High-performance liquid chromatographic separation of detritylated oligonucleotides on highly cross-linked poly-(styrene–divinylbenzene) particles

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ABSTRACT

Detritylated oligonucleotides were separated by reversed-phase high-performance liquid chromatography on highly cross-linked polystyrene-based particles having a mean particle diameter of 2.3 μm. The addition of poly(vinyl alcohol) during polymerization, which resulted in the presence of hydroxyl groups on the surface of the poly(styrene–divinylbenzene) beads, was necessary to obtain baseline resolution of phosphorylated oligodeoxyadenylic acids with a chain length of up to 30 bases. The impact of temperature was investigated and optimum resolution was achieved at 40°C. At pH 7.0, the retention times of oligonucleotides were found to depend on the ratio of bases and to increase in the order of C < G < A < T. Under the same conditions, it was possible to separate phosphorylated from dephosphorylated oligonucleotides, the former being eluted earlier. Recoveries ranged from 92 to 100%.

INTRODUCTION

Because of the increasing need for linkers, primers and probes in molecular biology, the demand for single-stranded oligonucleotides has grown rapidly over the past few years. Since the introduction of phosphoramidite chemistry [1], it has been possible to prepare them fairly easily in a short time by using an automated synthesizer. However, the samples synthesized usually contain many impurities together with the target oligonucleotides and hence purification is essential. Polyacrylamide gel electrophoresis [2] and conventional liquid chromatography [3] are mainly employed for this purpose, but they are time consuming and laborious. Therefore, it has been attempted to apply high-performance liquid chromatography (HPLC), in particular reversed-phase [4–6] and ion-exchange modes [7–9]. In reversed-phase HPLC separations, advantage is taken of the hydrophobic 5′-protecting group used during solid-phase synthesis, namely the dimethoxytrityl group, to resolve the desired product from 5′-hydroxylated failure sequences. This approach, however, requires the subsequent hydrolysis and extraction of the purified oligonucleotides before their use in molecular biological experiments. Ion-exchange chromatography, on the other hand, offers high resolution of oligonucleotides which have been already detritylated on the synthesizer, but requires subsequent desalting. Therefore, an optimum chromatographic purification protocol should employ a stationary phase that allows the separation of detritylated oligonucleotides using volatile buffer systems which can be readily evaporated.

Based on the recent demonstration that reversed-phase chromatography on highly cross-linked poly(styrene–divinylbenzene) (PS–DVB) particles is very effective for the rapid analysis of proteins with

high resolution [10], we have now investigated the applicability of PS–DVB for the separation of detritylated oligonucleotides.

EXPERIMENTAL

Instrumentation

The HPLC system consisted of two pumps (Model 114M, Beckman, Berkeley, CA, USA), a dynamic gradient mixer (Model 340, Beckman), a gradient controller (Model 421, Beckman), a sample injection valve (Model 7125, Rheodyne, Cotati, CA, USA) with a 20-μl sample loop, a variable-wavelength UV monitor (Model 484, Waters, Milford, MA, USA), a column oven (Model CTO-2A, Shimadzu, Kyoto, Japan) and an integrator (Model C-R6A, Shimadzu).

Chemicals

Styrene, divinylbenzene (DVB) and poly(vinyl alcohol) (PVA) were purchased from Riedel-de Haën (Seelze, FRG). HPLC gradient-grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Buffers were prepared using a stock solution of 2 M HPLC-grade triethylammonium acetate (TEAA) (Applied Biosystems, San Jose, CA, USA) and high-purity water (NANOpure; Barnstead, Newton, MA, USA).

Oligonucleotides

Standards of dephosphorylated oligodeoxyadenylic acids [d(A)$_{12}$, d(A)$_{12-18}$], phosphorylated oligodeoxyadenylic acids [pd(A)$_{12}$, pd(A)$_{14}$, pd(A)$_{16}$, pd(A)$_{12-18}$, pd(A)$_{19-24}$, pd(A)$_{25-30}$], phosphorylated oligodeoxycytidylic acids [pd(C)$_{12-18}$], phosphorylated oligodeoxyguanylic acids [pd(G)$_{12-18}$] and phosphorylated oligodeoxythymidylic acids [pd(T)$_{12-18}$] were purchased from Pharmacia (Uppsala, Sweden). Using phosphoramidite chemistry, the oligonucleotides listed in Table I were synthesized on a DNA synthesizer (Model 381-A, Applied Biosystems). Subsequently, they were purified by means of oligonucleotide purification cartridges (Applied Biosystems).

Column

For a 50 × 4.6 mm I.D. column, 1.1 g of the highly cross-linked PS-DVB particles [60% (v/v) DVB], which had been prepared by a two-step mi-

crosuspension method either in the absence or in the presence of 0.1% (w/v) PVA [11], were suspended in dioxane. The slurry was then sonicated and packed into the column at a pressure of 70 MPa with the use of an air-driven pump (Model Maximator MSF 111, Ammann Technik, Kölliken, Switzerland) and methanol as the driving solvent. Thereafter, methanol was replaced with water at the same inlet pressure.

Chromatographic conditions

Gradient profiles used for reversed-phase separations are given on each chromatogram. Isocratic runs at appropriate concentrations of organic solvent were made to determine the number of theoretical plates and resolution. The aqueous buffer was 0.1 M TEAA (pH 7.0) throughout. In order to keep the concentration of TEAA constant and not to be affected by volume contraction during mixing of organic solvents with water, the mobile phase was prepared as follows: for a 10% solution of acetonitrile in 0.1 M TEAA, 50 ml of the 2 M TEAA stock solution were added to 100 ml of acetonitrile in a 1000-ml volumetric flask and the final volume was made up to 1000 ml by the addition of water.

RESULTS AND DISCUSSION

Fig. 1 shows a scanning electron micrograph of the stationary phase particles made of a highly cross-linked styrene–divinylbenzene copolymer. Be-
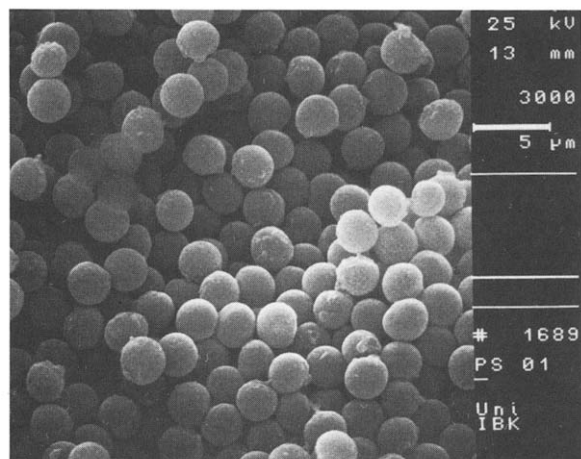

Fig. 1. Scanning electron micrograph of the cross-linked poly-(styrene-divinylbenzene) particles used.

cause of their uniform size, no sieving of the beads was required. The mean size and the specific surface area of the particles as assessed by mercury porosimetry were 2.3 μm and 1.3 m^2/g, respectively. No significant differences in size and surface area could be discerned between particles polymerized either in the absence or in the presence of PVA. Moreover, no pores larger than 30 Å could be detected. The advantage of a highly cross-linked PS–DVB adsorbent is that the totally organic polymer is operable over a wide pH range, typically 1–13, without any damage occurring to the packing. This results in a long column lifetime and allows the regeneration of a deteriorated column with aqueous sodium hydroxide, which is of great advantage in the analysis of biopolymers.

The chromatographic performance of PS–DVB batches polymerized without or with PVA is shown in Fig. 2a and b, respectively. It is evident that the addition of PVA during polymerization results in superior resolution of phosphorylated oligodeoxycytidylic and oligodeoxythymidylic acids 12–18 nu-
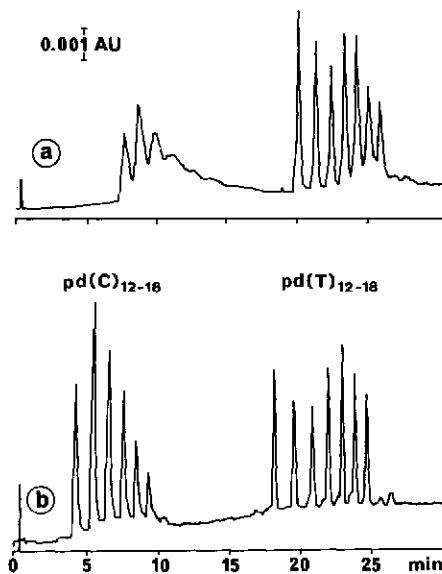
cleotides in length. PVA was used as a stabilizer during the polymerization step and it is known to be incorporated into the surface of PS beads [12]. Moreover, it has been demonstrated [13] that the adsorption of PVA at a polystyrene–latex surface yields layers of controlled thickness, thus creating both a more homogeneous and also, owing to the presence of hydroxyl groups, a less hydrophobic surface in comparison with PVA-free beads. Hence, the addition of PVA during polymerization led to both shorter retention times and enhanced resolution owing to improved mass transfer.

The effect of temperature on the separation of phosphorylated oligodeoxyadenylic acids 14 and 16 bases in length was studied under isocratic conditions. Optimum resolution (R_s = 5.8) was obtained at column temperature of 30–40°C. This is also reflected in the number of theoretical plates, which was about 2 · 10^4 m at 40°C with acetonitrile as organic modifier and which declined thereafter to 1 · 10^4/m at 60°C (Fig. 3). With methanol, the maximum resolution was observed at 22°C (R_s = 5.7) and the number of theoretical plates decreased steadily from 17 · 10^3 to 3 · 10^3/m at 22 and 60°C, respectively (Fig. 3). All subsequent separations were carried out with acetonitrile at 40°C because the time of analysis is shorter and the formation of secondary structures due to either self-complemen-
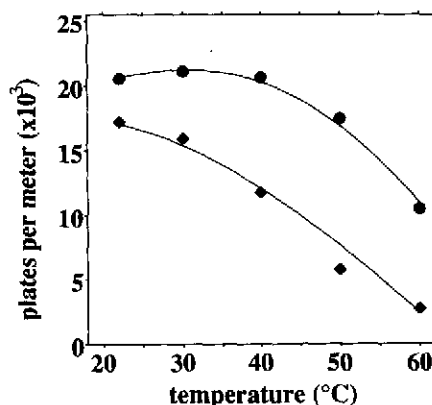

Fig. 2. Comparison of two batches of PS–DVB particles polymerized either (a) without or (b) with PVA. Column, PS–DVB (2.3 μm, 50 × 4.6 mm I.D.); eluents, (A) 0.1 M TEAA (pH 7.0) and (B) 0.1 M TEAA–acetonitrile (75:25), linear gradient from 10 to 50% B in 40 min; flow-rate, 1 ml/min; temperature, 40°C; detection, UV, 254 nm; sample, 0.5 μg each of pd(C)$_{12-18}$ and pd(T)$_{12-18}$.

Fig. 3. Number of theoretical plates as a function of temperature under isocratic conditions. Column, PS–DVB–PVA (2.3 μm, 50 × 4.6 mm I.D.); eluents, (●) 0.1 M TEAA (pH 7.0)–acetonitrile (95.3:4.7) and (♦) 0.1 M TEAA (pH 7.0)–methanol (86.8:13.2); flow-rate, 1 ml/min; detection, UV, 254 nm; sample, 0.165 μg of pd(A)$_{14}$.
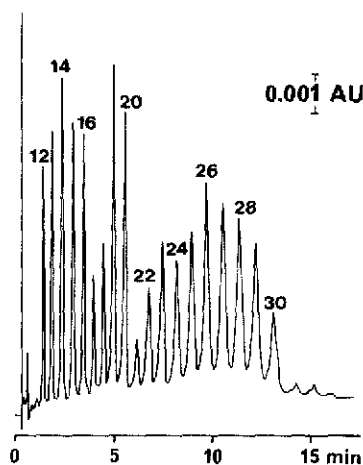
Fig. 4. Chromatogram of a mixture of oligodeoxyadenylic acids, pd(A)$_{12\text{-}30}$. Column, PS–DVB–PVA (2.3 µm, 50 × 4.6 mm I.D.); eluents, (A) 0.1 *M* TEAA (pH 7.0) and (B) 0.1 *M* TEAA–acetonitrile (90:10), linear gradient from 48 to 60% B in 3 min, followed by a 20-min linear gradient from 60 to 80% B; flow-rate, 1 ml/min; temperature, 40°C, sample size, 1.125 µg.

tarity or a high G content of the oligonucleotides [14] is reduced.

Using a 3-min linear gradient from 4.8 to 6.0% acetonitrile followed by a 20-min linear gradient from 6.0 to 8.0% acetonitrile, a ladder of phospho-
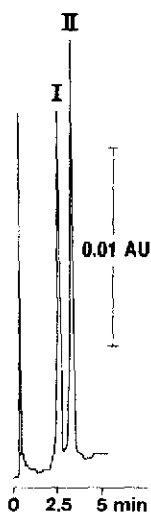

Fig. 5. Chromatogram of two detritylated heterooligonucleotides differing in length by one nucleotide. Column, PS–DVB–PVA (2.3 µm, 50 × 4.6 mm ID.); eluents, (A) 0.1 *M* TEAA (pH 7.0) and (B) 0.1 *M* TEAA acetonitrile (90:10), linear gradient from 60 to 80% B in 5 min; flow-rate, 1 ml/min; temperature, 40°C; detection, UV, 254 nm; sample size, (I) 0.29 µg and (II) 0.31 µg. For peak identification, see Table I.

rylated oligodeoxyadenylic acids ranging from 12 to 30 nucleotides in length could be resolved completely in less than 13 min (Fig. 4). Subsequently, the efficiency of PS–DVB–PVA particles in separating failure sequences from the desired heterooligonucleotide was evaluated. As shown in Fig. 5, a detri-
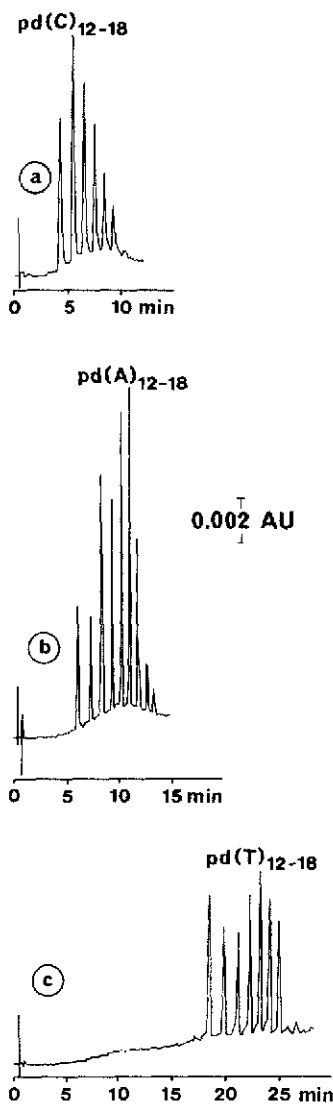

Fig. 6. Retention behaviour of various phosphorylated homooligonucleotides. Column, PS–DVB–PVA (2.3 µm, 50 × 4.6 mm I.D.); eluents, (A) 0.1 *M* TEAA (pH 7.0), and (B) 0.1 *M* TEAA–acetonitrile (75:25), linear gradient from 10 to 50% B in 40 min; flow-rate, 1 ml/min; temperature, 40°C; detection, UV, 254 nm; samples, (a) pd(C)$_{12\text{-}18}$, (b) pd(A)$_{12\text{-}18}$ and (c) pd(T)$_{12\text{-}18}$, 0.5 µg each.

TABLE I

SEQUENCES OF THE OLIGONUCLEOTIDES ANALYSED

Peak	Sequence (5'–3')	Length	C (%)	G (%)	A (%)	T (%)	k'^a
I	GCTCAGTGTAGCCCAGGAT	19	26.2	31.6	21.1	21.1	17.22
II	TGCTCAGTGTAGCCCAGGAT	20	25.0	30.0	20.0	25.0	18.39
III	CATGGGAGGGTTAGATAG	18	5.6	44.4	27.8	22.2	16.16
IV	AGTAGGTGGAAGATTCAG	18	5.6	38.9	33.3	22.2	17.37
V	GTGCTCAGTGTAGCCCAGGATC	22	27.3	31.8	18.2	22.7	18.52
VI	GTGCTCAGTGTAGCCCAGGATG	22	22.7	36.4	18.2	22.7	18.69
VII	GTGCTCAGTGTAGCCCAGGATA	22	22.7	31.8	22.7	22.7	19.08
VIII	GTGCTCAGTGTAGCCCAGGATT	22	22.7	31.8	18.2	27.3	19.39

^{a} Capacity factors were determined using a linear gradient of 3% to 10% acetonitrile in 0.1 M TEAA in 10 min; flow-rate 1 ml/min; see Fig. 5.

tylated 20-mer oligonucleotide could be separated sucessfully from a detritylated 19-mer oligomer.

Reversed-phase chromatographic separations of oligonucleotides have been found to depend significantly on base composition [15,16]. As can be seen in Fig. 6, the retention times of homooligonucleotides increased in the order C < A < T. Owing to their tendency to fold back and to form aggregates, oligodeoxyguanylic acids (n = 12–18) could not be separated successfully even under strongly dena-

Fig. 7. Effect of base composition on the separation of two hetero-oligonucleotides of the same chain length. Column, PS–DVB–PVA (2.3 μm, 50 × 4.6 mm I.D.); eluents, (A) 0.1 M TEAA (pH 7.0) and (B) 0.1 M TEAA–acetonitrile (90:10), linear gradient from 65 to 80% B in 2 min; flow-rate, 1 ml/min; temperature, 40°C; detection, UV, 254 nm; sample size, 0.28 μg each. For peak identification, see Table I.

turing conditions such as 2-min heating at 80°C in the presence of 2 M NaOH or 80% formamide, but were eluted as a single broad peak.

Based on this characteristic behaviour of reversed-phase chromatography, even isomeric oligonucleotides which differ only in respect of their base composition can be resolved. The sole difference between the two oligonucleotides separated in Fig. 7 is the replacement of one residue of adenine with a residue of guanine. As guanine is less retained than adenine, the oligonucleotide with the higher content of guanine is eluted earlier. In addition to base composition, the sequence of bases may also affect their retention behaviour on PS–DVB–PVA. This is corroborated by the k' values given in Table I. Whereas the exchange of the last base at the 3'-end does exert a small effect on the k' values of the oligonucleotides V–VIII in the order C < G < A < T, the far greater difference in k' values observed between the oligonucleotides III and IV is due to the presence of a cluster of six guanine bases in the former, which influences the retention behaviour far more than just the exchange of a single base.

For certain experiments in molecular biology, such as in situ hybridization, it is necessary to radiolabel oligonucleotides with [^{32}P]ATP. In order to avoid competitive hybridization of unlabelled probes to the DNA template, purification of the radiolabelled oligonucleotide is required [17]. As can be seen in Fig. 8, reversed-phase chromatography on PS–DVB–PVA particles permits the separation of a dephosphorylated from a phosphorylated 12-mer oligodeoxyadenylic acid. According to the sup-
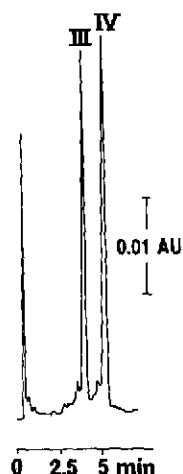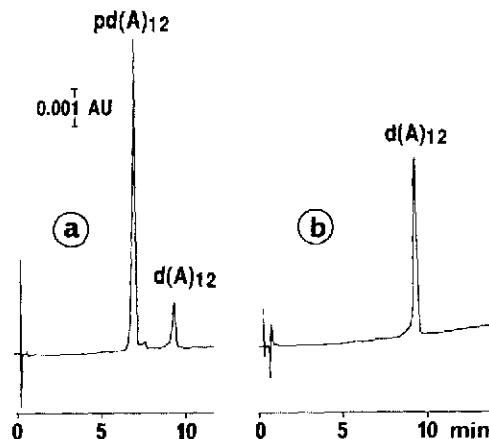
Fig. 8. Chromatograms of commercial preparations of (a) dephosphorylated and (b) phosphorylated 12-mer oligodeoxyadenylic acids. Column, PS–DVB–PVA (2.3 μm, 50 × 4.6 mm I.D.); eluents (A) 0.1 M TEAA (pH 7.0) and (B) 0.1 M TEAA–acetonitrile (90:10), linear gradient from 30 to 75% B in 20 min; flowrate, 1 ml/min; temperature, 40°C; detection, UV, 254 nm; sample size. 0.25 μg each.

plier, phosphorylated oligodeoxyadenylic acids are prepared first in the dephosphorylated form using phosphoramidite chemistry and, following purification, they are submitted to phosphorylation by polynucleotide kinase. From Fig. 8a, it can be seen clearly that the phosphorylated product contains non-phosphorylated parent material (Fig. 8b). Recoveries of different non-phosphorylated and phosphorylated oligonucleotides were in the range 92–100% (Table II).

TABLE II

RECOVERIES OF OLIGONUCLEOTIDES

Oligonucleotide	Amount injected (μg)	Recovery (%)
d(A)$_{12}$	0.75	98.7
II[a]	0.31	100.3
VII[a]	0.34	92.7
pd(A)$_{12}$	0.75	93.3
pd(A)$_{16}$	0.50	92.8
pd(T)$_{16}$	0.13	91.8

[a] For sequence, see Table I.

CONCLUSIONS

Detritylated oligonucleotides up to 30 nucleotides in length have been resolved within a few minutes on PS–DVB–PVA particles. Further, phosphorylated probes have been separated successfully from their non-phosphorylated analogues. The recoveries being excellent, HPLC on PS–DVB–PVA particles may prove a valuable tool in the purification of oligonucleotides, because they can be used in subsequent molecular biological experiments such as polymerase chain reaction and *in situ* hybridization without any further pretreatment after evaporation of the eluent.

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