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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AMINO ACIDS, PEPTIDES, PROTEINS AND POLYNUCLEOTIDES

XCIV[a]. SOLID-PHASE HYBRIDIZATION OF COMPLEMENTARY OLIGONUCLEOTIDES

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SUMMARY

The ability of synthetic oligonucleotides to specifically hybridize to a complementary oligonucleotide immobilized on an anionic stationary phase has been investigated. A sigmoidal melting curve was obtained when oligonucleotide duplex formation on the column was plotted against hybridization stringency over the ionic strength range 0.2–0.42 *M*. These studies confirm a rapid method for determining the relative melting temperature of hybridized oligonucleotide complexes and provide a basis for the selection of stringency conditions optimal for various synthetic oligonucleotide probes.

INTRODUCTION

Synthetic oligonucleotide probes complementary to specific regions of DNA and RNA have found wide application in molecular biology, including Northern and Southern blot analysis[1,2], screening of gene libraries[3], *in vitro* mutagenesis[4] and as primers for template-directed polymerization enzymes, such as DNA polymerases and reverse transcriptase[5,6]. The ease of preparing oligonucleotides as compared to cloning a cDNA fragment has resulted in their increased use. Successful application of such probes requires the establishment of appropriate hybridization conditions that preclude non-specific annealing. At present, criteria for hybridization stringency are established by calculating theoretical melting temperatures, using published formulae[7]. However, due to the inherent limitations of mathematically derived melting temperatures, these estimations can significantly differ from experimentally observed values and their use can account for ambiguous or misleading results obtained in Northern and Southern blots, *in situ* hybridization to RNA and DNA and the screening of transfected plaques[8,9].

During investigation on the high-performance ion-exchange (HPIEX) chromatographic purification of oligonucleotides, we observed that some oligonucleotides were eluted as a broad or asymmetrical peak, while others produced a second, highly

[a] For Part XCIII see ref. 14.

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retained peak. Closer examination of the sequences revealed partially complementary regions, suggesting that self-hybridization may have occurred, producing complexes with altered retention characteristics. It was therefore decided to investigate in detail the hybridization of complementary oligonucleotides in a solid-phase system. Two oligonucleotides were loaded sequentially onto an anion-exchange column, so that the first was immobilized on the solid support and was available to form a complex with the second during its passage through the column. The amount of duplex formed was found to be related to the ionic strength of the loading buffer, and the data derived from these experiments enabled the construction of a melting curve. We propose that this model of solid-phase oligonucleotide hybridization can be used to study the interaction of various matched and slightly mismatched probes in order to obtain relative hybridization stringency. Furthermore, it is envisaged that the procedure can be adapted to approximate closely the situation found in Northern, Southern and plaque blot experiments and establish quickly and conveniently the appropriate hybridization conditions.

EXPERIMENTAL

Oligonucleotide synthesis

Oligonucleotides complementary to unique sections of the acidic and basic form of bovine fibroblast growth factor (FGF-β[10] and FGF-α[11], respectively) gene and of the *int-2* gene[12] were synthesized on an Applied Biosystems (Foster City, CA, U.S.A.) Model 380A DNA synthesizer using phosphoramidite chemistry. The synthesized sequences were: 5′GA CAC AAC CCC TCT CTC TTC TGC TTG 3′ (probe 01, FGF-β), 3′CT GTG TTG GGG AGA GAG AAG ACG AAC 5′ (probe 02, complement of probe 01), 5′CAC CTC CCC CAC GCT TTC CGC ACT G 3′ (probe 03, FGF-α), 5′G CCG TTG AGC TCC TGG CCC 3′ (probe 04, *int-2*).

Chromatographic methods

Chromatographic separations were performed by HPIEX liquid chromatography, using a Waters Assoc. (Milford, MA, U.S.A.) system, incorporating a U6K injector, two M600A pumps and an M660 gradient programmer with a Zorbax Bio Series Oligo column (DuPont) (80 mm × 6.2 mm I.D.). Eluent A consisted of 0.02 *M* phosphate buffer (pH 7.0)–20% acetonitrile, while eluent B contained 0.02 *M* phosphate buffer (pH 7.0)–20% acetonitrile–1 *M* NaCl. Oligonucleotides were routinely purified and analysed on the column with a 0–100% eluent B linear gradient over 1 h at 1.0 ml/min.

The elution of oligonucleotides was monitored at 260 nm with a Lambda Max (Waters) M481 LC spectrophotometer.

Hybridization experiments

Solution hybridization was performed with a mixture of probe 01 and probe 02 at a molar ratio of 1:2. The probes, in eluent A, were boiled for 3 min, and the solution was allowed to cool slowly over 1 h to allow hybridization. The mixture was then loaded onto the column and oligonucleotides were eluted with a 1-h 0–100% eluent B linear gradient at a flow-rate of 1 ml/min.

Solid-phase hybridization was accomplished by loading probe 01 in eluent A at

a flow-rate of 1.0 ml/min, followed by a two-fold molar excess of probe 02 in either 0, 10, 2, 30, 35, 40 or 43% eluent B at a flow-rate of 0.1 ml/min for 30 min. Immediately prior to each run probe 01 and probe 02 were denatured in boiling water for 3 min and quenched on ice. Following the loading of probe 02, the flow-rate was increased to 1.0 ml/min and the column was subjected to a linear gradient, commencing with the loading elution conditions for probe 02 to 100% eluent B at a rate of 1.67% eluent B per min, thus ensuring that the slope of the gradients was identical in all experiments.

RESULTS AND DISCUSSION

In the course of investigations on the regulation of expression of mRNAs coding for growth factors and putative oncogene products[13], we utilised HPIEX chromatography to purify oligonucleotide probes 01, 02, 03 and 04. The chromatographic profile associated with the purification of probe 01 exhibited a highly resolved symmetrical peak (results not shown). A single peak with identical retention was observed on rechromatography, demonstrating the homogeneous composition of the eluted oligonucleotide (Fig. 1). Other oligonucleotides (*e.g.*, probes 03 and 04) exhibited anomalous retention and band-broadening behaviour. Similar profiles were obtained on rechromatography of the major peaks (Fig. 2a and b). Thus, it is probable that under ideal HPIEX conditions some synthetic oligonucleotides are resolved according to charge differences in a sequential fashion with little evidence of effects arising from secondary or higher-order hierarchical structures. However, in other cases, such simple retention versus net charge dependencies do not prevail. Thus, chromatographic behaviour can be characterized as 'regular' when the elution is mediated by net charge and the capacity factor is relatively constant over a range of

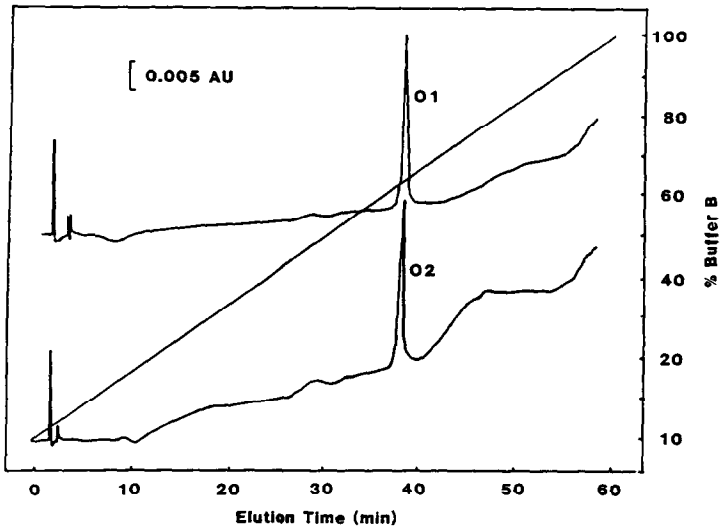

Fig. 1. Elution profiles of purified probe 01 and probe 02. Each oligonucleotide was fractionated on a Zorbax Bio Series Oligocolumn. Eluent A consisted of 0.02 *M* phosphate buffer (pH 7.0)–20% acetonitrile, while eluent B contained 0.02 *M* phosphate buffer (pH 7.0)–20% acetonitrile–1 *M* NaCl. A linear gradient of 0–100% eluent B was developed over 1 h at a flow-rate of 1.0 ml/min.

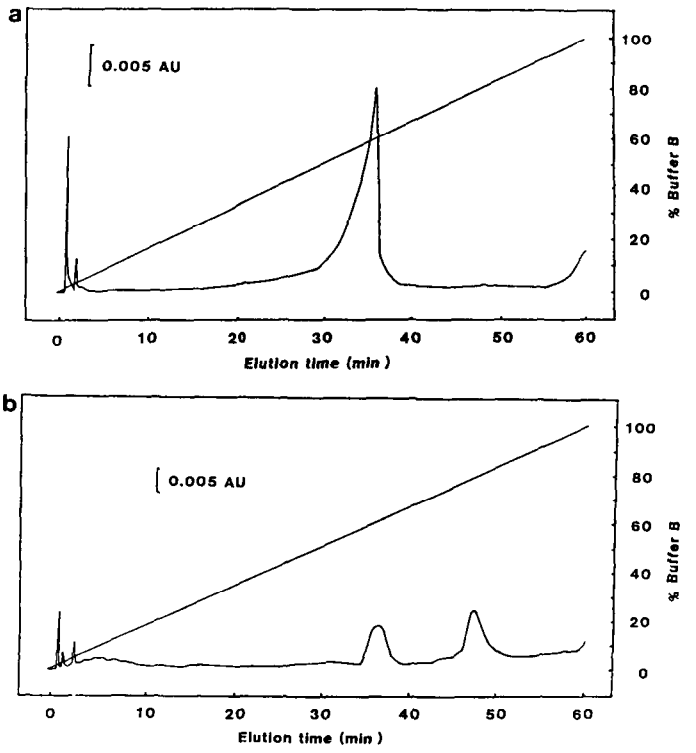

Fig. 2. Elution profiles of purified probe 03 (a) and probe 04 (b). Chromatographic conditions as described in Fig. 1.

mass loading conditions. Alternatively, chromatographic behaviour can be characterised as 'non-regular' when the elution is not mediated solely by net charge and the capacity factor, and the peak shape exhibits very complex dependencies on mass loading and concentration conditions. The latter behaviour may reflect aggregation or other slow, secondary chemical equilibrium processes involving the stationary phase surface. In the case of oligonucleotides, this behaviour could reflect internal autohybridization or inter-chain hybridization. This consideration led us to examine the possibility that chromatographic methods could be used to optimise hybridization conditions.

Two purified complementary 26-base oligonucleotide probes, probe 01 and probe 02, were used in the HPIEX chromatography hybridization experiments. The chromatogram of each probe exhibited a single sharp peak at 65% eluent B (Fig. 1). These oligonucleotides were also labelled at the 5′-end with [γ-^{32}P] dATP and found to migrate as a single band on polyacrylamide gel (data not shown).

Solution hybridization of a mixture of probe 01 and probe 02 was performed at a molar ratio of 1:2. The probes were boiled for 3 min in eluent A and allowed to cool slowly to ambient temperature. Fractionation of the sample by HPIEX chromatography on the Zorbax Bio Series Oligo column resulted in the elution of peaks A and B, as shown in Fig. 3a. While the monomeric forms of probes 01 and 02 are eluted

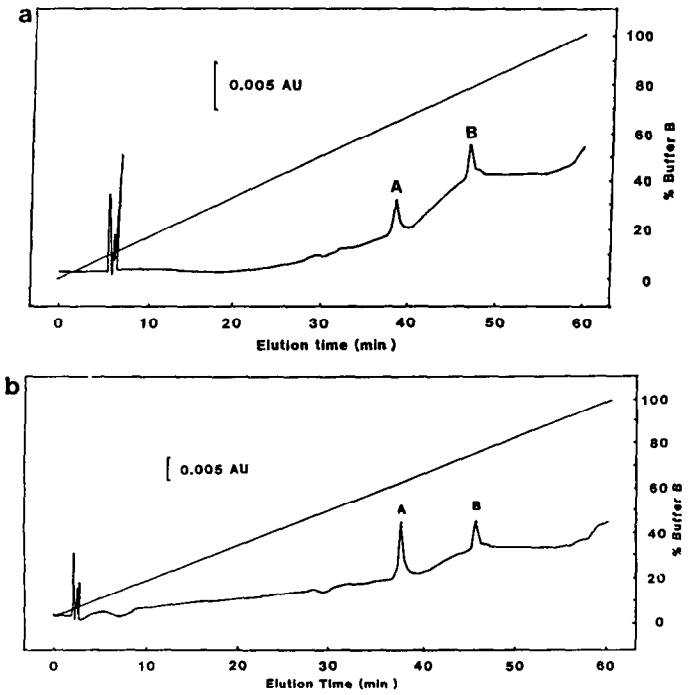

Fig. 3. Elution profiles of probe 01 and probe 02, hybridized in solution prior to loading (a). Peak B was collected, boiled in eluent B containing 50% deionized formamide and reloaded (b). Chromatographic conditions are as described in Fig. 1.

together as peak A at an eluent composition of 65% eluent B, an additional peak (B), containing duplex DNA, was eluted with 75% eluent B. Peak B was collected, boiled in eluent A containing 50% deionized formamide and reloaded. In addition to the expected elution of peak B an additional peak with a retention time identical to that of the individual oligonucleotide probes 01 and 02 was observed (Fig. 3b). Denaturation of the collected material and the subsequent appearance of monomeric probes 01 and 02, when rechromatographed, confirmed the duplex nature of peak B.

For solid-phase hybridization, probe 01 was immobilized on the ion exchanger prior to the loading a two-fold molar excess of probe 02. The salt gradient resulted in the elution of two peaks, 1 and 2 (Fig. 4a) from the column with retention times identical to those observed for peaks A and B, respectively, in Fig. 3. This suggests that the probing oligonucleotide, probe 02, hybridized to the immobilized target sequence, probe 01, in a manner indistinguishable from to that observed for solution hybridization when Fig. 3a is compared to Fig. 4a.

The ability of increasing salt concentrations to stabilize base pairing further and to facilitate more favourable hybridization stringency was investigated, using the same approach. Following loading of probe 01 onto the column, probe 02 was loaded in salt concentrations ranging from 0.0 to 0.43 *M* NaCl (Fig. 4a–g), and the magnitude of duplex formation at a particular ionic strength was calculated from the height/area of peak 2. In the absence of NaCl or at low NaCl concentrations, *e.g.* over the range

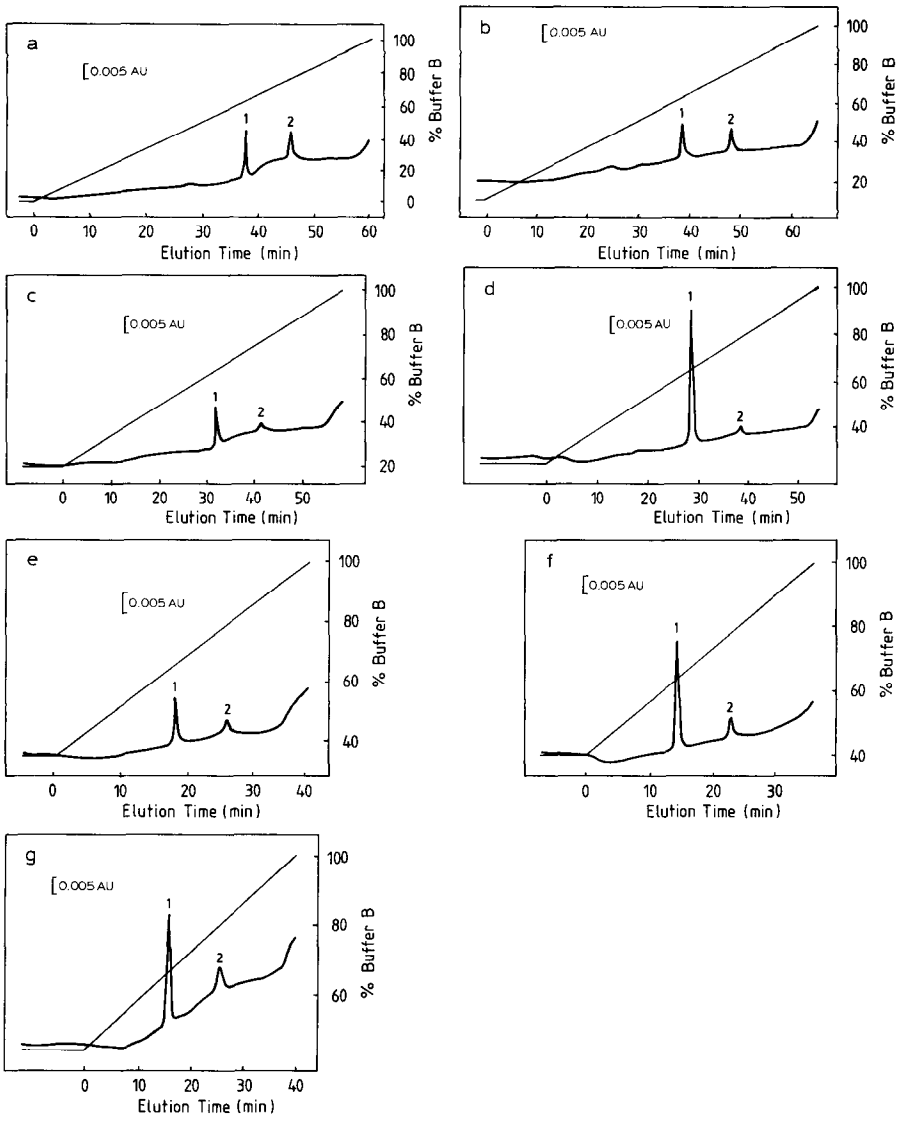

Fig. 4. Elution profiles of probes 01 and 02, allowed to hybridize on the column. After loading probe 01 in eluent A, a two-fold excess of probe 02 was loaded in (a) 0% eluent B, (b) 10% eluent B, (c) 20% eluent B, (d) 30% eluent B, (e) 35% eluent B, (f) 40% eluent B and (g) 43% eluent B at a flow-rate of 0.1 ml/min. The flow-rate was returned to 1.0 ml/min after loading probe 02, the oligonucleotides were eluted with a linear gradient from the loading conditions (a–g) of probe 02 and the gradient was developed to 100% eluent B at a rate of 1.67% eluent B per min. This elution protocol ensured that the slopes of the gradients were identical in all experiments.

0.0–0.1 *M* NaCl (Fig. 4a and b), significant hybridization occurred. With further increases of NaCl concentration up to 0.35 *M* (Fig. 4c–e) lower levels of complex formation were observed, but with high salt concentrations of 0.40 and 0.43 *M* (Fig. 4f and g) hybridization was facilitated, as indicated by the increased height of peak 2.

Data on the efficacy of hybridization above 0.43 *M* NaCl could not be obtained, due to the low retention of probe 01 on the column.

The significant degree of hybridization that was observed at low ionic strength (0.0–0.1 *M* NaCl) may, in part, be due to the high affinity of probe 01 for the column matrix under these conditions. The strength of this binding serves to retard the mobility of probe 01 and, therefore, to enhance the stability of the probe 01–probe 02 complex. Evidence for similar enhancement of duplex stability by solid phases is already available[8,9] from comparative data on the estimates of melting temperature, obtained by solution hybridization procedures and by probing of sequences immobilised on membranes.

In Fig. 5 is shown the derived melting curve for the probe 01–probe 02 hybrid, calculated by plotting the amount of duplex formation against the NaCl concentration. As is evident from Fig. 5, a number of interactive events occur at the different ionic conditions. Hybridization of the probing oligonucleotide to an immobilized oligonucleotide becomes energetically favourable, even at low salt concentrations, if the flexibility of the target oligonucleotide is constrained by the strength of its bonding to the solid phase. This matrix-mediated effect allows the weak interaction between complementary nucleotides to maintain the complex at low ionic strength. At intermediate ionic conditions, the target probe, although still interacting with the column materials exhibits considerable flexibility, and this behaviour tends to disrupt the bonds mediating hybridization.

At high ionic strength, hybridization is favoured, due to preferential bonding associated with the specific recognition. Although the target probe under these conditions would have considerable mobility and structural flexibility, the strength of the hybridization interaction nevertheless ensures the formation of a hybridized complex. A sigmoidal melting curve (comparable to other solid-phase melting curves generated when probing RNA and DNA on nitrocellulose, Zeta Probe and Gene Screen[13]) was obtained when oligonucleotide duplex formation on the column was

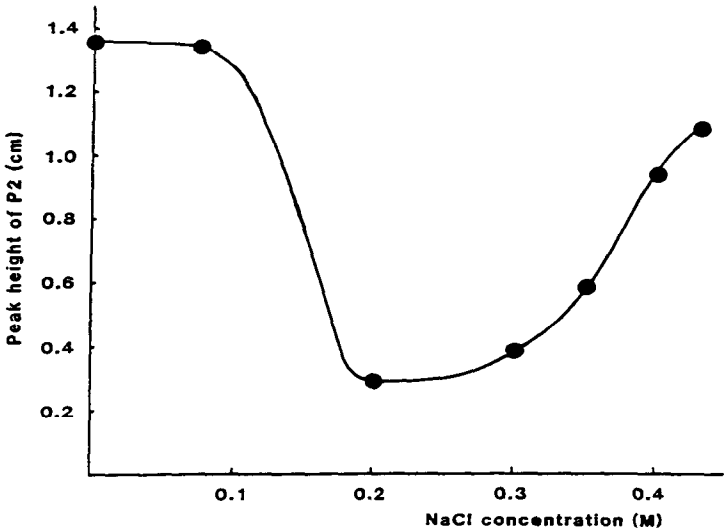

Fig. 5. Calculated melting point curve for the hybridization of probes 01 and 02.

plotted against hybridization stringency over the ionic strength range 0.2–0.42 *M*. While maximum hybridization could not be obtained due to the low retention of the oligonucleotides in high salt conditions the estimated melting temperature was considerably less than the theoretical value[7], *e.g.* up to *ca.* 20°C difference in melting temperature. Similar divergencies between the theoretical and experimental values of the 'melting temperature' are commonly found when dimethyl formamide is added to the buffer solutions with other solid-phase hybridization systems. This discrepancy could be due to the presence of acetonitrile added to the eluent to prevent hydrophobic interactions between the oligonucleotides and the column. Investigations currently underway will examine the ability of acetonitrile and other organic solvents to interface with the stacking of nucleotide bases. Nevertheless, the present results indicate that the described procedure is readily adaptable to examine the effects of one or more mismatches on the stability of the duplex derived from oligonucleotide probes. Results of these studies with additional oligonucleotides specifically designed to evaluate this question will be reported subsequently.

This paper shows that hybridization of oligonucleotide probes can be studied rapidly by the use of anion-exchange chromatography. With appropriate modification of instrumentation, including data handling standardisation, this procedure should be adaptable into a quantitative and predictive technique which will allow the investigation of hybridization of probes under a wide range of solvent/buffer conditions. Existing mathematical formulaes can predict optimum hybridization under a limited range of standard conditions (*e.g.* when performed with solutions containing NaCl, formamide and of pH 7.0–7.5). However, when probes are used as primers for template-directed polymerase enzymes, such as reverse transcriptase and DNA polymerases, conditions that ensure specific hybridization must be determined empirically, due to the unknown effects of such variables as pH or the salt type and its concentration. The technique described in this paper should enable the rapid assessment of the relative hybridization stringency over a range of such unique experimental conditions.

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