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# EFFECT OF G-RICH SEQUENCES ON THE SYNTHESIS, PURIFICATION, HYBRIDIZATION, CELL UPTAKE, AND HEMOLYTIC ACTIVITY OF OLIGONUCLEOTIDES

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**Abstract**: We designed G-rich oligonucleotides in which the position and length of the guanosine residues were modified and studied the effects of these contiguous guanosine residues on the synthesis, purification, hybridization, cell uptake, and hemolytic effect of oligonucleotides. Our results revealed that the hyperstructure formation of these oligonucleotides depended on the length and position of the guanosine residues and the flanking sequences. The phosphorothioate oligonucleotides formed much less hyperstructures than their phosphodiester counterpart. These hyperstructures were more stable towards nucleases, were taken up by cells efficiently, and showed pronounced polyanionic related side-effects.


Rationally designed oligonucleotides, such as antisense molecules, have been reported to inhibit gene expression in a sequence specific-manner.[1,2] These oligonucleotides bind to their target RNA by Watson-Crick base pairing. It was shown, however, that oligonucleotides can also exert their effect by a non-antisense mechanism. For example, oligonucleotides containing G-rich tracts can adopt a tetrameric G-quartet structure and inhibit gene expression by binding to proteins present in the serum and media. The G-quartet is a cyclic hydrogen-bonded array of four guanine nucleotides that can be formed by G-rich sequences in both DNA and RNA.[3-5] G-quartet structures can be intra- or inter-molecular four-stranded helices stabilized by planar Hoogsteen-paired quartet of guanosine.[3-5] The structures are stabilized by monovalent ions bound between two planes of G-quartets and coordinated to the carbonyl oxygen. A variety of different biological responses have been ascribed to oligonucleotides containing G-quartet sequences including antiproliferative responses, and inhibition of human immunodeficiency virus (HIV).[6-12] Although many biological roles for G-quartets have been proposed, there is no proven role for this structure in biological function. In this report, we designed G-rich oligonucleotides in which the position and length of the guanosine residues were modified, and studied the effect of these G-rich sequences on the synthesis, purification, hybridization, cell uptake and hemolytic activity of oligonucleotides.

Eight oligonucleotide sequences (**oligos 1-8, Table 1**) complementary to HIV RNA were synthesized. All the oligonucleotides had the same length (18-mer) except for **oligo 6**, which had two extra nucleotides at the 5'-end (20-mer). The position and length of the guanosine residues were

modified in each oligonucleotide.

| Oligo Number | Modification | Sequence (5' → 3') | Tm (°C) | Product (%) | Hyperstructures (%) |
|---|---|---|---|---|---|
| 1 | PO | TGTTTAAATCTTGTGGGG | 54.3 | 38.0 | 35.0 |
| 2 | PO | GTGTTTAAATCTTGTGGG | 55.1 | 45.0 | 20.0 |
| 3 | PO | GGTGTTTAAATCTTGTGG | 55.5 | 53.0 | 0.5 |
| 4 | PO | TGGTGTTTAAATCTTGTG | 53.3 | 57.0 | 0 |
| 5 | PO | ATGGTGTTTAAATCTTGT | 50.6 | 53.0 | 0 |
| 6 | PO | GGGTGTTTAAATCTTGTGGG | 57.1 | 51.0 | 7.5 |
| 7 | PO | GGGGTGGCTCCTTCTGAT | 62.3 | 10.5 | 54.0 |
| 8 | PO | ATCTTGCGGGGTGGCTCC | 62.8 | 50.0 | 3.0 |

**Table 1**: Sequences of the phosphodiester oligonucleotides. The synthesis and purification of the oligonucleotides were carried out as described previously.[13] The $T_m$s were measured using the complementary RNA as described previously.[14] The percentage of the product and hyperstructure in each case was determined by measurement of the area under the peaks in the reversed-phase HPLC of the crude oligos **1-8**

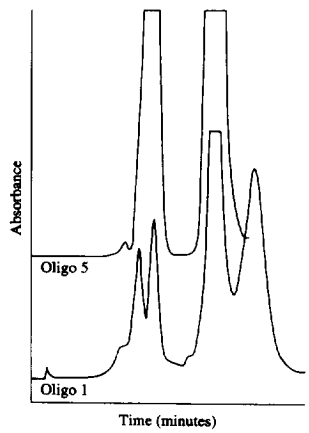


**Figure 1**: Reversed-phase HPLC profile of phosphodiester oligonucleotides **1** and **5**.

After the synthesis, the crude material containing the dimethoxytrityl (DMT) protecting group was analyzed by reversed phase HPLC.[13] Figure 1 shows the HPLC profile of two representative oligonucleotides **oligo 1** and **5**. **Oligo 5** which contained only two guanosine residues eluted as two peaks, the early eluting peak corresponding to the failure sequences and the late-eluting peak corresponding to the purified product respectively. In the case of the **oligo 1**, which contained four guanosine residues at the 3'-end, a third peak was observed, presumably corresponding to the hyperstructure, in the reversed-phase HPLC. The yields of the hyperstucture and the monomeric product were determined by peak integral ratio and is reported for each oligonucleotide sequence reported in Table 1. A comparison revealed that the yield of hyperstructures was dependent on the length and the position of the guanosine residues. Oligonucleotides that contained two guanosine residues did not form any detectable hyperstructures. **Oligo 8**, which had four contiguous guanosine residues in the middle, formed less hyperstructures than **oligo 1** or **oligo 7**, which contained four guanosine residues at the 5' or 3' ends. It is specially relevant to note that when the four guanosine were placed at the 5'-end of the oligonucleotide (**oligo 7**), larger proportion of hyperstructures was observed. Presumably, the presence of the DMT protecting group at the 5'-end facilitated the stacking of these hyperstructures. In order to see if the product can form these hyperstructures after purification, the peak corresponding to the product was collected, detritylated, and desalted (*vide infra*).[13]

Duplex stability of oligonucleotide sequences with complementary RNA indicates that there was no significant difference in melting temperatures ($T_m$s) (Table 1), suggesting that these oligomers bound to the target sequence. The oligonucleotides were then radiolabeled at the 5'-end with [$\gamma$-$^{32}$P] ATP and *T4*

*polynucleotide kinase*. The oligonucleotides were then analyzed by either nondenaturing or 8.3 M urea denaturing polyacrylamide gels. Figure 2 shows that the **oligos 1, 6, 7, and 8**, which contained three or four contiguous guanosine residues, were retarded in the gel while the oligonucleotides that had two Gs migrated as expected. The slow migrating hyperstructures observed in nondenaturing gel were resistant to denaturing conditions (50% formamide, 8.3 M urea). The difference in mobility of these hyperstructures suggested that they were different from each other.

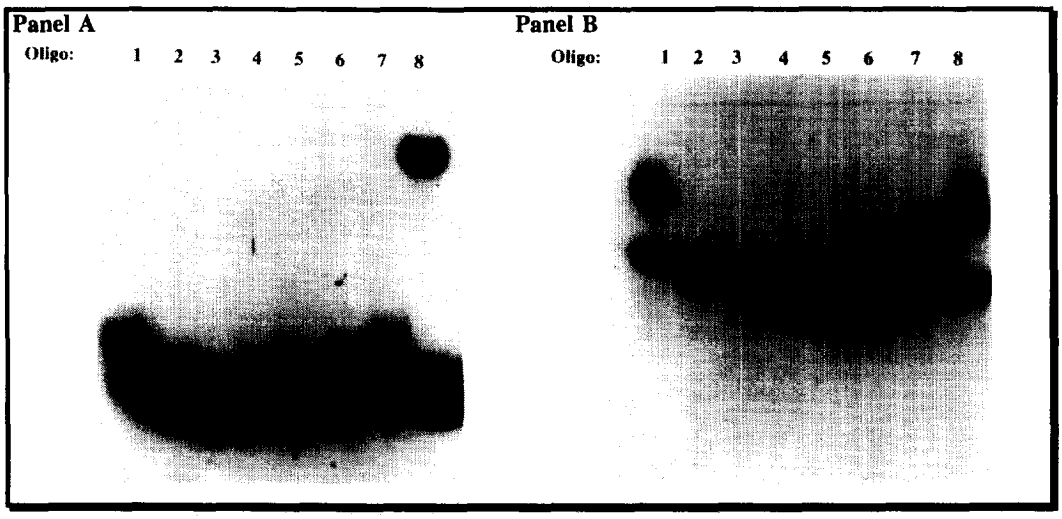


**Figure 2**: Analysis of the product by gel electrophoresis. **Panel A**. 10% Nondenaturing gel. **Panel B**. 20% polyacrylamide gel containing 8.3 M urea.

We also carried out the same studies using phosphorothioate oligonucleotides of similar sequences as in Table 1. As revealed in Table 2, similar profiles of product distribution and hyperstructures were observed as with phosphodiester oligonucleotides.

| Oligo Number | Modification | Sequence (5' → 3') | Product (%) | Hyperstructures (%) |
|---|---|---|---|---|
| 1 | PS | TGTTTAAATCTTGTGGGG | 41 | 17.0 |
| 2 | PS | GTGTTTAAATCTTGTGGG | 57 | 0.5 |
| 3 | PS | GGTGTTTAAATCTTGTGG | 42 | 0 |
| 4 | PS | TGGTGTTTAAATCTTGTG | 47 | 0 |
| 5 | PS | ATGGTGTTTAAATCTTGT | 60 | 0 |
| 6 | PS | GGGTGTTTAAATCTTGTGGG | 50 | 0 |
| 7 | PS | GGGGTGGCTCCTTCTGAT | 50 | 32.0 |
| 8 | PS | ATCTTGCGGGGTGGCTCC | 50 | 1.0 |

**Table 2**: Sequence of phosphorothioate oligonucleotides. The percentage of product and hyperstructures of the crude oligonucleotides following synthesis and deprotection were measured by reversed-phase HPLC.

The yield of hyperstructures was dependent on the length and the position of the guanosine residues. Phosphorothioate oligonucleotides, however, formed much less hyperstructures than phosphodiester oligonucleotides, which might reflect a reduced affinity towards its phosphorothioate counterpart. Analysis of the labeled oligonucleotide by gel electrophoresis after detritylation showed that

the product could also form hyperstructures (data not shown). Different oligonucleotides showed different hyperstructures as was the case with PO-oligonucleotides.

To examine the stability of the oligonucleotides, we incubated the radiolabeled oligonucleotides with *snake venom phosphodiesterase* (SVPD), a 3'-exonuclease.[15] As shown in Figure 3, we found that the slow migrating hyperstructures were resistant to degradation while the bands corresponding to the product were susceptible to degradation by SVPD. The **oligo 1** which formed hyperstructures, which involves 3'-end, remained stable against SVPD. The same result was obtained when we incubated the oligonucleotides in media containing 10% serum (data not shown).

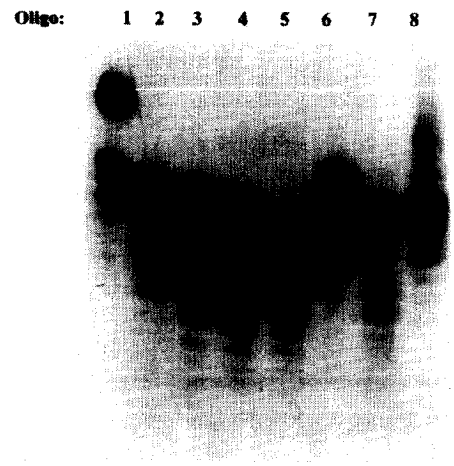


**Figure 3**: Stability of phosphorothioate oligonucleotides against *snake venom phosphodiesterase*. The samples were analyzed by polyacrylamide gel containing 8.3 M urea.

In order to examine the effect of the G-rich sequences on cell uptake, we labeled the oligonucleotides with fluorescein at the 5'-end. For this purpose, phosphorothioate oligonucleotides were used because phosphodiester oligonucleotides were degraded by enzymes present in the media. The fluorescein-tagged oligonucleotides were incubated with H9 cells for 4 h and the cellular uptake was measured by flow cytometry.[16] Figure 4 shows that the oligonucleotides which had the ability to form hyperstructures showed increased uptake. When three or four guanosine residues were present at the 3'-end (**oligo 1, 2**), the uptake increased by 3.5-fold. The presence of two guanosine residues increased the uptake by 1.5-fold. For **oligo 7** which contained four guanosine residues at the 5'-end and labeled by fluorescein at the 5'-end, showed no increase in uptake. This could be due to the interference of fluorescein in the formation of the hyperstructure. We, therefore, labeled the oligonucleotide at the 3'-end and studied its uptake. Indeed, there was increased uptake with this oligonucleotide (data not shown).

We also studied the effect of these oligonucleotides on complement and coagulation. In our earlier studies, we have shown that PS-oligonucleotides activate complement and prolong partial thromboplastin time (aPTT) [17,18] and may be due to the polyanionic nature of the phosphorothioate oligonucleotides.

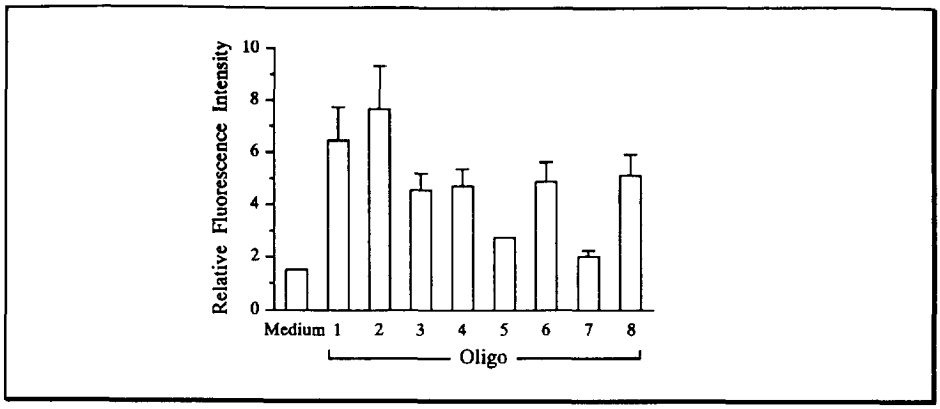


**Figure 4**: Cellular uptake. H9 cells were incubated with fluorescent-labeled PS-oligonucleotides. After 4 h incubation, aliquots of cell culture were removed and the fluorescence was detected using flow cytometry.

Since these hyperstructures are multimeric entities, they might further increase the polyanionic effect. In the event, human normal donor blood was spiked with the phosphorothioate oligonucleotides containing G residues and the hemolytic complement activity[17] and partial thromboplastin time (aPTT) were measured as described previously.[18] Serum treated with oligonucleotides containing G residues demonstrated further increased in aPTT (Figure 5). However, no significant increase in complement activation was observed (Figure 5).

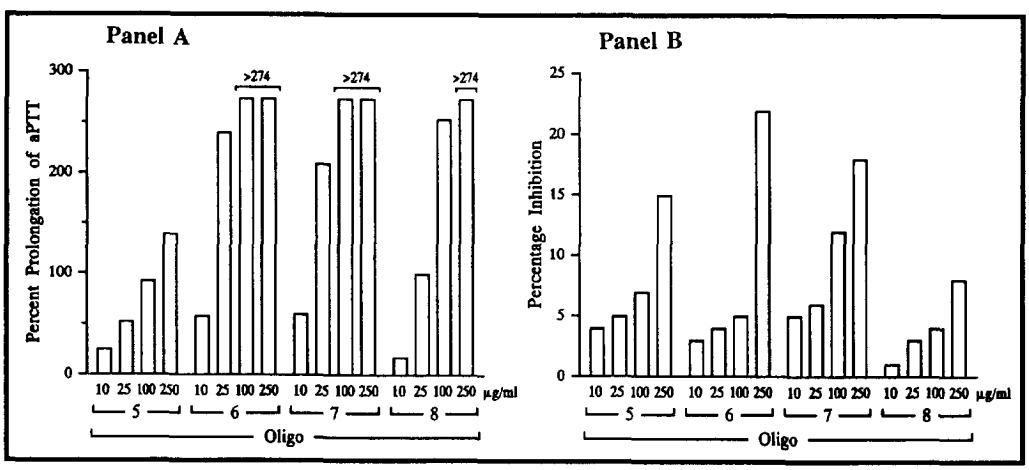


**Figure 5**: **Panel A**. aPTT effect: Percent prolongation of a PTT compared to untreated plasma. **Panel B**. Hemolytic activity: Percentage inhibition compared to untreated samples.

It is clear from these studies that oligonucleotides having contiguous Gs have the ability to form hyperstructures. The formation of these hyperstructures depended on the number of guanosine residues, their position in the oligomer, the flanking sequences, and on other factors such as concentration of salt,

oligonucleotide, and the amount of serum proteins. The hyperstructures formed were polyionic in nature and showed increased effect on aPTT compared to its linear counterpart. There have been many reports on the use of antisense oligonucleotides in which four Gs are present and both sequence specific and non-sequence specific biological effects have been observed.[6-12] Our study suggests that each G containing oligonucleotide is unique in its ability to form hyperstructures and therefore, it is not possible to predict that all oligonucleotides containing contiguous Gs will show similar biological properties.

**Acknowledgement**

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15. Phosphorothioate oligonucleotides (0.05 $A_{260}$ units) were incubated with 0.006 units of *snake venom phosphodiesterase* in a reaction mixture containing 10 mM Tris-HCl pH 8.5 and 10 mM $MgCl_2$ for 1 hr at 37°C.
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