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PURIFICATION OF HOMO- AND HETERO-OLIGONUCLEOTIDES USING HIGH-PERFORMANCE CHARGE-TRANSFER CHROMATOGRAPHY

PETER A. D. EDWARDSON*
Microbial Technology Laboratory, PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG (U.K.)
CHRISTOPHER R. LOWE
The Biotechnology Centre, University of Cambridge, Downing Site, Downing Street, Cambridge (U.K.)
and
TONY ATKINSON
Microbial Technology Laboratory, PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG (U.K.)
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SUMMARY

Oligonucleotides employed in molecular biology have previously been purified by gel electrophoresis, gravity flow chromatography and more recently, high-performance liquid chromatography. However, these techniques have a number of problems and for this reason we investigated high-performance charge-transfer chromatography using the dye acriflavin coupled to silica as the stationary phase. Numerous oligonucleotides were purified using this technique and in this report we present data on four such oligonucleotides two homo-oligonucleotides and two hetero-oligonucleotides. Homogeneity of oligonucleotides eluted from the acriflavin matrices was determined by electrophoresis on 20% polyacrylamide gels and in each case they were greater than 90% pure.

INTRODUCTION

The synthesis and purification of homo- and hetero-oligonucleotides is an area of growing interest and is required for the construction and use of specific gene probes and for diagnostic identification of, for instance, microbial infections. Oligonucleotide purifications can be achieved in a variety of ways; gel electrophoresis[1], gravity flow chromatography[2,3] or, more recently high-performance liquid chromatography (HPLC)[4–6]. Gel electrophoresis, however, is tedious and time consuming often resulting in poor yields, gravity flow chromatography is effective but has only been exploited for short oligonucleotides, whilst HPLC, both in the reverse-phase and ion-exchange modes has yielded irreproducible data.

We have investigated high-performance charge-transfer chromatography (HPCTC) for the separation of homo- and hetero-oligonucleotides using the charge-transfer dye acriflavin coupled to silica as the stationary phase.

The formation of neutral molecular complexes by electron displacement or transfer from one component, the donor to a second component, the acceptor, has been recognised for some time[7]. As charge-transfer complexes of this type commonly involve π–π interactions, desirable properties of the donor and acceptor depend to a large extent on the type and number of aromatic rings and on the presence or absence of electron donating and withdrawing substituents. Results on acriflavin-agarose matrices[3,8], and acriflavin-silica matrices[9] show that this dye exhibits excellent electron-acceptor properties towards nucleotides, particularly purines and therefore was selected for detailed investigation into the utility of HPCTC.

MATERIALS AND METHODS

LiChrosorb Si 100 (5 μm) (E. Merck) silica was epoxysilylated with γ-glycidoxypropyltrimethoxysilane and substituted with acriflavin as described previously[9–11].

All single stranded oligonucleotides were synthesised on a DNA synthesiser (Applied Biosystems, Model 380A). Oligonucleotides were detritylated and deprotected to provide crude product containing the desired oligonucleotide (n) in addition to $n-1$, $n-2$, $n-3$, $n-(n-1)$, which correspond to shorter oligonucleotides formed from incomplete coupling reactions during synthesis. Once deprotected the oligonucleotides were evaporated to dryness and dissolved in water (1 ml) to give a concentration of approx. 1–2 mg/ml.

Homogeneity of oligonucleotides eluted from the acriflavin-silica matrices was determined by electrophoresis on 20% polyacrylamide gels[1].

The acriflavin-silica matrices were packed into stainless-steel columns (150 mm × 4.5 mm) with the downward slurry packing technique[10] in methanol at 20.7 MPa (3000 p.s.i.). All chromatographic procedures were performed at ambient temperature (18–22°C) using Waters Assoc. (Hartford, Northwich, U.K.) HPLC equipment.

Buffers used in most chromatographic procedures were: 20 m*M* potassium phosphate, 20% acetonitrile (v/v) and 20 m*M* potassium phosphate, 20% acetonitrile (v/v), 1 *M* potassium chloride. Salt gradients comprised various curves, *i.e.* linear and non-linear, and pH values (3.2–7.3).

RESULTS AND DISCUSSION

Charge-transfer interactions have been recognised for some time[7] and it is well established that acriflavin interacts strongly with purines and to a lesser extent with pyrimidines. This interaction is related to the energies of delocalised π electrons in the highest occupied molecular orbitals[12]. The stability of complexes is determined by the energy difference between the highest occupied molecular orbitals of the donor and the lowest unoccupied molecular orbitals of the acceptor. In general, the shorter the distance between the energy levels the stronger the charge-transfer complex formed. However, the structure of acriflavin (Fig. 1) indicates that hydrophobic interactions between aromatic moieties of the support and the solute could occur. Furthermore, the positive charge on the nitrogen at position 9 (N^9) imparts a cationic character to the dye and can provide additional ionic effects at low ionic strength. Therefore, to minimise these undesired solute–matrix interactions an organic modi-

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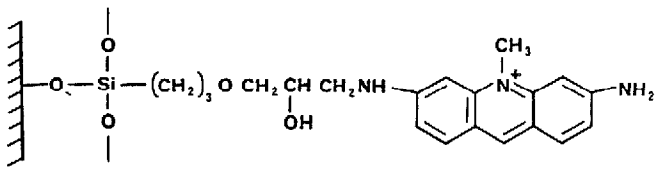

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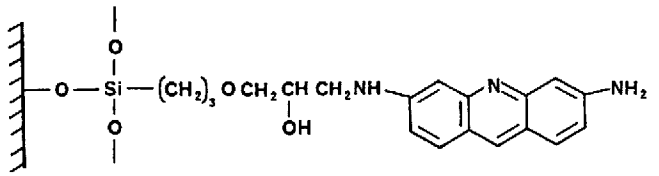

Fig. 1. (a) The structure of the 3,6-diamino-10-methyl acridinium-silica (acriflavin-silica) adsorbent. (b) The structure of the 3,6-diaminoacridinum-silica (proflavin-silica) adsorbent.

fier (acetonitrile) was employed in the buffer system and longer oligonucleotides were separated at a relatively high ionic strength (0.25–0.30 *M* potassium chloride).

Ligand choice and optimal ligand substitution levels

3,6-Diamino-10-methylacridinium chloride (acriflavin) and its non-methylated uncharged analogue, 3,6-diaminoacridinium chloride (proflavin) (Fig. 1) were individually attached to silica to investigate the importance of the methyl group and positive charge at N^9 position in the retention of oligonucleotides. Although acriflavin and proflavin exhibited similar retention behaviour for the bases adenine and thymine, oligonucleotides of $n = 2$ and greater (n = number of bases) were eluted in the void volume of the proflavin column, whereas they were retained on the acriflavin matrix.

Previous experiments employing acriflavin-agarose supports[3,8] indicated that a low ligand substitution level is favourable for separation of single stranded DNA (ssDNA). A number of matrices with a range of ligand substitution levels (3 μmol acriflavin/g silica–17 μmol acriflavin/g silica) were subsequently synthesised and their resolving power was investigated by calculating resolution of the homo-oligonucleotide dT10 under constant buffer conditions (A = 20 m*M* potassium phosphate, 20% acetonitrile, pH 6.5; B = 20 m*M* potassium phosphate, 20% acetonitrile, 1 *M* potassium chloride, pH 6.5; linear gradient 0–30% buffer B in 20 min).

Under these conditions, the optimal ligand substitution level was 7 μmol acriflavin/g silica (dry weight).

Optimal buffer conditions

Adsorption of oligonucleotides to the acriflavin matrix is favoured at low ionic strength[3], increasing the ionic strength decreased the extent of the interaction between the oligonucleotides and the immobilised dye. The equilibrating buffer comprised a

low ionic strength competing ion (20 m*M* potassium phosphate) and an organic modifier (acetonitrile). Phosphate buffers gave optimal resolution for the shorter oligonucleotides $n = 1$–5, possibly due to their competing with the phosphate groups on the oligonucleotides for electrostatic interactions with the positively charged ma-

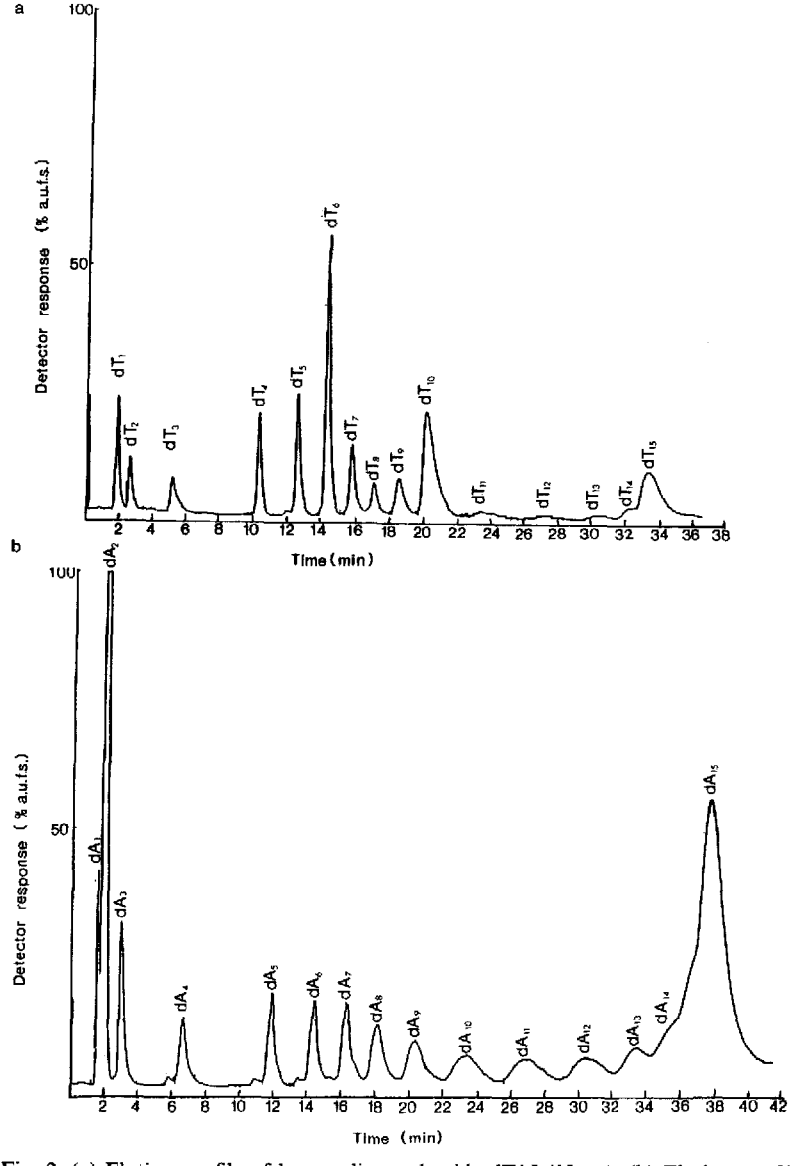

Fig. 2. (a) Elution profile of homo-oligonucleotide dT15 (10 μg). (b) Elution profile of homo-oligonucleotide dA15 (10 μg). Buffer conditions: A = 20 m*M* potassium phosphate, 20% acetonitrile (v/v), pH 6.5. B = 20 m*M* potassium phosphate, 20% acetonitrile, 1 *M* potassium chloride, pH 6.5. Gradient, 0–30% B in 40 min concave curve: flow-rate, 1 ml/min; pressure, 13.8 MPa (2000 p.s.i.); a.u.f.s., 0.2; temperature, ambient (18–22°C).

trix. Longer oligonucleotides were optimally eluted and separated with a salt gradient of potassium chloride.

The optimal pH was calculated as pH 6.0–6.5 using resolution data obtained from a series of pH values (pH 3.2–7.3).

These conclusions are contrary to those published by Boschetti *et al.*[3] who suggested an optimal pH of 4.0, and presented data to the effect that an increase in pH to 6.0 or above causes modification of the ionisation state of the support as well as that of the solute (modification of the electron density of the aromatic rings), which alters the elution molarity value for potassium chloride. Under high-performance conditions, we have experienced similar results, however, even though the k' and thus the elution molarity for potassium chloride is markedly decreased with high pH buffers, the resolution of oligonucleotides, especially longer oligomers (n = 15–17) is greatly increased at a higher pH (pH 6.0–6.5) and less peak broadening is observed.

Purification of oligonucleotides

Standard homo-oligonucleotides (deoxythymine 10-mer, dT10; deoxythymine 15-mer, dT15; and deoxyadenine, 15-mer, dA15) in addition to specific DNA probes were purified employing HPCTC.

Initial separations were performed on homo-oligonucleotides dT15 and dA15. Fig. 2a illustrates the purification of dT15 from a mixture also containing dT6 and dT10 using a concave gradient of 0–0.3 M potassium chloride pH 6.5 and Fig. 2b illustrates the purification of dA15 under the same conditions. As predicted from the theory of charge-transfer interactions, the purine oligonucleotide exhibited greater retardation than the pyrimidine dT15 suggesting that charge-transfer effects have a major role in the separation. Purified oligonucleotides were analysed by 20% polyacrylamide gel electrophoresis (Fig. 4) and both dT15 and dA15 were greater than 90% pure.

Oligonucleotides used in molecular genetics as DNA probes, however, are rarely homo-oligonucleotides[13]. Indeed, they are specific DNA sequences encoding certain amino acids which have unique codons (Met and Trp) or the other least ambiguous codons. Many hetero-oligonucleotides were investigated and as an illustration the separation of two heptadecamers are presented: DAB1 (CCA TGA AGT CGC TGG AA) and CAT1 (AGA CCC TTC AGC AT).

Fig. 3a illustrates the purification of CAT1 under the same elution conditions as above and Fig. 3b illustrates the purification of DAB1. Polyacrylamide gel analysis of these oligonucleotides (Fig. 4) confirmed that the heptadecamers purified by HPCTC were more than 90% pure.

CONCLUSIONS

In this report we have purified oligonucleotides up to a heptadecamer using HPCTC in approx. 40 min with high purity and reasonable yields. Oligonucleotides of length n = 18 have been purified using this technique, however, for $n > 19$, the resolution is greatly diminished. Prior to purification, desired oligonucleotides contained up to 30% contamination with shorter chain length oligonucleotides due to

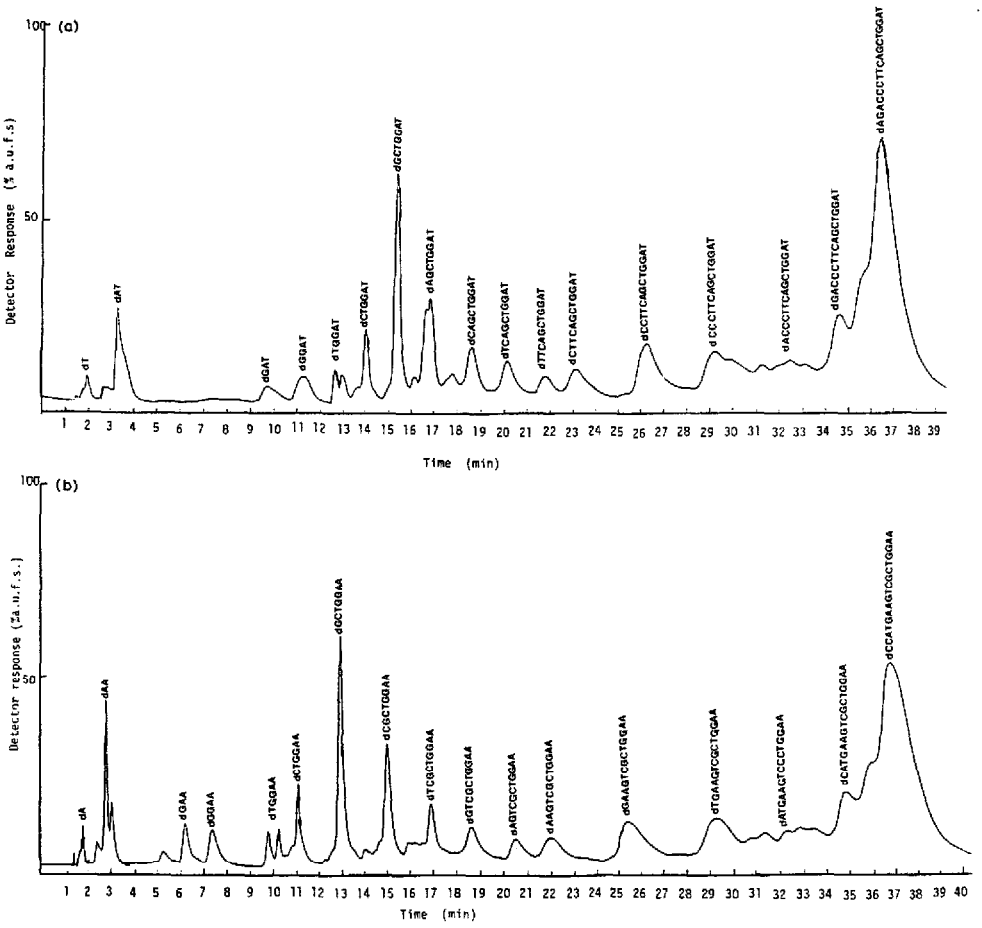

Fig. 3. (a) Elution profile of hetero-oligonucleotide CAT1 (10 μg). (b) Elution profile of hetero-oligonucleotide DAB1 (10 μg). Buffer conditions: A = 20 m*M* potassium phosphate, 20% acetonitrile (v/v), pH 6.5. B = 20 m*M* potassium phosphate, 20% acetonitrile, 1 *M* potassium chloride, pH 6.5. Gradient, 0–30% B in 50 min concave curve; flow-rate, 1 ml/min; pressure, 13.8 MPa (2000 p.s.i.); a.u.f.s., 0.2; temperature, ambient (18–22°C).

incomplete coupling reactions during synthesis. This contamination was eliminated by HPCTC of the samples.

The acriflavin-silica matrix exhibits both hydrophobic and ionic interactions with oligonucleotides because of the nature of its aromatic moieties and the cationic nitrogen (N^9). Hydrophobic interactions will probably occur between the aromatic rings of the acriflavin and the purine, pyrimidine and sugar moieties of the oligonucleotide. As the positive charge and methyl group at nitrogen (N^9) are important in the retardation of oligonucleotides, it is most likely that ionic interactions between the anionic phosphate groups on the oligonucleotide and the cationic dye are present.

Nevertheless, charge-transfer interactions play a major part in the resolution of oligonucleotides as is clear from the results shown in Fig. 2a and b, where the

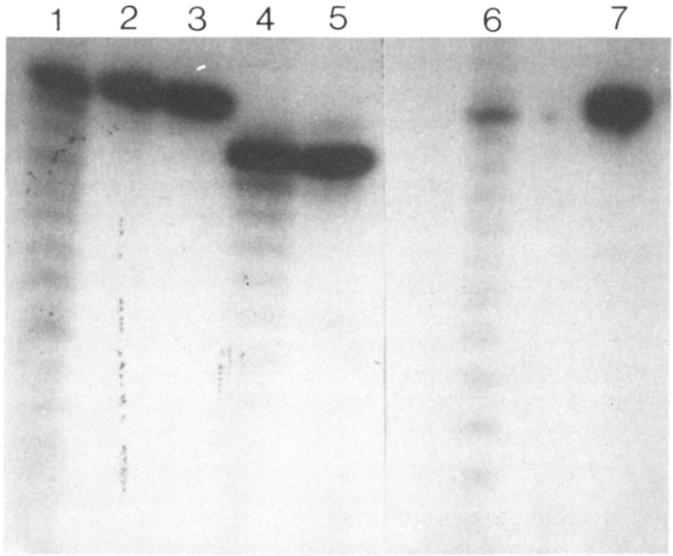

Fig. 4. Autoradiograph of DNA samples purified by HPCTC. Track 1: impure DAB1 (17-mer); track 2: purified DAB1 (17-mer); track 3: purified CAT1 (17-mer); track 4: impure dT15 (15-mer); track 5: purified dT15; track 6: impure dA15 (15-mer); track 7: purified dA15.

purine oligonucleotide dA15 is retarded longer than the pyrimidine dT15 as predicted by the theory of charge transfer[7].

Thus, the implication of cooperative adsorption effects on multi-functional stationary phases allows greater flexibility in the operation of this technique than is the case with other more conventional HPLC systems based on ion-exchange or reverse-phase. Resolution of single stranded oligonucleotides, particularly hetero-oligonucleotides, is more reproducible on the acriflavin-silica adsorbent than on the reversed-phase matrices and is comparable to that observed employing commercially available ion-exchange supports. Yields of 60–70% pure oligonucleotide are obtained from a single chromatographic run.

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