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Hydrophobic interaction chromatography of homo-oligonucleotides on derivatized Sepharose CL-6B Application of the solvophobic theory

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Abstract

This work describes the hydrophobic interaction chromatography of homo-deoxyoligonucleotides polyA, polyT and polyU with sizes up to 30 bases on a Sepharose gel derivatized with the 1,4-butanediol diglycidyl ether. The oligonucleotides interacted differently with the column according to the molecular mass, the hydrophobic character of the individual bases, the secondary structure of the molecule and the concentration of ammonium sulphate in the eluent. The retention factor, k', was determined from the chromatographic profiles at different concentrations of ammonium sulphate. A linear relationship between log k' and the concentration of ammonium sulphate in the eluent was found for all oligonucleotides at the higher concentrations (>1.0 M) of ammonium sulphate. The slope of these plots, termed the hydrophobic interaction parameter, was found to be an increasing function of the number of nucleotides. The same plots reveal that polyA molecules with high molecular mass have lower retention factors when compared with polyT, an observation that was not expected since the hydrophobicity of adenine is higher than that of thymine. This behaviour was due to the existence of secondary structures in polyA, which decrease the exposed hydrophobic area of the molecule.

Keywords: Hydrophobic interaction chromatography; Oligonucleotides

1. Introduction

Hydrophobic interaction chromatography (HIC) is a powerful separation technique for the purification of proteins [1]. This technique has also been recently used to purify plasmids for gene therapy applications [2]. In the cited study, a weak hydrophobic interaction chromatography gel was obtained by derivatisation of the cross-linked agarose matrix Sepharose CL-6B with 1,4-butanediol diglycidyl ether [3]. This chromatographic gel was able to separate double stranded supercoiled plasmid DNA from the more hydrophobic single stranded nucleic acid impurities from *Escherichia coli*. The present study uses synthetic homo-deoxyoligonucleotides of adenine (polyA), thymine (polyT) and uracil (polyU) with 6, 15 and 30 bases in order to clarify the mechanism of binding of single stranded molecules to the HIC gel. The results are interpreted on the basis of the solvophobic theory, which is briefly described next.

The effect of salt concentration on the interaction of biopolymers with weakly hydrophobic binding sites surfaces can be described through a simplified model. The magnitude of hydrophobic retention is determined by the free energy for the equilibrium

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distribution of the biomolecule between the bulk mobile phase and the stationary phase domains [4]. The corresponding equilibrium constant, K, can be written formally as:

$$\log K = -(\Delta G^{o}_{h\phi}/2.3RT) \quad (1)$$

where $\Delta G^{o}_{h\phi}$ is the Gibbs free energy change for the retention by hydrophobic interaction, R is the universal gas constant and T is the temperature [4]. The retention factor, k', which can be directly measured from chromatograms, is related to K according to:

$$k' = \phi K \quad (2)$$

where ϕ is the phase ratio.

In agreement with the solvophobic theory [5], the free energy change for the retention by hydrophobic interaction can be expressed as:

$$\Delta G^{o}_{h\phi} = \Delta G^{o}_{es} + \Delta G^{o}_{assoc} + \Delta G^{o}_{vdw} + \Delta G^{o}_{red} + N_{AV}(\gamma'_{S}A_{S} - \gamma'_{M}A_{M}) + RT\ln(RT/P^{o}V) \quad (3)$$

The term ΔG^{o}_{es} corresponds to the free energy change associated with electrostatic effects. In the absence of fixed charges at the surface of the stationary phase, the electrostatic contribution will be solely determined by this term, which vanishes at sufficiently high salt concentration [4]. The ΔG^{o}_{assoc} term corresponds to the biopolymer–ligand association in the absence of surrounding solvent, which means in the gas phase. ΔG^{o}_{vdw} corresponds to the Van der Waals interactions and ΔG^{o}_{red} corresponds to the reduction of the free energy due to the solvent–ligand and solvent–solute interactions not treated in the preceding terms. The next term corresponds to the net free energy changes associated with the cavity formation needed to accommodate the various species in the solvent. Variables γ'_{S} and γ'_{M} correspond to the surface tension of the stationary and mobile phase respectively, and A_{M} and A_{S} correspond to the molecular surface area of the free and bound molecule which is exposed to the mobile phase. The last term accounts for the entropy change arising from the change in the free volume [6], where P^{o} is the standard pressure and V is the mean molar volume of the solvent.

By extending the solvophobic theory to aqueous salts solutions, the free energy for retention in HIC can be related to the surface tension of aqueous solutions [6]. As an approximation, the surface tension of the mobile (γ'_{M}) and stationary (γ'_{S}) phase will be assumed to be equal, and equivalent to the surface tension of a salt solution, γ. Furthermore, we assume that γ can be expressed as a linear function of the molal surface tension increment of the salt, σ_{S}, through the equation:

$$\gamma = \gamma_{o} + \sigma_{S}m_{S} \quad (4)$$

where γ_{o} is the surface tension of the neat water and m_{S} the molal salt concentration. The parameter σ_{S} is thus a convenient measure of the propensity of a salt to increase the surface tension of aqueous solutions. In this way it is possible to simplify Eq. (3) by expressing $\Delta G^{o}_{h\phi}$ as a function of the molal concentration of the salt:

$$\Delta G^{o}_{h\phi} = \Delta G^{o}_{aq} - \Delta A'\sigma_{S}m_{S} \quad (5)$$

where ΔG^{o}_{aq} includes all the contributions to the retention free energy in the system except those which are due to the salt mediated hydrophobic interactions [4]. The term $\Delta A' = A_{M} - A_{S}$ corresponds to the change in the total surface area exposed to the mobile phase upon hydrophobic binding. The term ΔG^{o}_{aq} also includes the product $\Delta A'\gamma_{o}$ which accounts for the free energy differences of cavity formation in neat solvent in the absence of electrostatic effects.

In view of Eqs. (1), (2) and (5), the retention behaviour of biopolymers in hydrophobic interaction chromatography at sufficiently high salt concentrations can be described by a two-parameter equation:

$$\log k' = A + Cm_{S} \quad (6)$$

where

$$C = \Delta A'\sigma_{S}/2.3RT \quad (7)$$

and

$$A = \log\phi - (\Delta G^{o}_{aq}/2.3RT) \approx -(\Delta G^{o}_{aq}/2.3RT) \quad (8)$$

As seen by Eq. (6) the dependence of the $\log k'$ on the salt concentration is linear, as long as $\Delta A'$ is not a function of salt concentration. The slope C is termed the hydrophobic interaction parameter and is a function of the molecular surface area change upon

binding to the stationary phase surface ($\Delta A'$), and of the ability of the salt to increase the surface tension of the solution (σ_S) [4]. The value of C should increase when the hydrophobic surface area of the solute increases. The magnitude of this area depends largely on the primary structure, but also on the secondary and tertiary structure of the solute under the experimental conditions.

The intercept A on Eq. (6) also contains information about the hydrophobic interaction since the value of ΔG^{o}_{aq} is directly proportional to the molecular surface area change $\Delta A'$. Additionally, A contains information about effects other than hydrophobic interactions, namely Van der Waals and electrostatic interactions between the solute and the eluent which are accounted for in the terms ΔG^{o}_{aq} and consequently in $\Delta G_{h\phi}$ [4]. It is described [5] that different values of A can be obtained for closely related molecules mainly due to different electrostatic effects between the eluent and the biomolecule. The term that expresses this electrostatic effect entails namely the values of the dipole moment, the molecular volume and the polarizability of the biomolecule as well as the volume change occurring upon binding the solute to the ligand [5]. Taking into account that the sign of the parameter A is negative, the value of the intercepts will be more negative, for example, if the dipole moment of the biomolecule becomes greatest.

2. Experimental

2.1. Materials and methods

Lyophilised homo-deoxyoligonucleotides with different molecular mass were purchased from Interactiva (Ulm, Germany), resuspended in 10 m*M* Tris pH 8.0 and used without further purification. The HIC support [7] was a Sepharose derivative synthesised by covalent immobilization of the ligand 1,4-butanediol diglycidyl ether from Aldrich (Steinheim, Germany) on Sepharose CL-6B [3] purchased from Pharmacia (Uppsala, Sweden). The amount of epoxy groups immobilized on the support, determined after treatment of the activated matrix with sodium thiosulphate was around 500 μmol/g dry gel [7]. For the modulation of chromatography experiments analytical-grade ammonium sulphate and tris(hydroxymethyl)aminomethane from Merck (Darmstadt, Germany) were used.

2.2. Chromatography of the oligonucleotides

Chromatography was performed in a Pharmacia fast protein liquid chromatography (FPLC) system (P500 pump, LCC 500 chromatography controller). An XK 16/20 (20 cm×1.6 cm I.D.) column was packed with 28 ml of the HIC gel. The column was equilibrated with various concentrations of ammonium sulphate in 10 m*M* Tris–Cl (pH 8.0) at room temperature at a flow-rate of 1 ml min^{-1}. Five hundred μl of the various oligonucleotide samples were loaded onto the column and isocratic elution was carried out with the same concentration of ammonium sulphate. The absorbance of the eluate was continuously measured at 254 nm. After the chromatographic runs, the FPLC system was cleaned with 2 volumes of 1 *M* NaOH.

3. Results and discussion

3.1. Chromatographic experiments

Fifty μg of each oligonucleotide (polyA, polyT, polyU with 6, 15 and 30 bases) were injected in the HIC column and eluted with different concentrations of ammonium sulphate. A polyA molecule with 20 bases was also studied. The elution profiles obtained under the various conditions are shown in Fig. 1.

As observed and predicted by Eq. (6), the retention of the oligonucleotides increases with the salt concentration of the eluent. If a sufficiently high salt concentration is used, the oligonucleotides become strongly retained in the column, and can only be eluted by decreasing the salt concentration. The magnitude of the ionic strength necessary to promote this interaction depends on the hydrophobic character of the molecules.

In the presence of 1.0 *M* salt, polyA molecules with 20 (results not shown) and 30 (see Fig. 1) nucleotides were eluted from the column as two overlapping peaks. This behaviour suggests that

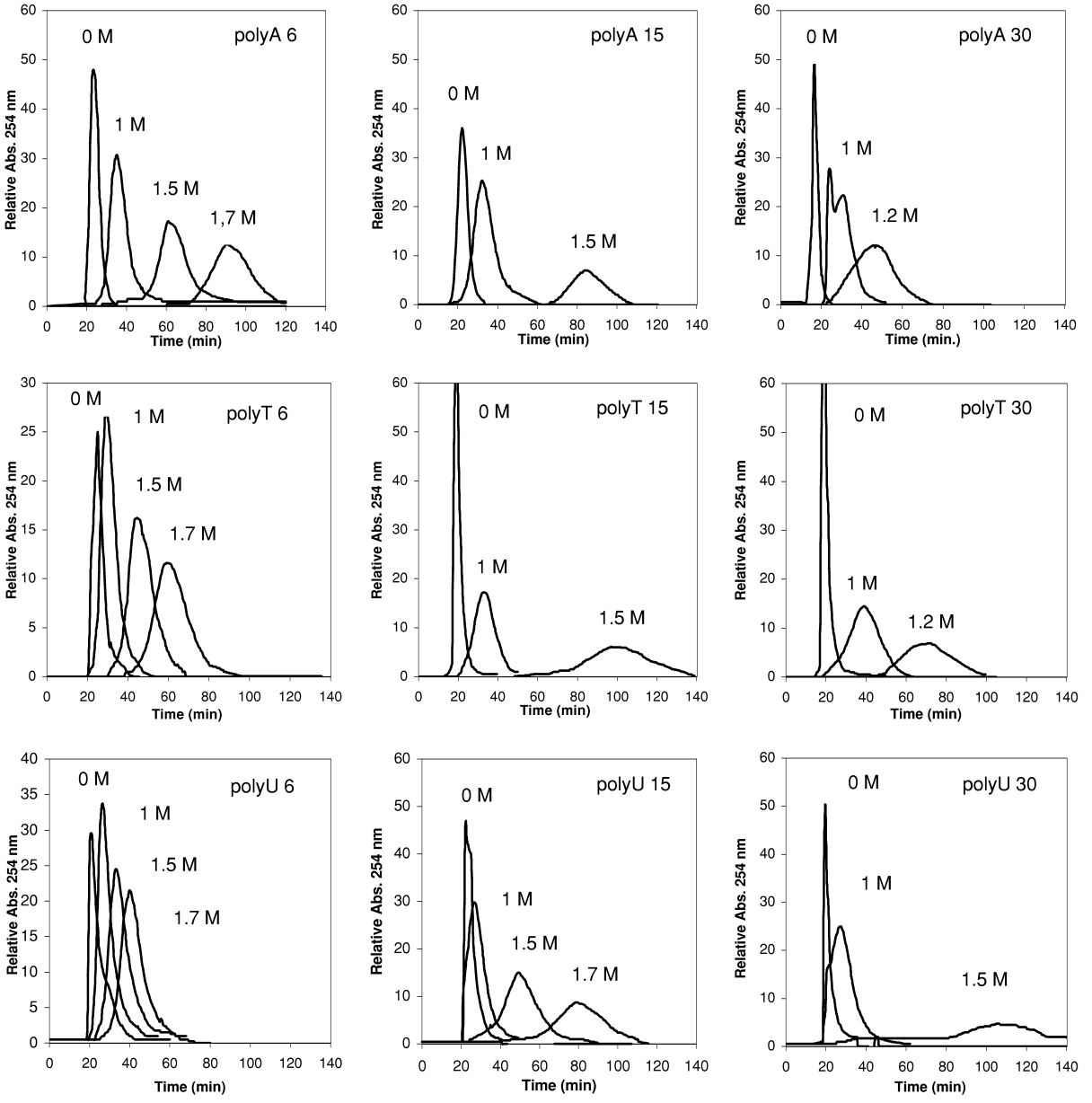

Fig. 1. Hydrophobic interaction chromatographic profiles of homo-deoxyoligonucleotides polyA (a), polyU (b) and polyT (c) on derivatized Sepharose CL-6B.

different isoforms of the oligonucleotide can co-exist [8]. These isoforms arise due to the formation of different secondary structures, a characteristic of certain homo-nucleotides (namely polyA) already described in a number of published studies [8]. At this salt concentration the two different retention times were not considered for the calculations of the parameters *C* and *A*.

Table 1
Logarithm of the retention factors of the different molecules at different ammonium sulphate concentrations

Conc. $(NH_4)_2SO_4$ (mol/l)	Molecular mass (number of bases)									
	PolyA				PolyU			PolyT		
	6	15	20	30	6	15	30	6	15	30
0	0	−0.058	−0.301	−0.301	−0.028	−0.137	−0.137	−0.038	−0.137	−0.283
0.2			−0.101	−0.176					−0.101	−0.15
0.5			−0.058	−0.079						−0.125
1	0.292	0.232	0.222	0.188	0.082	0.097	0.11	0.151	0.248	0.352
1.2	0.446	0.405	0.426	0.439		0.263			0.439	0.72
1.3	0.511	0.517	0.564	0.653		0.327		0.368	0.539	0.912
1.4			0.727	0.934						
1.5	0.624	0.796	0.944		0.253	0.528	0.907	0.455	0.829	
1.6		0.989								
1.7	0.824				0.368	0.778		0.602		
2					0.574			0.875		

3.2. Determination of the retention factors

The retention factors were determined from the chromatograms shown by calculating the quotient between the net retention volume and the void volume: $k' = V_N/V_o$ [7]. The net retention value is calculated by subtracting the void volume from the total retention volume ($V_N = V_R - V_o$). The value of the void volume was determined by injecting and eluting (with water at 1 ml min^{-1}) 500 μl of a 8.5 Kbp plasmid solution. Due to its high molecular mass, and to the fact that it is a highly hydrophilic molecule with the hydrophobic bases packed and shielded inside the double helix, the plasmid elutes with a retention volume equal to V_o. A value of 12 ml was thus estimated and used in all calculations. A list of the logarithm of the retention factors obtained for the different oligonucleotides at various molar concentrations of ammonium sulphate is shown in Table 1.

3.3. Determination of the hydrophobic interaction parameter, C

In view of Eq. (6), plots of the logarithm of the retention factor against the salt concentration were constructed (Fig. 2). The retention factor increased with the salt concentration for all the molecules studied. This increase was not linear at relatively low salt concentrations because under these conditions, electrostatic interactions between the charged surface

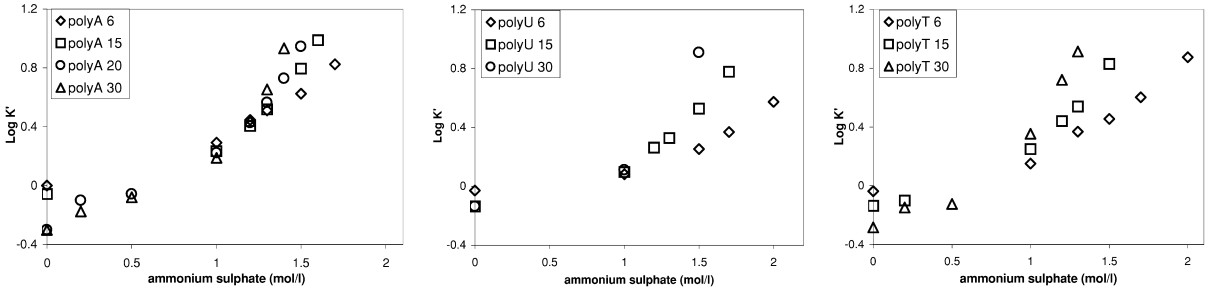

Fig. 2. Plots of the logarithm of the retention factors of homo-deoxyoligonucleotides polyA (a), polyU (b) and polyT (c) on derivatized Sepharose CL-6B against the ammonium sulphate concentration in the mobile phase.

of the oligonucleotides and the surface of the ligand are dominant over hydrophobic interactions. When these effects are at play, Eq. (6) can not be applied because the sum of the interactions will not be a linear function of the salt concentration. The prediction of a linear behaviour only holds when the Gibbs free energy of association of the solute with the hydrophobic ligand is exclusively governed by the hydrophobic effect [5]. This is observed for relatively high salt concentrations, under which electrostatic effects vanish and hydrophobic interactions dominate. Thus, Eq. (6) was fitted to adequate ranges of experimental data in order to obtain linear correlations (Fig. 3). The hydrophobic interaction parameter C was calculated and plotted as a function of the number of nucleotides (Fig. 4).

As shown in Fig. 4, the hydrophobic parameter increased with the number of nucleotides, and was found to depend on the base composition.

According to Eq. (7), C depends both on the molecular surface area change upon binding to the stationary phase surface ($\Delta A'$), and on the molal surface tension of (σ_S) the salt used. Since the salt used was always the same (ammonium sulphate), C depends only on $\Delta A'$, which is proportional to the hydrophobic contact surface area of homo-oligonucleotides [4]. Thus, and generally, the value of the hydrophobic interaction parameter is expected to increase with the total hydrophobic contact surface area, which in turn will increase with the molecular mass of the solute [4].

In the particular case of a homo-oligonucleotide with n bases, the net hydrophobicity H of the molecule can be regarded as a sum of the contributions of all the constituent nucleotides [9]:

$$H = \sum_{i=1}^{n} B_i + (n-1)P \quad (9)$$

where the hydrophobicity of each nucleotide can be defined as a sum of two parts [9]. The first term of the equation accounts for the hydrophobicity of the aromatic base (B_i) and is proportional to its hydrophobic contact surface area. The second term adds the contribution (negative) of the polar phosphodiester groups (P) to the net hydrophobicity [10]. While the first term is base dependent, the second is considered to be constant for all nucleotide residues in the oligomer. The value of B_i is positive and usually very large when compared with the value of P.

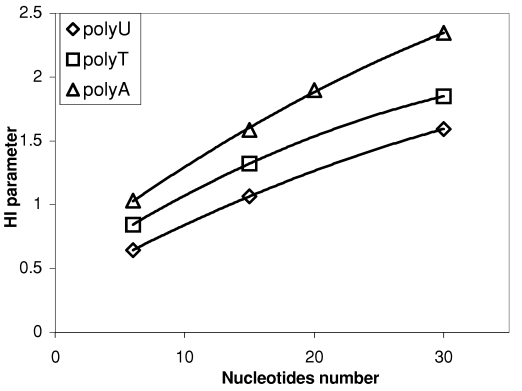

Fig. 4. Hydrophobic interaction parameter (parameter C) against the number of nucleotides of the molecules.

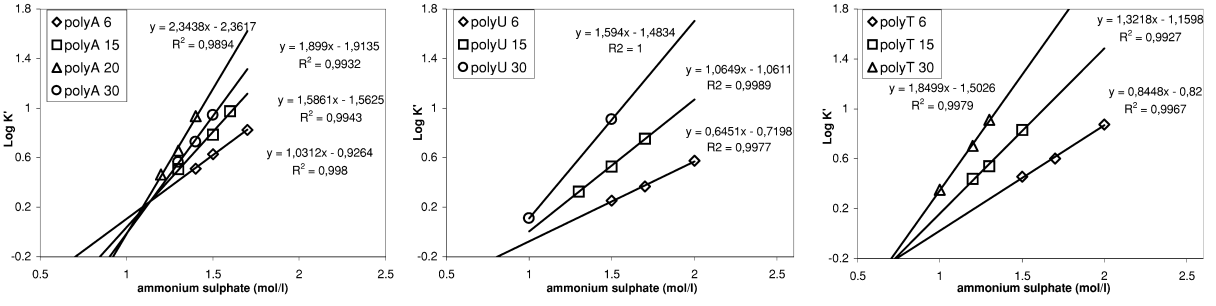

Fig. 3. Linear trendline of the plots of logarithm of the retention factors of homo-deoxyoligonucleotides polyA (a), polyU (b) and polyT (c) on derivatized Sepharose CL-6B against the ammonium sulphate concentration in the mobile phase.

The effect of the base composition on the hydrophobic interaction parameter can be explained on the basis of the relative hydrophobicity of the individual nucleotides, and of the primary structure of the oligonucleotides. Previous studies using reversed-phase chromatography report that the hydrophobicity of A (B_A) is higher than the hydrophobicity of T (B_T) which in turn is higher than the hydrophobicity of U (B_U) [10]. The difference between A and T is probably the result of the existence of two aromatic rings in the case of the adenine and the difference between T and U is due to the presence of an additional hydrophobic methyl group in the thymine base [9]. Thus, and according to Eq. (9), the order of hydrophobicity of the homo-oligomers will be the same than that of the individual nucleotides: $H_{nA} > H_{nT} > H_{nU}$. However, according to the data obtained, this order of hydrophobicity is only valid for the case of the lower molecular mass oligonucleotides, as can be observed in Fig. 7 for the molecules with 6 nucleotides. In fact, for the concentration of 1.3 mol/l ammonium sulphate, under which the retention is governed by hydrophobic interaction, the retention factor for polyA 30 is significantly lower than the retention factor for polyT 30. This means that the hydrophobicity of polyT 30 is higher then that of polyA 30, which contradicts the value of the parameter *C* obtained for these molecules. In this case the parameter *C* does not seem to be a correct measure of the hydrophobicity of the molecules, as observed before in some cases [4].

3.4. Determination of the parameter A

Parameter *A* was calculated from Fig. 3 and plotted against the number of nucleotides of the molecule (Fig. 5). As shown in Fig. 5, *A* decreased with the number of nucleotides, and was found to depend on the base composition.

A comparison of different oligomers with the same number of nucleotides shows that *A* is more negative in the case of polyA. The values of *A* for polyT and polyU are always very close because the only difference in these molecules is the presence of an additional methyl group in the nucleotides of polyT. The more negative value in the case of polyA can be attributed to a higher dipole moment of adenine, which is responsible for stronger ion–dipole

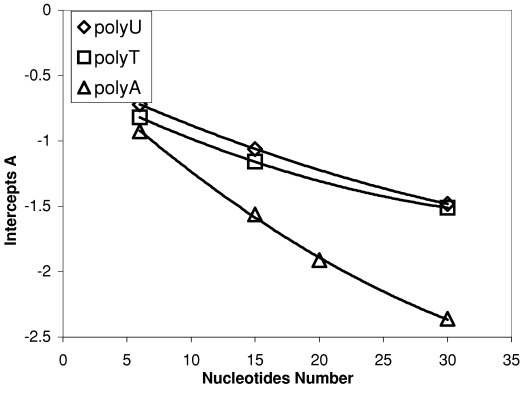

Fig. 5. Intercepts of the trendlines in Fig. 3 (parameter *A*) against the number of nucleotides in the molecules.

interactions between the solute and the eluent. It is known that families of solutes containing amino groups have large dipole moments [5], which is the case for the adenine base. In fact, the solvophobic theory predicts that differences between the retention factors of different families of solutes originate mainly from the electrostatic contribution to the free energy change [11]. This contribution is governed by the static dipole moment of the solutes. In the case of polyA, the electrostatic interactions seem to be very important because they are dominant over hydrophobic interactions until relatively high concentrations of ammonium sulphate. As seen in Fig. 1, at a concentration of 1.0 *M* of ammonium sulphate, the interaction of polyA with the HIC support is stronger in the case of the lower-molecular-mass oligonucleotides. This is not the expected behaviour when hydrophobic interactions are dominant because the global hydrophobic character should increase with the molecular mass.

A comparison of homo-oligonucleotides with the same base but different molecular masses shows that the values of the intercepts become more negative when the molecular mass increases. This is probably due to a larger electrostatic interaction brought about by a larger number of dipoles in the molecule. In fact, it is known that the contributions of these electrostatic forces are additive [12]. Additionally, the decrease in the value of the intercepts with the molecular mass is more pronounced in the case of poly A, which is probably a consequence of the higher dipole moment of adenine.

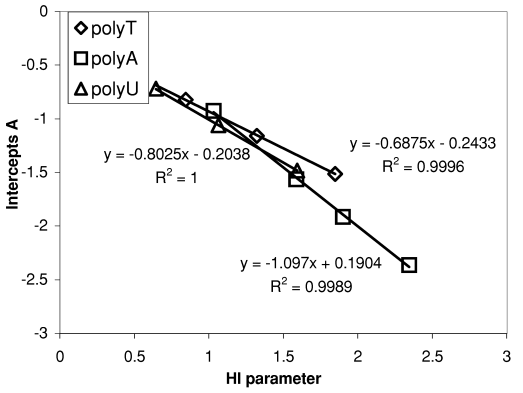

Fig. 6. Values of parameter *A* against the values of parameter *C* for all the molecules studied.

As already mentioned, *A* should be proportional to $\Delta A'$, which means that it should be proportional to *C* [4]. This conclusion is confirmed by Fig. 6 which shows a linear relationship between the parameters *A* and *C* for polyA, polyT and polyU. As observed, the value of the intercept *A* decreases with an increase in the hydrophobic parameter *C*.

3.5. Structural effects on the values of the retention factors

Another interesting analysis of the results can be made by plotting log k' against m_S in the linear zone (Fig. 7). When comparing the behaviour of oligonucleotides with six bases, the polyA retention factor is always higher than that of polyT which is higher than that of polyU. As expected, this elution order reflects the order of hydrophobicity of the molecules. In the case of the molecules with 30 bases, however, the retention factors are higher in the case of polyT. This behaviour can be explained due to the fact that the intercept *A* in the case of the polyA molecule with 30 bases has a very high negative value which renders the magnitude of log k' lower when compared with polyT. However, the decrease in the value of the intercept can also be related to the secondary structures for the polyA molecules. In fact, it is described in the literature that the value of the parameter *A* is more negative when the hydrophobic character of the molecule when measured by $\Delta G^{o}_{h\phi}$ decreases [4]. The secondary structures can shield the hydrophobic bases which will reduce the exposed hydrophobic surface area ($\Delta A'$). A reversal of the elution order is described in the literature in reverse phase chromatography for the series d(TBT) and d(TBTT), where B is adenine or thymine [13], when compared with the nucleotides adenine and thymine alone. In the two cases referred above, the oligomer first eluted was that where B corresponded to adenine. This behaviour was related to secondary structural factors and it was concluded that the influence of these factors should be taken into account when determining the net hydrophobicity of a molecule, *H*.

The existence of secondary structures for the case of nucleic acids single strands is described in the literature [8]. In fact, various studies have proved that single strands of nucleic acids can be highly ordered structures, and at least locally helical. In the specific case of the homo-polynucleotides, at lower temperatures, the optical properties become dramatically different from the corresponding monomer properties [8]. This result has been obtained for molecules such as polyA, and is due to the high

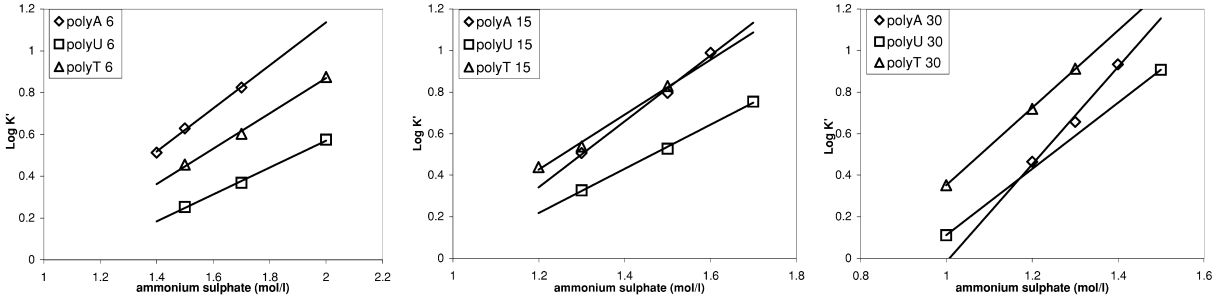

Fig. 7. Plots of the logarithm of the retention factors of homo-deoxyoligonucleotides with 6 (a), 15 (b) and 30 (c) nucleotides on derivatized Sepharose CL-6B against the ammonium sulphate concentration in the mobile phase.

stacking propensity of adenine bases. On the contrary, the optical activity of poly U is not very different from its monomer. Thus, polyU may have little, if any, ordered local structure, and this conclusion can be extrapolated to the case of polyT due to structural similarities. In fact, the stacking propensity of the bases was found to be as follows: adenine and guanine stack more than cytosine, and uracil stacks the least [13]. In conclusion, the polyA propensity for the formation of secondary structures, in opposition to polyT and polyU, may be responsible for the low retention factors of the polyA 30 oligomer when compared with polyT 30.

4. Conclusions

The results presented in this work show that the interaction of homo-oligonucleotides with the HIC support is dependent on the hydrophobic character of the individual bases, the molecular mass and the secondary structures of the molecules and the concentration of ammonium sulphate in the eluent.

The interaction for all the molecules studied increases with the concentration of ammonium sulphate, as expected for hydrophobic interactions. At relatively high salt concentrations, when the hydrophobic interactions are the dominant mechanism, the values of log k' increase in a linear fashion with the salt concentration. In this case, the slope of the log k' versus salt concentration plot corresponds to the hydrophobic interaction parameter, C, which is a measure of the hydrophobicity of the molecule.

At relatively high salt concentrations of ammonium sulphate, the interaction also increases with the molecular mass of the oligonucleotides, as predicted for hydrophobic interactions. In fact, the hydrophobic character, described earlier as H, increases with the number of nucleotides in the molecule. For low ionic strengths, however, electrostatic interactions between the biomolecules and the solvent are dominant over hydrophobic interaction and this behaviour may be inverted. The electrostatic interactions are especially important in the case of the polyA oligonucleotides, because the adenine as an amino group which contributes to the relatively high dipole moment of the molecule.

For the low molecular mass oligonucleotides the log k' of the various molecules at high ionic strengths increased with the hydrophobicity of the individual bases. As described in the literature, the hydrophobicity of adenine is higher than thymine, which is higher then uracil. In fact, the strength of the interaction between the hydrophobic support and the lower-molecular-mass oligonucleotides followed this sequence. For the higher-molecular-mass polyA, however, other influences such as the existence of secondary structures and the high dipole moment of the adenine reduced the interaction of this oligonucleotide with the support. This information is entailed in the value of A, the intercept of the log k' versus salt concentration curve. This value is more negative for a larger dipole moment of the biopolymer or when the exposed hydrophobic surface area is smaller, as is the case due to the formation of secondary structures.

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