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Hydrophobic, Pellicular, Monolithic Capillary Columns Based on Cross-Linked Polynorbornene for Biopolymer Separations

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Monolithic capillary columns were prepared by transition metal-catalyzed ring-opening metathesis copolymerization of norborn-2-ene and 1,4,4a,5,8,8a-hexahydro-1,4,5,8-*exo,endo*-dimethanonaphthalene inside a silanized 200-*μ*m-i.d. fused-silica capillary using a mixture of toluene and 2-propanol as porogen and $Cl_2(PCy_3)_2Ru(=CHPh)$ as initiator. The synthesized columns allowed the rapid and highly efficient separation of single- and double-stranded nucleic acids by ion-pair reversed-phase high-performance liquid chromatography and of proteins by reversed-phase high-performance liquid chromatography. Compared to 3-mm-i.d. analytical columns synthesized from an identical polymerization mixture, a considerable improvement in the peak widths at half-height of oligonucleotides in the order of 60−80% was obtained. Significant differences in morphology between the capillary column, where the surface of the monolith was rather soft and rugulose, and the analytical column, where the surface was very sharp and smooth, were observed, most probably due to differences in polymerization kinetics. The synthesized monoliths were successfully applied to the separation of the diastereomers of phosphorothioate oligodeoxynucleotides. To confirm the identity of the eluting compounds on the basis of their intact molecular masses, the chromatographic separation system was on-line hyphenated to electrospray ionization mass spectrometry.

After more than 35 years of development of stationary phases for high-performance liquid chromatography (HPLC), the field has not yet come to saturation and intensive efforts are still put into the advancement of column packing materials that meet the requirements of high-speed, high-resolution liquid chromatography.[1] The idea of micropellicular stationary phases lacking pores accessible only by diffusional mass transport for HPLC of biological compounds was born in the mid 1960s[2] and revived in the 1980s when their potential for rapid, high-resolution separations of biological macromolecules was recognized.[3,4] Likewise, the monolithic column configuration, in which the chromatographic bed consists of a single piece of a porous polymer,[5,6] has turned out to offer distinctive advantages for liquid chromatographic separation of biopolymers, including simple preparation and functionalization,[1] enhanced mass transfer, column efficiency,[7] and robustness.[8] Recently, it was realized that monolithic stationary phases of micropellicular configuration are eminently suited for highly efficient chromatography of peptides, proteins, and nucleic acids.[9−11]

The most common method for the preparation of monolithic columns is free radical polymerization of suitable monomer mixtures such as styrene/divinylbenzene,[12] acrylic acid/methylenebisacrylamide,[13] glycidyl methacrylate/ethylene dimethacrylate, or acrylamide/methylenebisacrylamide[14] in the presence of inert solvents that form the pores. Although the influence of experimental conditions during preparation of the monoliths on morphology has been studied in detail,[14−17] the control of final structure and chromatographic properties remain tricky presumably due to the free radical mechanism of polymerization.

Recently, however, ring-opening metathesis polymerization (ROMP) has been proposed for the preparation of monolithic separation media based on norbornene.[10] Due to the living character of this type of polymerization, well-defined stationary

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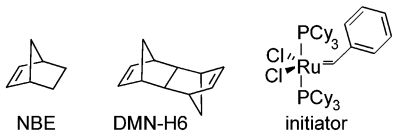

Figure 1. Chemical structure of the monomers norbornene (NBE) and hexahydrodimethanonaphthalene (DMN-H6) and the transition metal-based initiator used to prepare the monoliths (Cy = cyclohexyl).

phases can be synthesized under strictly controlled conditions. The microstructure and morphology of the poly(norbornene/hexahydrodimethanonaphthalene) (NBE/DMN-H6) monoliths were optimized in a reproducible way upon variation of the polymerization conditions, including monomer and porogen stoichiometry and additional phosphine concentration.[18,19]

So far, norbornene-based monolithic structures have only been realized in analytical column dimensions of 3 mm or more inner diameter.[10,18,19] The downscaling to smaller inner diameters characteristic for capillary- and nano-HPLC might seem trivial, but as this study will demonstrate, significant differences between morphology and column performance are observed with monoliths of different dimensions. Thus, the major motivation of this work was to explore as to what extent ROMP is suitable for the fabrication of monolithic structures in the capillary format, which enables the rapid and efficient separation of biopolymers, including oligonucleotides, proteins, and double-stranded DNA fragments. As a consequence of miniaturization, the separation systems can be readily interfaced to electrospray ionization mass spectrometry (ESI-MS), representing a very potent tool for the characterization of biological compounds.

EXPERIMENTAL SECTION

Chemicals and Samples. Acetonitrile (HPLC gradient grade) was obtained from Riedel-de Haen. Acetic acid (analytical reagent grade), butyldimethylamine (analytical reagent grade), triethylamine (analytical reagent grade), norbornene (NBE), and the initiator $Cl_2Ru(PCy_3)_2(=CHC_6H_5)$ (Cy = cyclohexyl) were purchased from Fluka (Buchs, Switzerland). 1,4,4a,5,8,8a-Hexahydro-1,4,5,8-*exo*,*endo*-dimethanonaphthalene was prepared according to the literature.[20] The chemical structures of the monomers and the initiator are depicted in Figure 1. Bicyclo[2.2.1]-hept-2-enyl-5-methyldichlorosilane was purchased from ABCR (Karlsruhe, Germany). Reagent grade tetrahydrofuran, 2-propanol, and toluene were distilled over sodium under argon. Pyridine was distilled over calcium hydride under argon.

A 1.0 M stock solution of triethylammonium acetate was prepared by mixing equimolar amounts of acetic acid and triethylamine, and a 0.5 M stock solution of butyldimethylammonium bicarbonate was obtained by passing carbon dioxide gas (AGA, Vienna, Austria) through a 0.50 M aqueous solution of the amine at 5 °C until pH 8.9 was reached. All standard proteins were obtained from Sigma (St. Louis, MO). Standards of oligodeoxythymidylic acids (dT_{12-18}) were purchased as sodium salts from Pharmacia (Upsala, Sweden) and the pBR322 DNA−*Hae*III digest was from Sigma. Phosphorothioate oligodeoxynucleotides were ordered from Microsynth (Balgach, Switzerland) and used without further purification.

Preparation of Monolithic Columns. Monolithic columns of 3-mm i.d. were prepared exactly according to the protocol described in ref 18. For the preparation of 200-μm-i.d. capillary columns, a 200-cm piece of 200-μm-i.d. fused-silica capillary (Polymicro Technologies, Phoenix, AZ) was etched with 1 M sodium hydroxide for 5 min. After repeated washing with water, the capillary was dried in vacuo. Silanization was performed at 60 °C overnight using a mixture of pyridine/toluene and bicyclo-[2.2.1]hept-2-en-5-ylmethyldichlorosilane (molar ratio 3:2:1). The silanized capillary was washed consecutively with acetone, water, and ethanol and dried under reduced pressure at ambient temperature.

The polymerization mixture used for preparation of the monoliths comprised 25% NBE, 25% DMN-H6, 40% 2-propanol, 10% toluol, 0.4% initiator, and 40 ppm triphenylphosphine. Two different solutions, one containing NBE, DMN-H6, and 2-propanol and the other containing toluene, triphenylphosphine, and initiator, were prepared and cooled to −30 °C. After mixing of the two solutions for a few seconds, the polymerization mixture was injected into a 30-cm piece of the silanized fused-silica capillary by means of a plastic syringe. The capillary was kept at room temperature for 1 h and subsequently rinsed with methanol/ethyl vinyl ether/toluene (volume ratio 45:45:10) to remove the initiator.

High-Performance Liquid Chromatography and Coupling with Electrospray Ionization Mass Spectrometry. The system used for HPLC experiments with UV detection consisted of a high-precision pump (model 480 GT, Gynkotek, Germering, Germany), 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 sample injection valve (Rheodyne Inc., Cotati, CA) with a 500-nL internal sample loop, a UV detector (model 254 nm Knauer, Berlin, Germany) with a Z-shaped capillary detector cell (ULT-UZ-N10, 3-nL cell, LC Packings, Amsterdam, The Netherlands), and a PC-based data system (Chromeleon 4.30, Dionex-Softron, Germering, Germany). A primary flow of 500 μL/min was split with the help of a tee-piece and a 50-μm-i.d. fused-silica capillary.

The system used for HPLC/ESI-MS experiments consisted of a low-pressure gradient micropump (model Rheos 2000, Flux Instruments, Basel, Switzerland) controlled by a personal computer, a vacuum degasser (Knauer), 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), and a microinjector (model C4-1004, Valco Instruments Co. Inc., Houston, TX) with a 500-nL internal sample loop. A primary flow of 100−200 μL/min was split with the help of a tee piece and a 50-μm-i.d. fused-silica capillary.

ESI-MS was performed on a quadrupole ion trap mass spectrometer (LCQ, Thermo Finnigan, San Jose, CA), equipped with an electrospray ion source. A syringe pump equipped with a 250-μL glass syringe (Unimetrics, Shorewood, IL) was used for postcolumn addition of 2.0 μL/min acetonitrile through a microtee piece (Upchurch Scientific, Oak Harbor, WA). For analysis with pneumatically assisted ESI, an electrospray voltage of 3.4 kV and

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a nitrogen sheath gas flow were employed. The temperature of the heated capillary was set to 250 °C. Total ion chromatograms and mass spectra were recorded on a personal computer with the Xcalibur software version 1.1 (Thermo Finnigan).

RESULTS AND DISCUSSION

Characterization of the Monolithic, Norbornene-Based Capillary Columns. Polymer-based monolithic supports are usually prepared by in situ polymerization of a mixture of suitable monomers and porogens within a tube acting as a mold. The permanent porosity in the monolith is created upon phase separation of the solid polymer from the liquid porogens during the course of polymerization. Size and morphology of the pores strongly depend on several factors, including polymerization kinetics and solvency of the porogens for the resulting polymer. Microporogens, having good solvency for the polymer, favor the formation of small pores, whereas macroporogens that have only poor solvating capabilities for the polymer ensure the formation of large pores.[17]

One of the major problems during the manufacture of monolithic columns is the volume contraction upon polymerization of the liquid monomer mixture, leaving undesirable voids between the chromatographic bed and the wall of the column tube. Moreover, porous polymeric stationary phases often swell and shrink during the gradients of organic solvent commonly used in reversed-phase HPLC, and the polymer may also be deformed upon contact with organic solvents as a consequence of the pressure gradients normally encountered in HPLC. These problems can be addressed by mechanically compressing the monolithic bed after polymerization,[5] by using solvents that swell the polymer, or by forming covalent chemical bonds between the polymer and the surface of the column tube.[21] The latter is difficult to realize with the stainless steel or polymeric PEEK tubing commonly utilized for columns of analytical and preparative dimensions. Consequently, tubes of borosilicate glass, which offer conventional silane chemistry for covalent immobilization, have been used.[10] Nevertheless, such tubes are mechanically stable only up to pressures of 10 MPa, which precludes their application for many high-performance applications, where high column efficiency can only be accomplished with densely packed chromatographic beds having relatively low permeability.

Polyimide-coated fused-silica capillary tubing is available in a wide range of diameters and may be operated at pressures exceeding 100 MPa. Since their inner surface can be readily functionalized using the silanol groups as anchoring functions, fused-silica capillaries represent an almost ideal tubing material for the fabrication of monolithic capillaries. Figure 2 compares the separations of homooligonucleotides in monolithic columns of capillary and analytical dimensions. It is clearly seen that the chromatographic performance of the 200-μm-i.d. capillary column significantly surpasses that of the 3-mm-i.d. analytical column, although both were prepared from identical polymerization mixtures (25% norbornene, 25% hexahydrodimethanonaphthalene, 40% 2-propanol, 10% toluene, 0.4% initiator, and 40 ppm triphenylphosphine). Even a systematic variation in the synthetic procedure did not enable the fabrication of 3-mm columns having a performance comparable to that of the 200-μm-i.d. columns. The

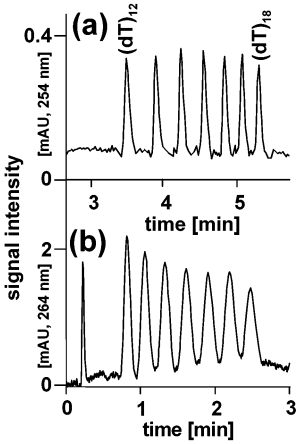

Figure 2. Comparison of oligodeoxynucleotide separation in monolithic columns of (a) 200-μm-i.d. capillary and (b) 3-mm-i.d. analytical dimensions. Columns, NBE/DMN-H6 monolith, (a) 60 × 0.20 mm i.d. and (b) 100 × 3 mm i.d.; mobile phase, (A) 0.1 M TEAA, pH 7.0 and (B) 0.1 M TEAA, pH 7.0, 20% acetonitrile; linear gradient, (a) 40−80% B in 10 min and (b) 55−80% B in 10 min; flow rate, (a) 9 μL/min and (b) 2 mL/min; temperature, 20 °C; detection, UV, (a) 254 and (b) 264 nm; and sample, (a) 2 ng, (b) 0.1 μg $(dT)_{12-18}$.

Table 1. Comparison of the Resolution Values for $(dT)_{12-18}$ Using Norbornene-Based, Monolithic Columns with 3-mm and 200-μm i.d.

	resolution	
compounds	0.2-mm-i.d. column	3-mm-i.d. column
$(dT)_{12}-(dT)_{13}$	3.47	1.44
$(dT)_{13}-(dT)_{14}$	3.14	1.42
$(dT)_{14}-(dT)_{15}$	2.88	1.42
$(dT)_{15}-(dT)_{16}$	2.75	1.35
$(dT)_{16}-(dT)_{17}$	2.35	1.27
$(dT)_{17}-(dT)_{18}$	2.27	1.25

peak widths at half-height of oligonucleotides ranged from 3.4 to 4.3 s in the capillary column, while those in the 3-mm-i.d. monolith were 5.5−7.9 s. The resolution of all peaks (Table 1) was better by 80−140% in the capillary column compared to the analytical column. Although the chromatographic efficiency is not as high as that observed with monolithic capillary columns based on PS/DVB,[11] it is comparable with that of micropellicular, octadecylated PS/DVB particles, a commercially available granular stationary phase representing one of the most efficient phases for oligonucleotide separations (see Figure 8 in ref 22).

There may be several reasons for the improved efficiency observed with capillary columns. First, columns in the capillary format are generally believed to be more efficient than analytical columns because of a more homogeneous column bed.[23] Additionally, differences in the optimized preparation protocols, especially in the temperature control during polymerization, may result in morphological differences between monoliths prepared from identical polymerization mixes. The monolithic capillary columns were prepared at room temperature by injecting the polymerization mix cooled to −30 °C into the fused-silica capillary and keeping the capillary at room temperature for 1 h. The heat of polymerization is efficiently dissipated in this experimental con-

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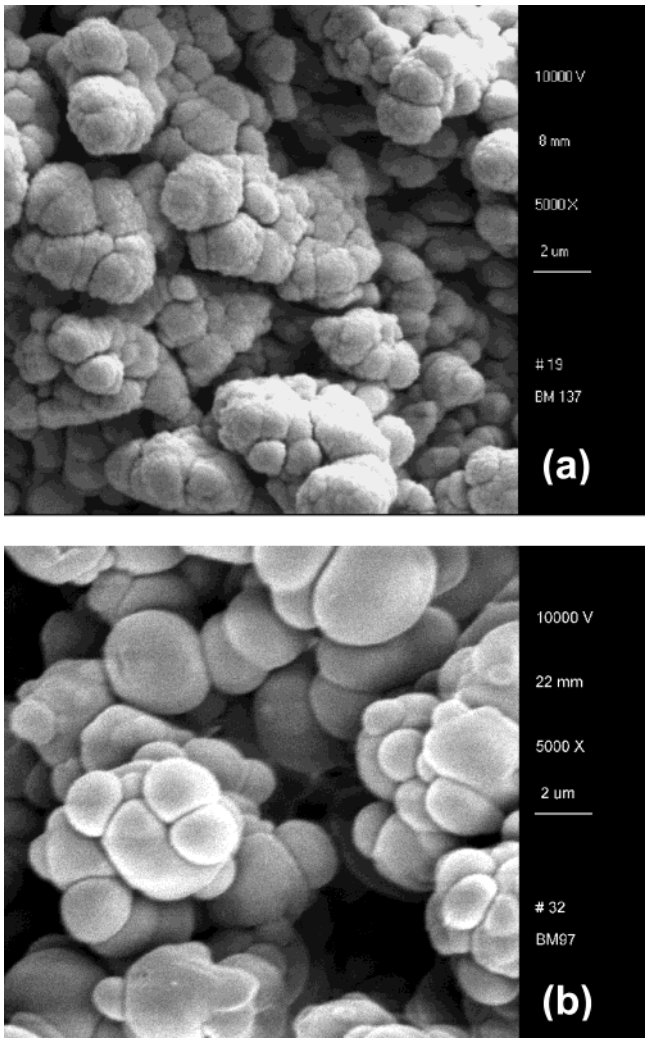

Figure 3. Electron micrographs of (a) capillary monolith in 0.2-mm-i.d. fused-silica and (b) analytical monolith 3-mm-i.d. borosilicate glass column.

figuration because of the very low polymerization volume, the thin capillary wall, and the large surface-to-volume ratio of the fused-silica capillaries. On the contrary, the 3-mm-i.d. glass columns had to be chilled to 0 °C before filling with the −30 °C polymerization mixture and kept for 30 min at 0 °C followed by 30 min at room temperature in order to avoid excessive heating of the polymerization mixture. The cooling is necessary because of the rather restricted transfer of a comparatively larger amount of polymerization energy through the thick walls of the borosilicate tube.

Electron microscopy revealed significant differences in morphology between the capillary column and the analytical column, as illustrated by the scanning electron micrographs of Figure 3. While the surface of the monolith prepared in the fused-silica capillary was rather soft and rugulose (Figure 3a), the surface of the 3-mm monolith was very sharp and smooth (Figure 3b). The finding that soft surfaces enhance the chromatographic efficiency correlates with earlier observations made with PS/DVB particles or monoliths, where only a rugulose surface facilitated highly efficient separations of proteins[24] or nucleic acids.[25] The difference

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Table 2. Dependence of Column Back Pressure of Flow Rate with Different Percolation Solvents

solvent	flow rate range (μL/min)	regression line[a] *a*	correlation coefficient
water	1.91−14.2	1.832	0.9983
acetonitrile	3.35−21.60	0.7192	0.9995
methanol	3.63-21.25	1.180	0.9991
tetrahydrofuran	0.64−1.60	23.63	0.9978

[a] Back pressure (MPa) = $a \times$ flow rate (μL/min), column dimensions, 60 × 0.2 mm i.d.

in the surface morphologies is most probably due to different polymerization kinetics and temperatures in the monoliths of different dimensions.

Subsequently, the monolithic capillary columns were characterized by the dependence of pressure drop across the column of flow rate with different percolation solvents (Table 2). Because of the high mechanical stability of the fused-silica capillary tubing, pressures far beyond 10 MPa, which is the upper pressure limit of 3-mm monoliths in borosilicate tubing, were tolerable in these experiments. Moreover, the covalent immobilization of the porous monolith at the capillary wall and the high surface-to-volume ratio in the capillary had a positive effect on the stabilization of the chromatographic bed. Excellent linear correlations with regression factors R^2 of better than 0.998 indicated that the monolithic rod is not compressed even at high pressures up to 40 MPa. Tetrahydrofuran represents a good solvent for the polymer and causes extensive swelling of the polymer, resulting in low column permeability even at low flow rate. Methanol and acetonitrile did not cause any considerable swelling due to their poor solvency for polynorbornene. Hence, the monolithic columns could be operated over a wide range of flow rates of at least 1−15 μL/min with eluents commonly utilized for gradient elution in the reversed-phase (RP) and ion-pair reversed-phase (IP-RP) chromatographic modes of HPLC.

As expected, the capillary monoliths showed column permeabilities similar to those of 3-mm-i.d. analytical monoliths,[18] which indicates that the total channel volume and channel dimensions are very similar. Nevertheless, the permeability of the NBE/DMN-H6 monoliths was considerably higher compared to the permeability of PS/DVB monoliths (~5 MPa at a flow of 2.6 μL/min water at 22 °C versus 18−20 MPa at a flow of 2.6 μL/min 100 mM aqueous triethylammonium acetate/5% acetonitrile, 50 °C[25]). This suggests that the channels for convective flow in the NBE/DMN-H6 monoliths are larger than those in the PS/DVB monoliths,[24] which probably bears the potential for further improving the efficiency of the NBE/DMN-H6 monoliths.

Separation of Biopolymers in Norbornene-Based Monoliths. Nonpolar polymeric surfaces without functionalization are applicable as stationary phase for two major chromatographic modes that are commonly utilized for HPLC of biopolymers: RP-HPLC and IP-RP-HPLC. While the former is the method of choice for high-resolution separations of peptides and proteins, the latter is eminently suited for the separation of single- and double-stranded nucleic acids.[26] A practical example for the separation

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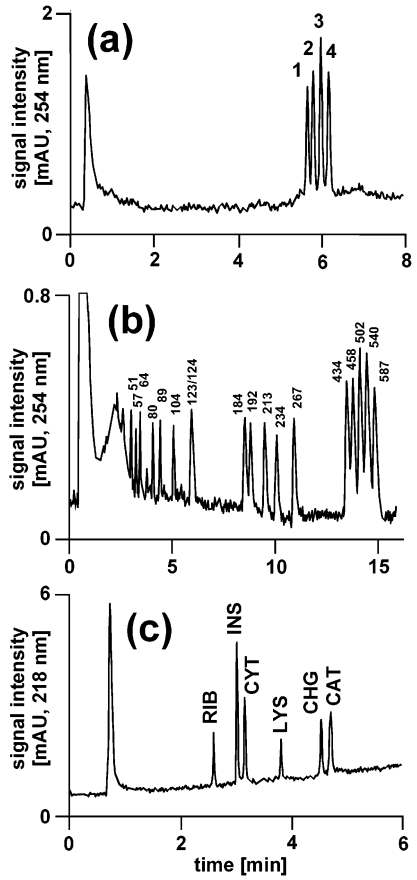

Figure 4. High-resolution separations of (a) oligodeoxynucleotides, (b) double-stranded DNA fragments, and (c) proteins in norbornene-based capillary monoliths. Column, NBE/DMN-H6 monolith, (a, b) 60 × 0.20 mm i.d., (c) 220 × 0.20 mm i.d.; mobile phase in (a), (A) 0.1 M triethylammonium acetate, pH 7.0 and (B) 0.1 M triethylammonium actetate, pH 7.0, 20% acetonitrile; mobile phase in (b), (A) 0.1 M triethylammonium acetate, 1% methanol, pH 7.0 and (B) 0.1 M triethylammonium actetate, pH 7.0, 1% methanol, 20% acetonitrile; mobile phase in (c), (A) 10% acetonitrile, 0.1% TFA in water and (B) 0.1% TFA in acetonitrile; gradient, (a) 20−80% B in 10 min (b) 20−50% B in 1 min, 50−80% B in 14 min and (c) 0−100% B in 10 min; flow rate, (a) 5, (b) 3, and (c) 6 μL/min; temperature, (a, b) 50 and (c) 20 °C; detection, UV, (a, b) 254 and (c) 218 nm; sample in (a), (1) GTGCTCAGTG TAGCCCAGGA TGCC; (2) GTGCTCAGTG TAGCCCAG**T**G ATGCC, (3) GTGCTCAGTG TAGCCCAG**TT** GATGCC, and (4) GTGCTCAGTG TAGCCCAG**T TT**GATGCC, 0.5 ng each, (b) pBR322 DNA−*Hae*III digest, 12 ng, and (c), RIB = ribonuclease A, INS = insulin, CYT = cytochrome *c*, LYS = lysozyme, CHG = α-chymotrypsinogen A, and CAT = catalase, 2 ng each.

of single-stranded oligodeoxynucleotides by IP-RP-HPLC in a monolithic NBE/DMN-H6 capillary column is illustrated in Figure 4a. Four homologous oligodeoxynucleotides, ranging in length from 24 to 27 nucleotides and differing from each other by the insertion of one, two, and three thymidines after position 18 of the 24-mer, could be baseline separated within 7 min with a gradient of 4−16% acetonitrile in 10 min in 0.1 M triethylammonium acetate. The single-nucleotide resolution demonstrated in this chromatogram is essential for the applicability of IP-RP-HPLC in the quality control of synthetic oligonucleotides.

Separation and fraction of double-stranded DNA fragments is a key element in various molecular biological experiments, including cloning, DNA sequencing, genome fingerprinting, DNA hybridization, and mutation detection. The mixture of fragments generated by the enzymatic cleavage of DNA with restriction endonucleases may range from a few base pairs to thousands of base pairs, depending on DNA size, DNA sequence, and restriction enzyme used. Figure 4b depicts the analysis of a mixture of DNA fragments obtained by digestion of the pBR322 plasmid with the restriction enzyme *Hae*III. The fragments ranging in size from 51 to 587 base pairs were separated by capillary IP-RP-HPLC applying a gradient of 4−10% acetonitrile in 1 min, followed by 10−16% in 14 min in 0.1 M aqueous triethylammonium acetate containing 1% methanol. The addition of methanol to the eluent was previously found to improve the separation efficiency especially for the larger DNA fragments on norbornene-based monoliths.[19]

The excellent separation efficiency of NBE/DMN-H6 monoliths for double-stranded DNA is documented in peak width at half-height of 3.1−8.5 s for the fragments up to ∼250 base pairs. Longer DNA fragments eluted with peak widths at half-height around 10−12 s due to the shallower gradient required to resolve them. This separation is a second example for the improved separation performance of norbornene-based capillary monoliths compared to their analogues in the analytical 3-mm format. While the separation of the 184/192-base pair fragments was incomplete on the 3-mm monolith (compare Figures 3 and 4 in ref 19), the two fragments were almost separated to baseline in the 200-μm capillary monolith (Figure 4b).

Finally, the hydrophobic stationary phase was tested for the separation of some proteins by RP-HPLC, which represents one of the most common and powerful chromatographic separation modes for peptides and proteins.[34] Even though most commercial RP-HPLC columns are based on silica gel as the support material, organic polymeric stationary phases offer some advantages such as improved chemical stability at extreme pH and the absence of silanols causing unwanted secondary interactions with the analytes. Figure 4c illustrates the separation of six proteins by capillary RP-HPLC applying a gradient of 10−100% acetonitrile in 10 min in 0.1 aqueous trifluoroacetic acid at a flow rate of 6 μL/min. Although the application of a steep gradient ensued the rapid elution of the proteins as extremely sharp peaks with peak widths at half-height of 1−2 s, the selectivity was high enough to separate all components of the mixture to baseline within less than 5 min and still left space for additional peak capacity.

Separation of Diastereoisomeric Phosphorothioate Oligodeoxynucleotides in NBE/DMN-H6 Monoliths. Synthetic DNA or RNA analogues, particularly those that have been modified at the phosphorus internucleotidic linkage, have become indispensable tools for research and therapy in the field of antisense nucleic acids, where blockage of protein expression can be effected by Watson−Crick binding of a short (15−30 nucleotides) strand to an appropriate complementary sequence within the target mRNA.[27] Replacement of a nonbridging oxygen atom

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in the phosphodiester group by sulfur yields oligodeoxynucleotide analogues known as phosphorothioate oligodeoxynucleotides. The modified backbone of antisense phosphorothioate oligonucleotides prevents their rapid degradation by ubiquitous ribonucleases and improves their transport through the cell membranes. The creation of a chiral center on the phosphate group upon incorporation of the sulfur atom changes the physical and chemical properties of the modified molecule and leads to the formation of a set of 2^n diastereomeric products, where n is the number of chiral internucleotide linkages.

While both anion-exchange chromatography and IP-RP-HPLC using granular stationary phases have been applied to the separation and purification of phosphorothioate oligodeoxynucleotides, we are not aware of any investigations demonstrating the suitability of monolithic stationary phases for the separation of this type of biopolymer. Anion-exchange chromatography has not been reported so far to be able to resolve diastereomeric phosphorothioates,[28] but IP-RP-HPLC using octadecyl silica as stationary phase succeeded in separating some of the distereoisomers, depending on the total length of the oligonucleotide.[29,30] Nevertheless, in both chromatographic modes, the peaks of phosphorothioate oligonucleotides are usually significantly broader than those of the phosphodiester analogues due to partial separation of diastereomers.[31]

Figure 5a shows the separation of the diasteroisomers of $T_{PS}T_{PS}T$ by IP-RP-HPLC in a NBE/DMN-H6 monolith. Three peaks were observed for the four possible isomers, and the relative peak areas suggest that two isomers coelute in the third peak. The elution order of the diastereomers may be assumed to be the same as determined by Zon et al., using a μBondapak C18 column in combination with 0.1 M triethylammonium acetate/acetonitrile as mobile phase, namely, $R,R < S,R < S,S \approx R,S$. The situation becomes much more complicated for a 5-mer phosphorothioate of the sequence $T_{PS}T_{PS}T_{PS}T_{PS}T$, where 16 diastereoisomers are possible in principle. The chromatogram depicted in Figure 5b reveals the partial separation of at least eight peaks, with shoulders on some of the peaks indicating the presence of even more compounds.

To confirm the identity of the eluting compounds as stereoisomers of the phosphorothioate oligodeoxynucletides, their intact molecular mass was determined by on-line hyphenation of IP-RP chromatographic separation to ESI-MS. The molecular masses obtained by deconvolution of the raw ESI mass spectra yielded molecular masses of 882.8 and 1524.0, respectively (see insets in Figure 5a and b), which corresponded excellently to the theoretical molecular masses of the all-phosphorothioate 3- and 5-mer (882.57 and 1522.92). Hence, the presence of truncated sequences, partially protected species, or oxidation products could be excluded.

Liquid Chromatography/Mass Spectrometry of Antisense Phosphorothioate Oligodeoxyribonucleotides and Oligoribonucleotides. Antisense oligonucleotides are a rather new class of therapeutic agents that possess large potential to provide effective therapies for a variety of diseases.[32,33] The therapeutic significance of modified oligodeoxynucleotides requires a high level of quality control to ensure sample integrity. In such quality assurance protocols, chromatographic analysis of raw and final products is necessary to ensure a dynamic range that enables the detection of contaminations at concentration levels down to at least 1% relative to the target product. Moreover, a combination with spectroscopic techniques is highly desirable for the identification or structural elucidation of byproducts and degradation products coming from production.[28,34] In clinical studies employing antisense oligonucleotides, high sensitivity and low sample requirement are obligatory for the identification of metabolites.[35] The on-line combination of high-resolution separation in capillary monoliths with identification by ESI-MS is eminently suited to fulfill the above-mentioned criteria for high-throughput analysis.[36]

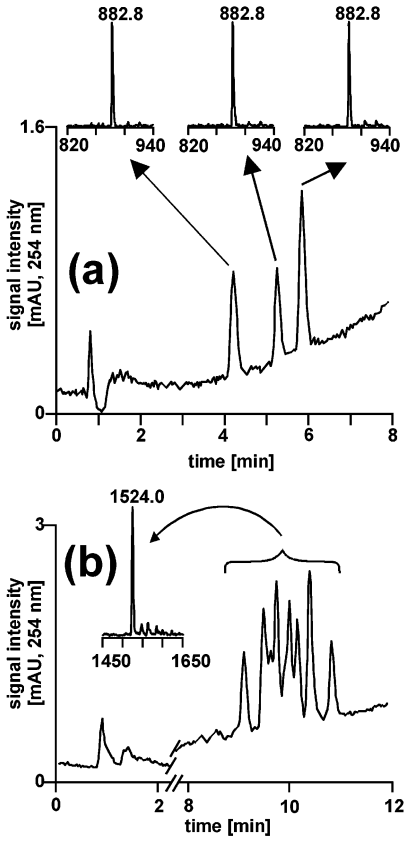

Figure 5. IP-RP-HPLC of short, (a) 3- and (b) 5-mer, diastereomeric phosphorothioate oligodeoxynucleotides in an NBE/DMN-H6 monolith. Column, monolithic NBE/DMN-H6 monolith, 60 × 0.20 mm i.d.; mobile phase, (A) 0.1 M triethylammonium acetate, pH 7.0 and (B) 0.1 M triethylammonium acetate, pH 7.0, 20% acetonitrile; linear gradient, 20−80% B in 10 min; flow rate, 3 μL/min; temperature, 50 °C; detection, UV, 254 nm; sample, (a) $T_{PS}T_{PS}T$ 2 ng; (b) $T_{PS}T_{PS}T_{PS}T_{PS}T$, 2 ng (PS = phosphorothioate linkage).

Figure 6 illustrates the analysis of antisense oligodeoxynucleotides of mixed sequence by IP-RP-HPLC/ESI-MS. While the 8-mer eluted as a broad, more than 1-min-wide peak due to partial separation of the diastereoisomers (Figure 6a), the peaks of the 16- (Figure 6b) and 24-mer (Figure 6c) became increasingly narrow with increasing length of the oligonucleotides under identical chromatographic conditions. This indicates that the relative differences in the chromatographic properties between the different stereoisomers decrease with increasing size of the analytes. Nevertheless, compared to the separations of phosphodi-

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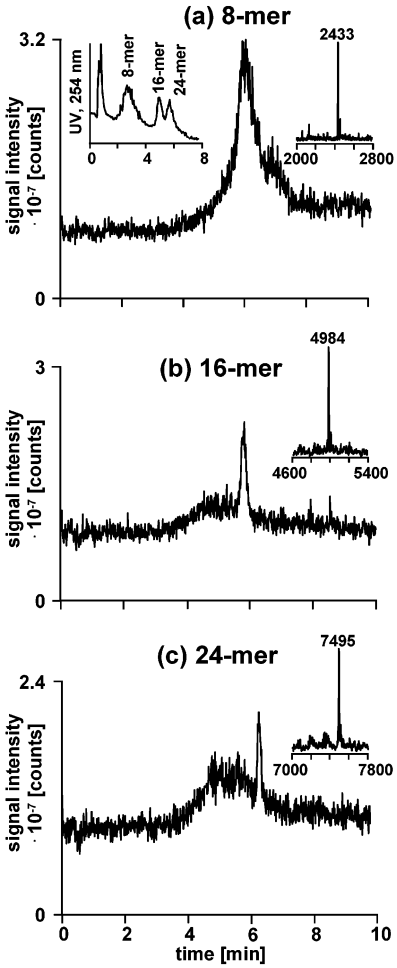

Figure 6. IP-RP-HPLC/ESI-MS analysis of mixed-sequence phosphorothioate oligodeoxynucleotides. Column, NBE/DMN-H6 monolith, 60 × 0.20 mm i.d.; mobile phase, (A) 25 mM butyldimethylammonium bicarbonate, pH 8.9 and (B) 25 mM butyldimethylammonium bicarbonate, pH 8.9, 80% acetonitrile; linear gradient, 5−70% B in 10 min; flow rate, 4 μL/min; temperature, 60 °C. sample, (a) $C_{PS}C_{PS}T_{PS}G_{PS}C_{PS}T_{PS}C_{PS}C$, 20 ng, (b) $C_{PS}C_{PS}T_{PS}G_{PS}C_{PS}T_{PS}C_{PS}C_{PS}C_{PS}C_{PS}C_{PS}T_{PS}G_{PS}$ $G_{PS}C_{PS}T$, 15 ng, and (c) $C_{PS}C_{PS}T_{PS}G_{PS}C_{PS}T_{PS}C_{PS}C_{PS}C_{PS}C_{PS}C_{PS}C_{PS}T_{PS}G_{PS}G_{PS}C_{PS}T_{PS}C_{PS}C_{PS}C_{PS}T_{PS}G_{PS}C_{PS}T_{PS}C$, 25 ng. (PS = phosphorothioate linkage). The left inset in (a) shows the UV chromatogram obtained with a gradient of 4−40% acetonitrile in 0.1 M triethylammonium acetate, pH 7.0 in 15 min; the right insets show the deconvoluted ESI mass spectra of phosphorothioate oligonucleotides.

ester oligonucleotides in the NBE/DMN-H6 monoliths, the peaks of the phosphorothioates are still broader and single-nucleotide resolution is impossible even with very shallow gradients (compare left inset in Figure 6a with Figure 4a).

Mass spectra of high quality from mid-femtomole amounts of the analytes were obtained for the three oligonucleotides, from which their molecular mass was deduced with high mass accuracy (measured molecular masses, 2433, 4984, and 7495; theoretical molecular masses, 2433.41, 4984.78, and 7496.13). With detectabilities in the mid-femtomole range, the on-line IP-RP-HPLC/ESI-MS method is highly suited both for quality control and for metabolic profiling.

Despite the increased resistance of all-phosphorothioate oligonucleotides to nuclease digestion, they show nonspecific binding to proteins causing toxic side effects. This problem may be

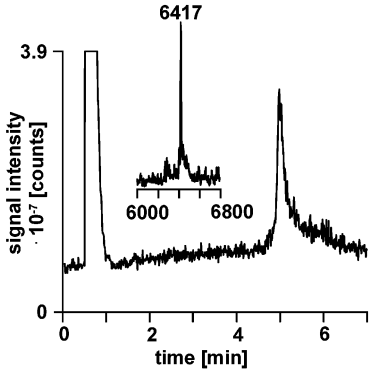

Figure 7. Analysis of RNA with phosphorothioate modifications by IP-RP-HPLC/ESI-MS. Column, monolithic NBE/DMN-H6, 60 × 0.20 mm i.d.; mobile phase, (A) 25 mM butyldimethylammonium bicarbonate, pH 8.9 and (B) 25 mM butyldimethylammonium bicarbonate, pH 8.9, 80% acetonitrile; linear gradient, 5−70% B in 10 min; flow rate, 4 μL/min; temperature, 60 °C; sample, $A_{PS}G_{PS}A_{PS}A_{PS}$AAGGGGG-$GACU_{PS}G_{PS}G_{PS}A_{PS}$A, 50 ng + 30 mM EDTA (PS = phosphorothioate linkage; remaining linkages are phosphodiesters).

alleviated by the use of mixed backbone oligonucleotides. Currently, all-phosphodiester-, all-phosphorothioate, and mixed phosphodiester/phosphorothioate oligodeoxyribonucleotides can be prepared consistently and with excellent purity by automated solid-phase synthesis.[37] Phosphorothioate oligoribonucleotides, however, are more difficult to synthesize because of the presence of an extra hydroxyl group at the 2′-position, requiring a different strategy of protecting groups.[38,39]

Figure 7 shows the analysis of a synthetic oligoribonucleotide containing mixed phosphodiester and phosphorothioate linkages employing the monolithic capillary columns and ESI-MS. The chromatogram certifies that the target oligoribonucleotide is of high purity and does not contain significant amounts of failure sequences. Moreover, the intact molecular mass of 6417 calculated from the ESI-MS spectrum correlates excellently with the expected molecular mass of 6418.52, which proves that the correct sequence has been assembled, that the modified phosphorothioate linkages have been incorporated, and that the protecting groups have been completely removed. The incomplete hydrolysis of protecting groups usually remains undetected by conventional gel electrophoresis, chromatography, or hybridization assays, and only mass spectrometry has been shown to readily reveal such modifications.[40] However, the presence of protecting groups may significantly alter the binding properties of antisense oligonucleotides, and hence, their detection by mass spectrometry is essential in order to ensure maximum product quality. With analysis times of less than 10 min, IP-RP-HPLC/ESI-MS is suitable for the fast and efficient characterization of synthetic oligonucleotides of mixed backbone.

CONCLUSIONS

It has been demonstrated that the chromatographic separation performance of cross-linked, norbornene-based, monolithic capil-

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lary columns prepared via ROMP indicates good separation capabilities for single- and double-stranded nucleic acids as well as for proteins. Columns in the capillary format offer better chromatographic performance compared to their analogues in the analytical format presumably due to different surface morphologies as the result of different polymerization kinetics. Nevertheless, the fact that identical polymerization mixtures can be used for the preparation of both 200-μm and 3-mm-i.d. monlithic columns clearly demonstrates the reliability of the metathesis-based polymerization technology. Diastereoisomers of short phosphorothioate oligonucleotides are separable, while the longer oligomers coalesce into a single peak, with peak widths decreasing with increasing length of the oligonucleotides. With analysis times of less than 10 min, IP-RP-HPLC on-line interfaced to ESI-MS is suitable for the fast and efficient characterization of synthetic oligodeoxyribonucleotides and oligoribonucleotides.

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