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Separation of G structures formed by a 27-mer guanosine-rich oligodeoxyribonucleotide by dye–ligand affinity chromatography

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Abstract

G-DNA structures, formed by a 27-mer guanosine-rich oligodeoxyribonucleotide, AACCCGGCGTTCGGGGGGTACCGGGTT, were isolated and studied by dye–ligand affinity chromatography, using a Reactive Green 19–agarose resin (RG19–aga) and gel electrophoresis. The experiments were performed in the presence of Li^+, Na^+ and K^+, which are able to stabilise the G structures to different extents. Desalting procedures followed by affinity chromatography, performed in the presence of Li^+, gave us information on the relationships among the species isolated and their stability. The results show that the more stable species were those obtained in the presence of K^+, while in the presence of Li^+, the formation of G structures was negligible and the oligonucleotide was almost exclusively present as a stem–loop structure recognised by the RG19–aga affinity resin. Electrophoretic and denaturation and renaturation experiments supported the affinity chromatography results.

Keywords: Dye ligands; Nucleotides; DNA; Metal cations

1. Introduction

It is well known [1] that, under particular conditions, G-rich oligodeoxyribonucleotide sequences can form structures, which are generally called G structures, characterised by the formation of G quartets, as shown in Fig. 1a. According to this model, the DNA folds into stacks of square planar quartets of deoxyguanosine residues, each dG forming Hoogsteen base pairs with its neighbours in the plane of the quartet [2]. The formation, stability and interconversion of the G structures are dependent on the cations present in the solution and their concentration. The different cations, depending on their ionic radius and charge, affect the G structures because the two G quartet base pair assemblies fold around the ion to form a sandwich-like 'cryptand' complex as in Fig. 1b. It is reported that K^+ induces transitions to exceptionally stable forms, which is reasonable due to its ionic dimensions [3–6].

Guanine-rich sequences, often short runs of guanines in longer repeated sequences, occur in immunoglobulin switch regions, in gene promoters, and in chromosomal telomeres. It has been suggested that the self-recognition of guanine-rich motifs of DNA, at physiological salt concentration, serves to bring together, and to zipper up in register, the four homologous chromatids during meiosis [7–9].

Evidence for the formation and interconversion among the G structures was obtained by electrophoretic and spectroscopic experiments, although the

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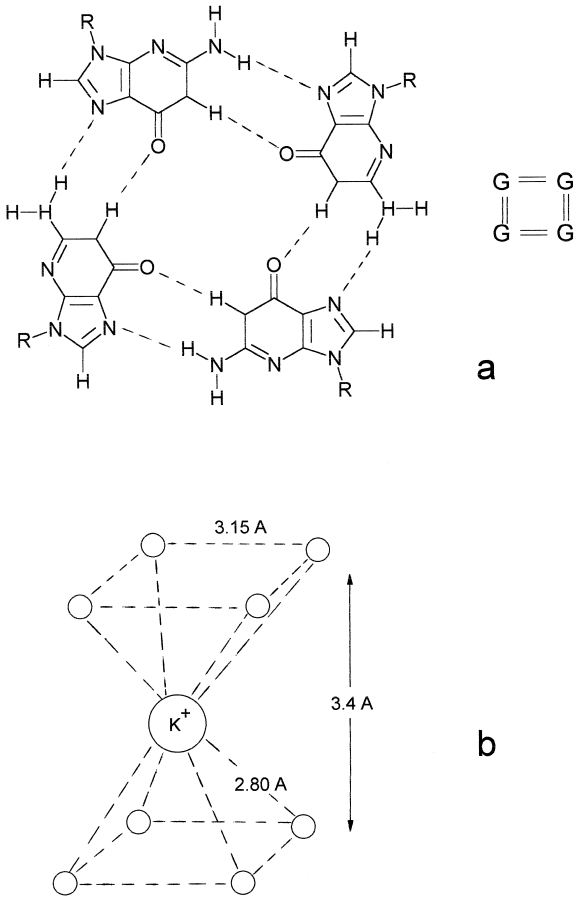

Fig. 1. (a) Model structure of a Hoogsteen hydrogen-bonded guanine tetrad. (b) Tridimensional structure of a K^+ cation–quadruplex complex. The eight small circles represent the oxygen atoms of two stacked guanine tetrads.

spectroscopic data were often related to mixtures enriched in various species varying the temperature and/or the cation.

Generally, nucleic acid purification is performed by ion-exchange [10,11], reversed-phase [12,13] or mixed mode [14,15] HPLC and size-exclusion chromatography [16,17], with separations based on differences in size, charge, hydrophobicity, shape, base sequence and base composition.

Affinity chromatography is one of the most powerful techniques employed in the purification of molecules used in pharmaceutical and biotechnological applications as well as in molecular biology [18–21]. It offers more advantages than the other purification methodologies in terms of economy, time for performing a purification procedure and purity. Among the general affinity ligands the reactive triazine dyes are preferred over the biological ones because they are inexpensive, easy to synthesise and immobilise to a wide range of matrices via their functional group, resistant to biological and chemical degradation [22]. They show high adsorbent capacity and high selectivity which, in principle, could be further improved by modifying their structure in such a way to mimic more accurately natural biological ligands (biomimetic dyes) [23–25].

There are reports in the literature that dye-binding DNA sequences were isolated from a chemically synthesised pool of random sequence DNAs by repeated cycles of dye affinity chromatography followed by polymerase chain reaction (PCR). Some DNAs, selected using a Reactive Green 19–agarose (RG19–aga) column, were studied to search for sequence similarities. Variants of an 18-nucleotide (nt) consensus sequence were found in some oligomers. Furthermore, it was found that the 18-nt sequence was not retained on the Reactive Green column unless it formed a stem–loop structure in which the lower portion of the stem was stabilised by the formation of 2–5 additional Watson–Crick base pairs by flanking nucleotides [26].

In the experiments reported here we used the oligonucleotide in Fig. 2 in which 10 of the 18 nucleotides forming the consensus sequence are Gs, to check its capacity to form G-DNA structures and to isolate them by RG19–aga affinity chromatography working in the presence of different cations.

2. Experimental

2.1. Materials

The instrument and all the reagents needed for the synthesis of the 27 oligodeoxyribonucleotide sequence were purchased from Biosearch (San Rafael, CA, USA). The reagents used for purification by HPLC and for the electrophoretic experiments were provided by Fluka (Buchs, Switzerland). The RG19–aga resin was provided by Sigma (Milan, Italy). The Sephadex G10 resin was purchased from Pharmacia LKB (Uppsala, Sweden). The gel electrophoresis experiments were performed at 5°C using a Beck-

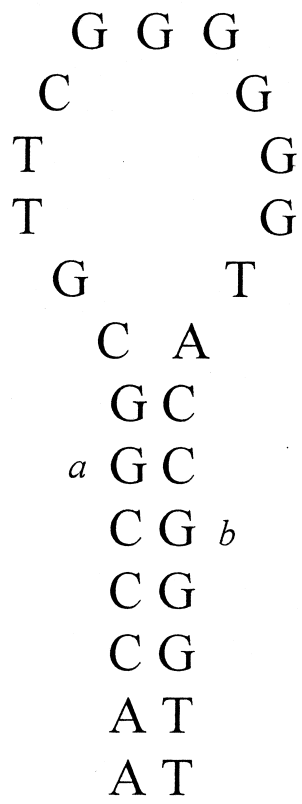

Fig. 2. 27-Mer oligodeoxyribonucleotide sequence, from *a* to *b* is designed the *consensus* sequence.

mann electrophoresis apparatus (Palo Alto, CA, USA). All the reagents used to prepare eluents for the affinity chromatography and to reveal the gels were of analytical grade. All chromatographies were performed using an Amicon glass column. The spectrophotometer used for the UV measurements was a Perkin-Elmer Lambda 2S.

2.2. Synthesis and purification of the oligodeoxyribonucleotide

The 27-mer oligodeoxyribonucleotide, AACCCGGCGTTCGGGGGGTACCGGGTT, was synthesized by phosphoramidite chemistry on a Cyclone DNA synthesizer (Biosearch), using a 15 mm column, according to the manufacturer's procedure. The instrument was programmed to remove the last DMT (dimethoxytrityl) protecting group of the 5′ end deoxyribose unit. The oligomer was detached from the silica support and protecting groups were removed by incubating the silica-bound sample in concentrated ammonium hydroxide (5 h, 55°C). After concentration under vacuum, the sample was purified by HPLC on a Varian 1050 chromatograph equipped with a 1050 Varian variable-wavelength UV–Vis detector set at 260 nm, using a Bondapak RP18 column, with a gradient of CH_3CN in 0.1 *M* triethylammonium acetate (pH 7, 0 to 20% in 90 min, flow-rate 4 ml/min). The purity of the product obtained by HPLC was checked by classical 19% denaturing polyacrylamide gel electrophoresis.

2.3. Dye–ligand affinity chromatography

For each affinity chromatography experiment small amounts of the oligomer ($1.7–2.2 \cdot 10^{-4}$ g) were suspended in 0.5 ml of high-salt buffer (0.5 *M* XCl, where X^+ could be Li^+, Na^+ or K^+, 20 m*M* Tris–Cl, pH 7.6) and stored for 24 h at 4°C. The sample was then applied onto the RG19–agarose column (7 cm×1 cm, 5.5 ml) and allowed to equilibrate for 20 min (preliminary affinity chromatography experiments showed that a 5-min equilibration was sufficient to allow a complete binding of the oligomer). The column was washed first with four volumes of the high-salt buffer solution used to suspend the oligomer and then with four volumes of water at a flow-rate of 0.7 ml/min. The fractions collected were checked at 260 nm with an UV variable-wavelength spectrophotometer

2.4. Electrophoretic experiments

All native horizontal gel electrophoresis experiments were performed in 20% polyacrylamide gels using a solution of 0.5× TBE (Tris–borate-EDTA), from SIGMA-ALDRICH, as the running buffer. Both the gels and the running buffer were 0.2 *M* in CH_3COOX ($X=Li^+$, Na^+ or K^+). The gels were run (5°C and 8 V/cm) in a Beckmann electrophoresis apparatus in which the gel was completely immersed in the buffer tank which was in turn cooled by a heat exchanger connected to a circulating cold bath. DNA samples were prepared concentrating small volumes eluted from the affinity chromatography column, mixed with 1 μl of 30% glycerol solution containing marker dyes (Bromophenol Blue and Xylene Cyanol

FF). The oligonucleotide spots were revealed using the Silver Stain method. Under these conditions Bromophenol Blue migrated as a 12-mer and Xylene Cyanol FF as a 46-mer. A 30-mer, TACGG TGGC TT CAT GACC TCCGAGAAGAGC, and a 15-mer, GACGTGTCATCATTA, oligodeoxyribonucleotides were also used as DNA markers.

3. Results

In Fig. 3 are reported the elutions of the 27-mer oligonucleotide in the presence of Li^+, Na^+ and K^+. We indicate with prefix 1 the species eluted in the fraction range 5–12, with prefix 2 those eluted in the fraction range 16–28 and with prefix 3 the species eluted with water in the fraction range 41–46.

In the presence of Na^+ or K^+, two species were detected both eluting at high salt concentration, 1Na, 2Na and 1K, 2K, respectively. With Lithium, again two species were detected, one eluting in high-salt buffer, 1Li, but the other, 3Li, in a higher amount, eluting when the column was washed with deionised water. The two species obtained in the presence of the Li^+ cation (1Li, 3Li) were denatured and renatured separately (heating the samples to 95°C for 10 min and slowly cooling to room temperature), applied to the affinity column, allowed to equilibrate for 20 min, and then eluted as above in the presence of Li^+. This experiment showed that each species was capable to form from each other.

Samples of the species eluted with the high-salt buffer in the presence of Na^+ and K^+, namely 1Na, 2Na and 1K, 2K, were separately subjected to a desaltification procedure through a Sephadex G-10 column (29 cm×1.5 cm, 51 ml) eluting with water at a flow-rate of 2 ml/min and checked at 260 nm. The desalted samples were resuspended in a high-salt

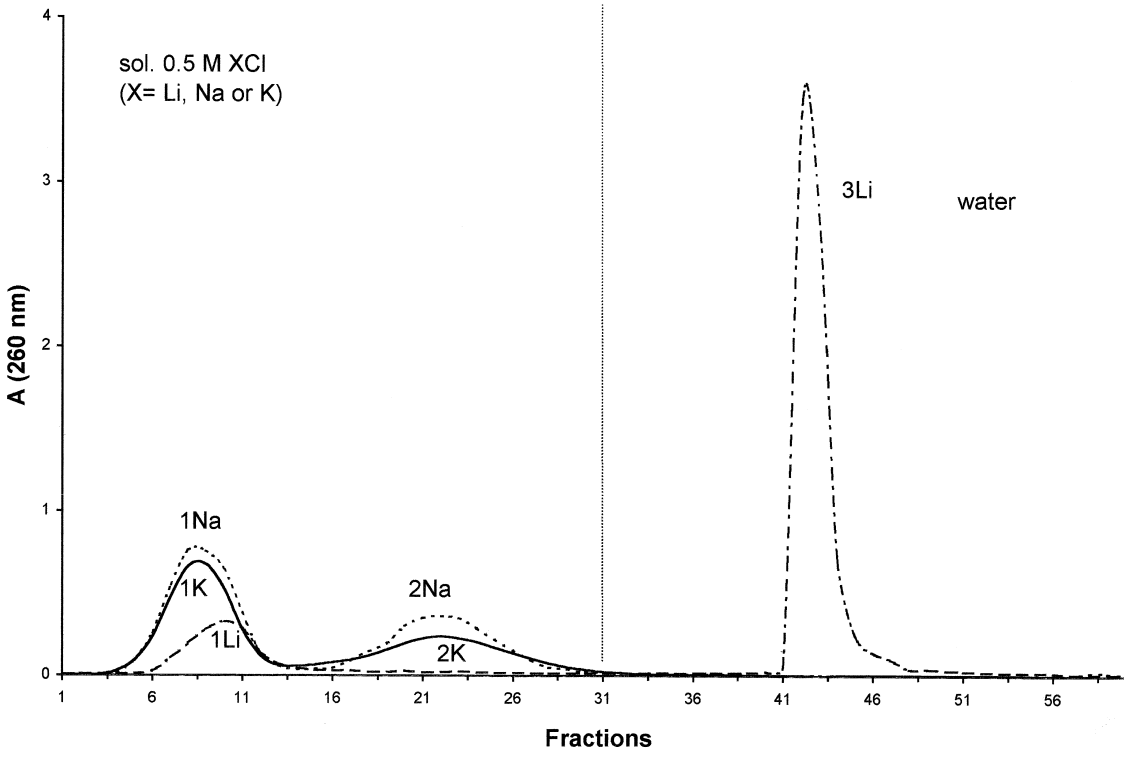

Fig. 3. RG19–agarose affinity chromatography of the 27-mer in the presence of Li^+ (– · –), Na^+ (- - -) and K^+ (———). The vertical line indicates the change in the solvent composition. The prefixes 1, 2 and 3, here and in Figs. 4 and 5, indicate the species eluted in the fraction ranges 5–13, 16–28 and 41–46, respectively.

buffer, 0.5 *M* LiCl, and stored at 4°C for 24 h. Each sample was then loaded onto the affinity chromatography column, allowed to equilibrate for 20 min and then eluted with four volumes of a high-salt buffer containing Li^+ and with four volumes of water at a flow-rate of 0.7 ml/min as in the preceding experiment. In all cases two species were eluted: one with the high-salt buffer, 1Na1, 1K1, which were not further analysed, and the other with deionised water, 3Na1, 3K1, reported in Fig. 4, indicating with suffix 1 that the species 1Na and 1K, respectively, in the preceding experiment were treated. A similar pattern was obtained in the elution, under the same conditions, of the species 2Na and 2K, obtaining the species 1Na2, 1K2 and 3Na2, 3K2 reported in Fig. 5. Obviously, all these last species, after equilibration with Li^+, contain this cation as ligand irrespective that they were named as Na or K.

Native polyacrylamide gel electrophoresis experiments showed that:

1. water-eluted species (3Li, 3Na1, 3K1, 3Na2, 3K2) migrated at a position corresponding to about half the size of the linear single-stranded form observed with denaturing polyacrylamide gel electrophoresis
2. species 1Li, 1Na and 1K migrated at a position corresponding to about four times the size of the stem–loop oligonucleotide
3. species 2Na and 2K showed almost the same molecular mass of the oligomer folded into the stem–loop structure (3Li) even if they moved slightly faster than the latter species.

4. Discussion

In each dye–ligand affinity chromatography reported, the sample was always loaded onto the column in high-salt buffer to allow the negatively charged DNA molecules to approach the negatively charged dye molecules bound to the agarose. Under these conditions the DNA species eventually, non-

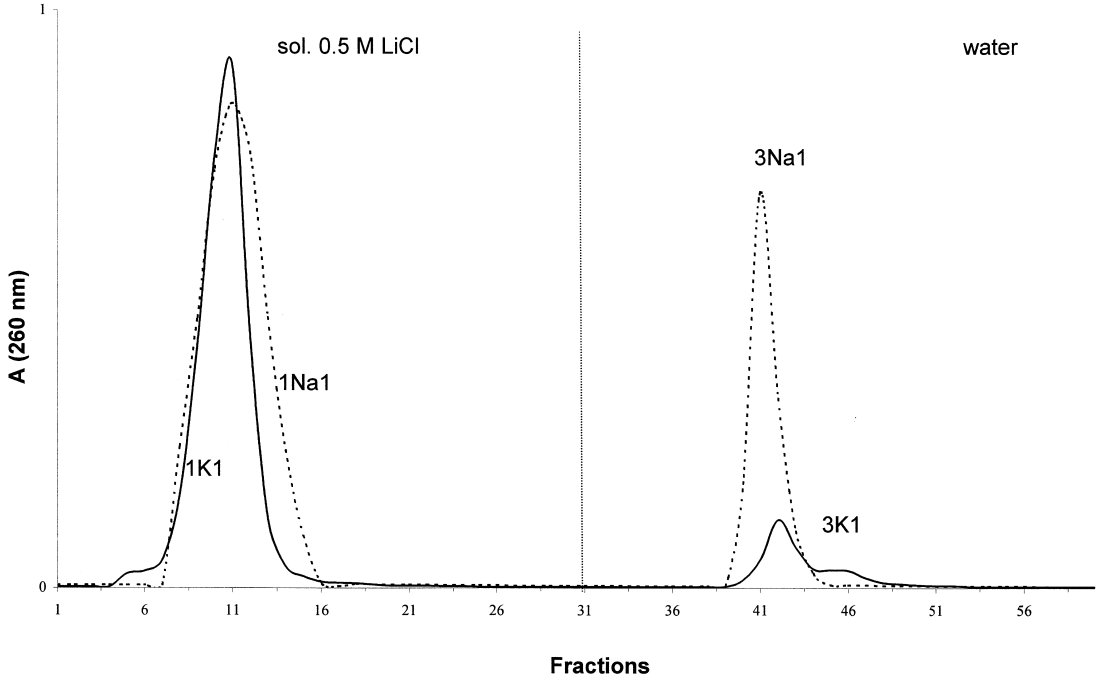

Fig. 4. Affinity chromatography of 1Na (- - -) and 1K (———) which were desalted, resuspended in high-salt buffer (20 m*M* Tris–Cl pH 7.6) 0.5 *M* LiCl and stored at 4° C for 24 h. The percent conversion in the stem–loop structure was 1Na=24.2% and 1K=11.1%. Suffix 1 indicates that the species with prefix 1 in Fig. 3 were treated.

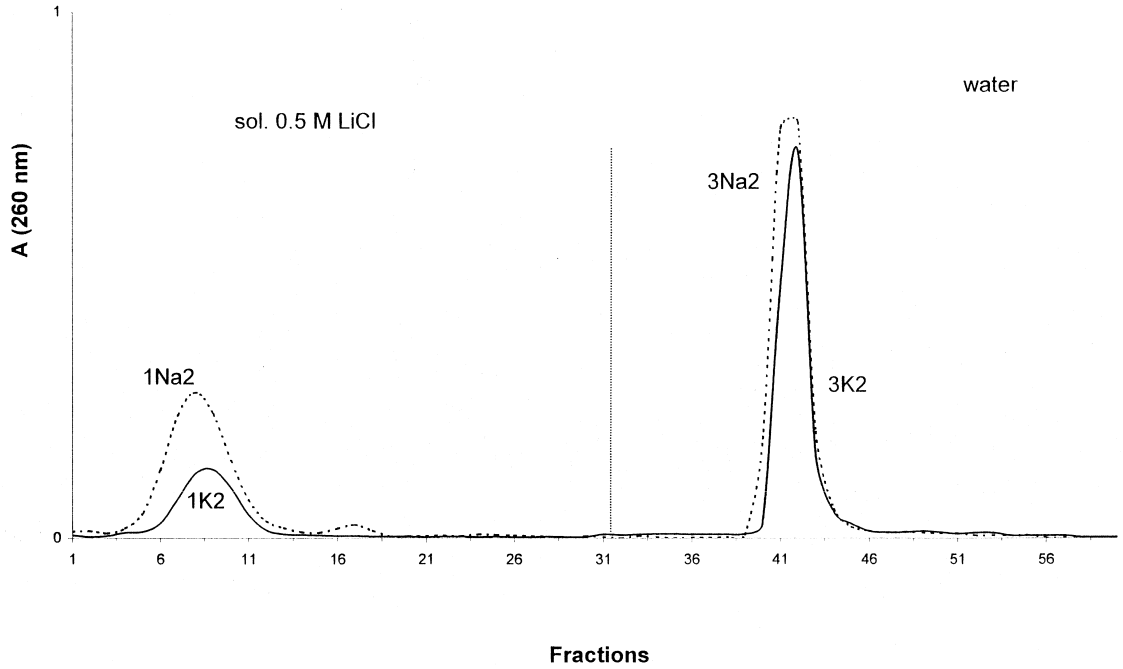

Fig. 5. Affinity chromatography of 2Na (- - -) and 2K (———) which were desalted, resuspended in high-salt buffer (20 m*M* Tris–Cl pH 7.6) 0.5 *M* LiCl and stored at 4°C for 24 h. The percent conversion in the stem–loop structure was 2Na=73.5% and 2K=62.9%. Suffix 2 indicates that the species with prefix 2 in Fig. 3 were treated.

recognized or retained with non-specific interactions, were washed away with the high-salt buffer used in the first step of the elution, while the recognised species was retained and eluted after with water. This indicates that the retained species, recognised by the bound dye, is the 27-mer in its stem–loop structure containing in the inner of the loop the lithium cation that stabilises that structure [26]. These species can be converted into each other as demonstrated by denaturation and renaturation experiments performed on species 1Li and 3Li, described above.

The oligonucleotide used for these studies had a stem–loop structure in which the lower portion of the stem was stabilized by Watson–Crick base pairs by flanking nucleotides. As mentioned above, the 18-nt consensus sequence, in the particular conformation induced by the stem, was recognized by the Reactive Green 19 dye immobilized to the resin used for the affinity chromatography experiments. The dye–ligand affinity chromatography experiment in Fig. 3 shows that, in the presence of the Li^+ cation, about 90% of the sample was present in its stem–loop structure, 3Li, whilst only a small percentage of the sample was washed away with the high-salt buffer. In the presence of both Na^+ and K^+ cations, only the species labeled 1Na, 2Na, and 1K, 2K that were eluted with the high-salt buffer solution were observed, with no formation of species with the stem–loop structure.

However, when these last four fractions were, separately, desalted and resuspended in a high-salt buffer containing Li^+ cations, hence chromatographied on the RG19–aga resin, they were shown to be capable of forming the stem–loop structure labeled 3Na1, 3K1 in Fig. 4 and 3Na2, 3K2 in Fig. 5, although to different extents depending on the stabilisation induced by the cation.

In fact, from Fig. 4, only 11.1% of 1K and 24.2% of 1Na were converted into the stem–loop structure 3K1 and 3Na1, respectively, and from Fig. 5, 62.9% of 2K and 73.5% of 2Na were converted into the stem–loop structure 3K2 and 3Na2, respectively. This indicates that the stabilisation induced by the

K^+ cation is greater than that induced by the Na^+ cation; moreover, from these data it can be deduced that the four-stranded species (1K, 1Na) were more stable than the 'monomer' species (2Na, 2K).

These findings were confirmed by the electrophoretic approach . In fact, the migration data for 3Li under (i) are in accordance with the stem–loop structure assumed by the oligonucleotide. The data under (ii) for the species 1Li, 1Na and 1K indicate that these species came from an interstrand association realised through the formation of a G structure, formed by four oligonucleotide strands, stabilised by Hoogsteen base pairs; and the migration data under (iii) for the species 2Na and 2K support the hypothesis that these species are 'monomers' and came from an intramolecular rearrangement of the oligomer which was not recognized by the affinity column and thus eluted with the high-salt buffer solution.

5. Conclusions

In this work it was reported that different structures, formed by a 27-mer oligonucleotide in the presence of Li^+, Na^+ and K^+, could be isolated by a Reactive Green 19–agarose resin. In the presence of Li^+ the oligonucleotide was almost exclusively present as a stem–loop structure recognised by the dye immobilised to the affinity resin. In the presence of Na^+ and K^+ two new different non-recognised species were isolated. At present the dye–ligand affinity chromatography is successfully employed for proteins purification. It could probably be used for nucleic acids or nucleic acid structure separation.

The experiments performed showed that, in both cases, the first-eluted species had four times the molecular mass of the oligonucleotide and it was obtained by the aggregation of four oligonucleotide molecules stabilised by Hoogsteen bonds. The species eluted in the presence of K^+ was the most stable. The second-eluted species resulted from an intramolecular rearrangement and, in this new form, the oligonucleotide was not recognizable by the dye immobilized to the affinity column. The second-eluted species formed in the presence of K^+ is more stable than that eluted in the presence of Na^+.

The results obtained here lead us to stress the importance of evaluating the dye–ligand affinity chromatographic approach for nucleic acid purification.

Furthermore, this technique could simplify the studies of G structures, which are generally carried out as mixtures of different G forms, so that the interpretation of the spectroscopic data obtained is usually very difficult. It is evident that an effective separation of the different possible structures that a generic oligomer could form would give the possibility of an exhaustive spectroscopic study for a better comprehension of them.

Thus the dye–ligand affinity chromatographic technique could provide an important contribution for the resolution of this problem.

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