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Synthesis and characterization of a bunchy oligonucleotide forming a monomolecular parallel quadruplex structure in solution

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Abstract—A cluster of four d(TG_4T) hexanucleotides with the 3′-ends linked together by a bunchy spacer has been synthesized and shown to form a monomolecular parallel quadruplex **5** in solution characterized by four G-quartets. ^{1}H NMR spectroscopy and CD thermal denaturation experiments indicate the monomolecular quadruplex (**5**) to be more stable than the tetramolecular counterpart $[d(TG_4T)]_4$.

Oligodeoxyribonucleotides (ODN) can form an unexpected number of secondary structures.[1,2] Among these, quadruple helices based on G-quartets (G-quadruplexes) have aroused widespread interest not only for their substantiated presence in many biologically important regions of the genome[3,4] but also because such structures form the scaffold of several aptamers provided with useful biological properties.[5–8] G-quadruplexes can be classified depending on the number of self-associating strands that form the structure (one, two or four strands) and are further distinguished by the orientation of the strands to each others (parallel or antiparallel) as well as the glycosidic conformation of the guanosine residues (*anti* or *syn*). Even though most of the G-quadruplex structures involved in biomolecular events belong to the antiparallel type, yet evidences have been provided that some proteins are able to interact with parallel G-quadruplex as well.[9–12] Biological relevance of such structures has further risen due to the discovery of ODNs forming parallel quadruplexes, including aptamers provided with anti-HIV activity[13–15] and hematoporphyrin binding aptamers.[16] However, intermolecular formation of parallel structures in vitro is very slow and may require high ODN concentrations.[17] These unfavorable kinetic and thermodynamic parameters could be disadvantageous in view of their potential therapeutic use. In order to overcome these drawbacks, we devised a new class of ODN analogues, which we called bunch-ODNs, capable to form monomolecular parallel quadruplex structures. The structural feature of these analogues is the presence of four ODN strands whose 3′-ends are linked together by a bunchy spacer. This paper deals with the chemical synthesis and the structural characterization of bunch-$[d(TG_4T)]_4$, which represents the first example of a new class of quadruplex forming ODNs. The sequence of the chains was chosen mainly because d(TG_4T) forms a stable tetramolecular helix, already well characterized by thermodynamic measurement as well as NMR, X-ray crystallography and CD spectra.[18–21] This quadruplex possesses a fourfold symmetry with all strands parallel to each other and all nucleotides in an *anti*-conformation. CD and NMR spectroscopies have been used to investigate the solution structure of bunch-$[d(TG_4T)]_4$ in the presence of potassium or sodium ions in comparison with the natural counterpart $[d(TG_4T)]_4$.

Our synthetic strategy uses the commercially available Controlled Pore Glass (CPG) support **1** and the

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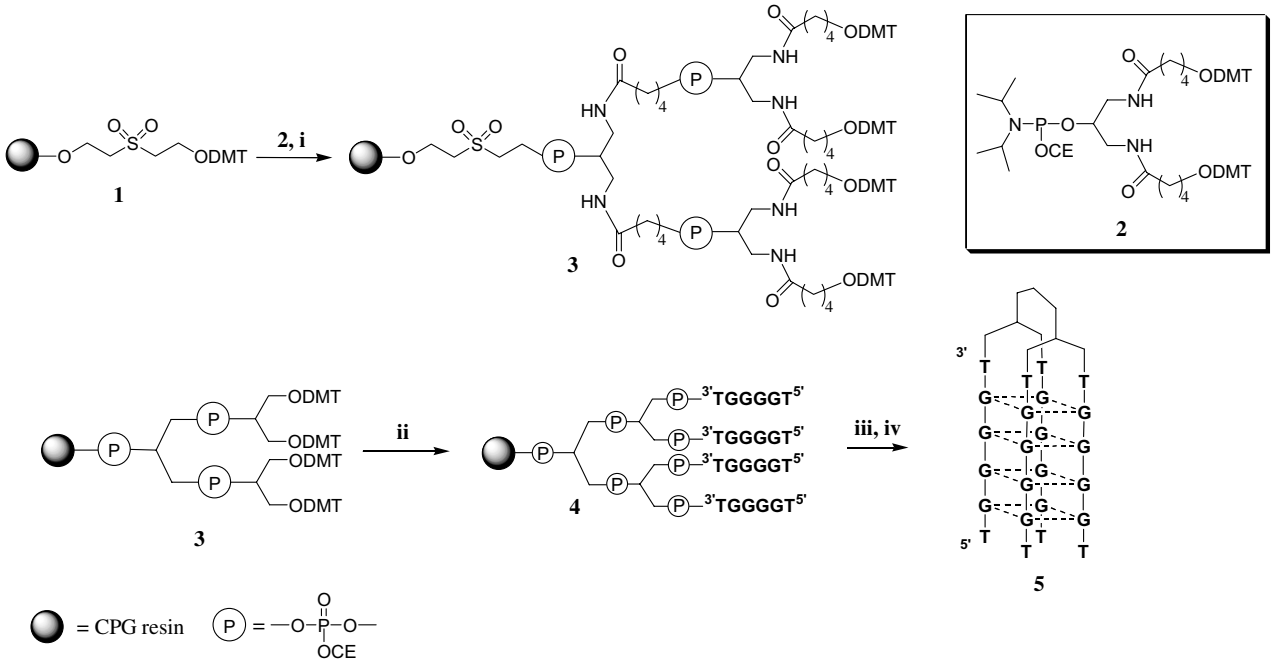

Scheme 1. Reagents and conditions: (i) two coupling procedures with **2**; (ii) ODN synthesis; (iii) detachment and deprotection by NH_4OH concn 32% (7 h, 55 °C); (iv) HPLC purification and annealing.

phosphoramidite derivative of the di-functional linker **2**. On support **1** (0.048 meq/g), performing two coupling cycles with **2** by an automatic DNA synthesizer, we obtained support **3** that bears four symmetrical protected primary alcoholic functions, prone to ODN-chain assembly (0.085 meq/g of OH functions, by DMT spectrophotometric test). ODN chains were assembled on **3** utilizing 3′-phosphoramidite nucleotide units, thus obtaining the polymer bound ODN **4** (coupling yields >98% per cycle). The presence of a β-sulfone phosphodiester function in the linker connecting the bunchy ODN to the solid support allows the release of the phosphomonoester group via a β-elimination under alkaline treatment (concd NH_4OH, 7 h, 55 °C), affording the bunch-ODN **5**. Crude **5** was purified by HPLC[†] on an anionic exchange column and the collected peaks were desalted on a RP18 column thus furnishing **5** (>95% pure, by HPLC and PAGE). In a typical synthetic cycle, starting from 100 mg (0.0085 meq) of support **3** 135 OD_{260} units yield of pure **5** were obtained. The structure of **5** was confirmed by ^1H NMR data and by ESI mass spectrometry,[‡] which showed pattern peaks indicating a molecular weight at 8762.6 a.m.u. (calcd. 8763.7) (Scheme 1).

In order to demonstrate that **5** can adopt a G-quadruplex structure and, in case, to estimate its stability, we performed circular dichroism (CD) studies and CD thermal denaturation experiments in comparison with the tetramolecular G-quadruplex $[d(TG_4T)]_4$. CD spectra were performed at 20 °C at quadruplex concentrations in the range $4.0–4.5\times10^{-5}$ M in K^+ or Na^+ buffers.[§] Particularly, bunch-ODN **5** and $[d(TG_4T)]_4$ showed almost identical CD profiles in both saline conditions (Fig. 1A), characterized by a positive and a negative Cotton effect at 264 and 249 nm, respectively, diagnostic of a G-quadruplex structure involving four parallel strands.[22] CD thermal denaturation experiments (Fig. 1B) were performed monitoring the CD values (mdeg) at 264 nm in the range 20–80 °C with 1 °C/min heating rate. In K^+ buffer both **5** and $[d(TG_4T)]_4$G-quadruplexes undergo irrelevant CD_{264} variation up to 80 °C, thus not showing the typical descendant sigmoidal curves indicative of a melting process. On the other hand, in Na^+ buffer, the increase of temperature leads to a significant reduction of CD_{264} values for both complexes, thus indicating the fusion to occur. The complex $[d(TG_4T)]_4$, showed a defined sigmoidal curve with a derivatizable T_m value (56 °C at 4.5×10^{-5} M concentration). In the case of **5** the melting curves regis-tered at concentrations 1.0×10^{-5}, 2.0×10^{-5} and 4.5×10^{-5} M did not show well-defined changes of convexity up to 80 °C

[†] Column: Nucleogel SAX (Macherey–Nagel 1000-8/46); buffer A: 20 mM KH_2PO_4 aq solution pH = 7 containing 20% CH_3CN; buffer B: 1 M KCl, 20 mM KH_2PO_4 aq solution pH = 7 containing 20% CH_3CN; linear gradient from 0% to 100% B in 30 min, flow rate 1 mL/min. The isolated oligomer was desalted on a Sep-Pak (C18) column.

[‡] ESI mass spectra were recorded with an API 2000 (Applied BioSystem) instrument used in negative mode.

[§] The sample, dissolved in the buffer, was annealed heating the solution at 85 °C for 20 min and keeping the sample, after slow cooling, at 10 °C for 12 h. K^+ buffer: 10 mM KH_2PO_4, 70 mM KCl, 0.2 mM EDTA, pH 7.0; Na^+ buffer: 10 mM NaH_2PO_4, 70 mM NaCl, 0.2 mM EDTA, pH 7.0.

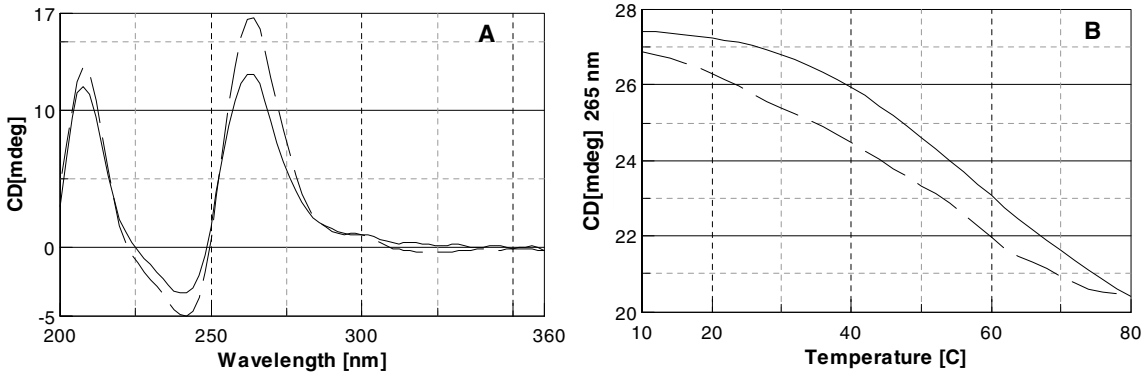

Figure 1. CD spectra (A) and melting profiles (1 °C/min) (B) of **5** (—) and d[TG_4T]$_4$ (- - -) in Na^+ buffer[§] (4.0×10^{-5} M).

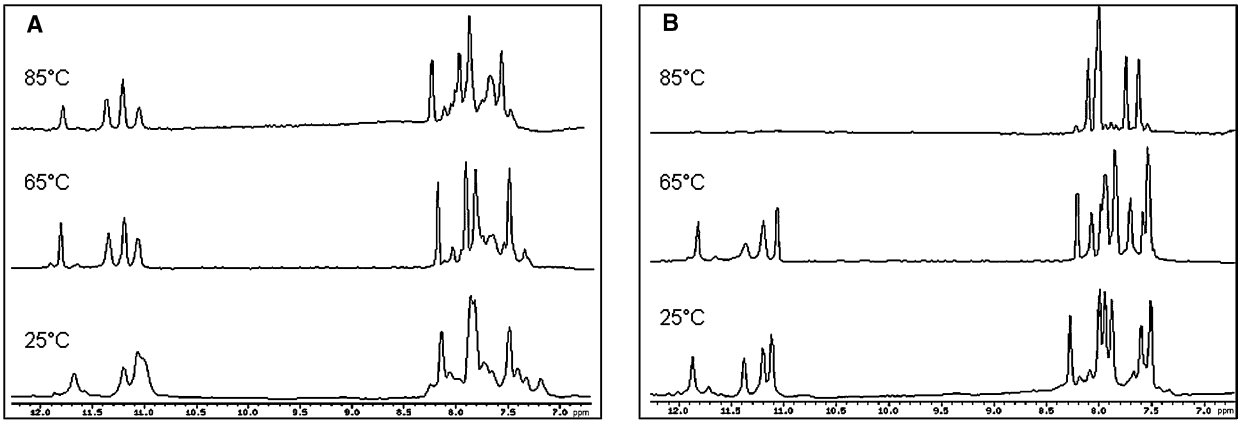

Figure 2. Aromatic and imino proton regions of the 500 MHz ^{1}H NMR spectra of **5** (A) and [d(TG_4T)]$_4$ (B) registered at approximate concentrations 0.5 mM (H_2O/D_2O, 9:1, v/v) in Na^+ buffer.[§]

thus suggesting that only partial fusions occur. Furthermore, it is noteworthy that the melting curve profiles of **5** are not concentration dependent thus indicating that the complex is mainly present as a monomolecular species. The whole of data suggests that **5,** in Na^+ buffer, is characterized by a higher stability than its tetramolecular counterpart [d(TG_4T)]$_4$, most likely due to the favourable entropy of the monomolecular complex.

^{1}H NMR studies (500 MHz) on both complexes confirmed the data obtained by CD. Spectra were recorded in Na^+ and K^+ buffers at 25, 65 and 85 °C using pulse field gradient WATERGATE for H_2O suppression. The spectrum of **5**, in Na^+ buffer at 25 °C (Fig. 2A), shows the presence of four singlets in the range 10.5–12.0, two of them partially overlapping, confidently assigned to exchange protected imino protons involved in Hoogsteen N(1)H/O(6) hydrogen bonds of G-quartets[23,24] also on the basis of the comparison with the corresponding region of the spectrum of [d(TG_4T)]$_4$ (Fig. 2B), which displays a very similar peak profile. Even if a detailed ^{1}H NMR study has not been yet accomplished, it is not unreasonable at this stage to hypothesize that **5** is characterized by a quadruplex structure containing four G-quartets with a pseudo fourfold symmetry, so that the imino protons of the four oligonucleotide chains resonate as they were magnetically equivalent. NMR spectra in Na^+ buffer at higher temperatures indicate that the above structure of **5** is preserved at least up to 85 °C, whereas the tetramolecular complex [d(TG_4T)]$_4$ is destroyed at this temperature, as indicated by the disappearance of the imino protons signals. The ^{1}H NMR spectra in K^+ buffer, for both complexes, displayed quite similar signal patterns. However, it was not possible to observe any quenching of the imino protons up to 85 °C also for [d(TG_4T)]$_4$.

Even though bunch-[d(TG_4T)]$_4$ is not the first monomolecular parallel quadruplex ever investigated,[25] nevertheless, thanks to its unique structural features, it can be regarded as the prototype of a new class of quadruplex forming ODNs, potentially useful for a number of interesting applications, considering the increasing number of ascertained or supposed biological roles in which quadruplex structures are involved.[3,4] As a matter of fact, monomolecular quadruple helices formed by bunch-ODNs are provided with more favourable thermodynamic and kinetic parameters than their tetramolecular counterparts.

Therefore, bunch-ODNs with suitable sequences and predetermined strand orientations could be used as aptamers or decoys with improved properties. Furthermore, bunch-ODNs may be important tools for structural studies of alternative, less stable quartets, such as A-tetrads,[26,27] C-tetrads,[28] T-tetrads,[29] mixed

tetrads[30] as well as tetrads formed by modified nucleosides.[31,32] Syntheses of several parallel and/or antiparallel stranded bunch-ODNs, also containing such types of unusual tetrads, are currently in progress in our laboratory.

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