS) utilizing collisionally induced dissociation (CID) provides valuable information about the base sequence of oligodeoxynucleotides.[44,45] In this example for the application of monolithic capillary columns in nucleic acid analysis, the feasibility of performing on-line MS/MS experiments on oligodeoxynucleotides upon liquid chromatographic separation was examined. To evaluate the performance of IP-RP-HPLC-ESI-MS/MS for oligodeoxynucleotide sequencing, a 5-mer oligodeoxynucleotide (sequence 5′-ATGCG-3′) was ordered from Microsynth. The IP-RP-HPLC-ESI-MS analysis of the unfragmented 5-mer gave a molecular mass of 1805.00, which exceeded the expected mass value of 1503.04 by 301.96 mass units. This mass difference could be attributable to an additional thymidine residue (which probably entered into the synthesis automat by accident) or to a 5′-terminal dimethoxytrityl protecting group (that had not hydrolyzed after the last coupling cycle). Substantially increased retention in the chromatographic analysis was indicative of the latter assumption. The presence of a dimethoxytrityl protecting group as well as the total sequence of the oligodeoxynucleotide was confirmed using IP-RP-HPLC-ESI-MS/MS (Figure 8). The ESI-MS/MS experiment was performed by isolating the $[M - 2H]^{2-}$ charge state at m/z 901.37 and collisional activation at 19% relative collision energy. Assignments and masses for the fragment ions observed in the tandem mass spectrum (Figure 8b) are listed in Table 4. In addition to the parent ion, all four ions that show loss of one nucleobase are observed. The most diagnostic ions, however, arise from fragmentation which produces w series ions that are used to determine the 3′→5′ sequence and the a_n-B_n series ions, that are used to determine the 5′→3′ sequence.[44] The complete w series is present in the MS/MS spectrum, and the masses correspond to those expected for an oligodeoxynucleotide with the sequence 5′-ATGCG-3′, proving that the 3′ terminus is unmodified. The a_n-B_n series, however, shows a mass shift of +302 from the expected mass, corresponding to the presence of the dimethoxytrityl protecting group at the 5′ terminus. Finally, the presence of the protective group was confirmed by cleavage with 2% formic acid at room temperature for 5 min, yielding the oligodeoxynucleotide ATGCG, with the expected mass of 1502.98.

CONCLUSIONS

It has been demonstrated that the chromatographic separation performance of monolithic capillary columns for single- and double-stranded nucleic acids significantly surpasses that of capillary columns packed with microparticulate stationary phases. Careful control of the synthetic conditions is obligatory in order to obtain a porous polymer structure of high permeability, which permits a rapid mass transfer between the mobile and the continuous stationary phase. With characteristic flow rates in the 2−3 μL/min range and the application of volatile mobile-phase components, IP-RP-HPLC using monolithic 200-μm i.d. capillary columns is eminently suited for the direct hyphenation to ESI-MS.

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