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Abstract

The focus of this research is to discern the structural and biophysical features of small CpG containing deoxyoligonucleotides that have significant biological properties including the inducement of Toll-like receptor 9 (TLR9) in the mediation of cellular invasion. Cell invasion (metastasis) is a significant problem in the control and treatment of breast cancer. Recent research from our laboratory has demonstrated enhanced cellular invasion in the MDA-MB-231 breast cancer cells by ODN-M362, a 25-base single-stranded CpG-containing deoxyoligonucleotide. The mechanism(s) for this induction remain unknown; however, our studies reveal key insights into the structural and sequence requirements for DNA activation of this cellular invasion process. The deoxyoligonucleotides that are effective in eliciting an invasion response have been shown to adopt multiple structural motifs including stem-loops, hairpins, or duplex structures. Sequence modifications were designed to probe base sequence, structure, and stabilities that are required for initiating TLR-9 mediated cellular invasion. Our results demonstrate that these small deoxyoligonucleotides and their structures play a pivotal role as biological response modifiers in this invasion process. Hence, this research is focused on determining the relationship(s) between biological activity and secondary structure of the deoxyoligonucleotides. We have applied a number of biophysical methods including DSC, CD, and computational methods towards understanding the structure and stabilities of these deoxyoligonucleotides.

Introduction

Deoxyribonucleic acid (DNA) was characterized as the repository of genetic information in 1944. Since then, DNA has become a useful tool in identification and diagnostics, in the development of drugs to treat disease, and in gene therapy. More recently, researchers have found that DNA also plays an important biological role through involvement in cellular defense mechanisms and cellular immunity. In this paper, we investigate the interactions of oligodeoxynucleotides (ODNs) with Toll-like receptor 9 (TLR9), which have significant biological activity and play an important role in the control and treatment of breast cancer.

TLR9 is a mediator of the innate immune system that recognizes both microbial and vertebrate DNAs. Members of the Toll-like receptor (TLR) family all contain leucine rich repeat domains in the extracellular portion and an intracellular TIR (Toll-1L-1R) domain. There are at least ten members in the TLR family that can be divided into five subfamilies, one of which is TLR9 (including TLRs 7, 8 and 9). These proteins recognize pathogen derived RNAs and DNAs. Non-specific endocytosis of ODNs is required to activate antigen-presenting cells (APCs). Members of the TLR9 subfamily are expressed intracellularly in the endosomal-lysosomal compartment as opposed to other subfamilies that are bound to the cell surface. Upon recognition of vertebral or microbial DNA, TLR9 induces an inflammatory response mechanism (Wagner 2004).

Over the past decade, there have been a number of studies conducted to examine the effects of base sequence on the binding of ODNs to TLR9. It is commonly accepted in the literature that the ODN sequence must contain CpG nucleotides in order to bind to and activate TLR9. Rutz, et al, have demonstrated binding of CpG ODNs to TLR9 via surface plasmon resonance (SPR) biosensor technology (2004). A sequence-specific recognition of and response to a specific CpG containing ODNs by TLR9 has been reported in many studies (Bauer 2001). The ODN known as M362 is a 25-base long sequence that has been well characterized as a TLR9 agonist.

In this study, we propose that the activation of TLR9 is not only dependent on a specific CpG containing sequence, but also on the secondary structure of the ODN. ODN M362 contains a central 16-base long sequence that is self-complementary. This enables the ODN to adopt a hairpin conformation with dangling ends (four base overhang on the 5' end and five base overhang on the 3' end). In addition, the ODN may exist as a bimolecular duplex. We have performed a series of sequence modifications to determine if a particular secondary structure of the ODN is necessary to activate TLR9. Furthermore, we have applied several biophysical techniques, including differential scanning calorimetry (DSC) and circular dichroism (CD) spectrophotometry studies, in order to investigate the secondary structure of the ODN under various salt and pH conditions.

Methods

Sample Preparation:

Oligonucleotide sequences were purchased from Midland Reagents and used without further purification. They were prepared in Tris EDTA buffer at pH 7.5 for use in invasion assays. The oligonucleotides were prepared in 10 mM BPES buffer at varying sodium chloride concentrations and pH values for biophysical analysis.

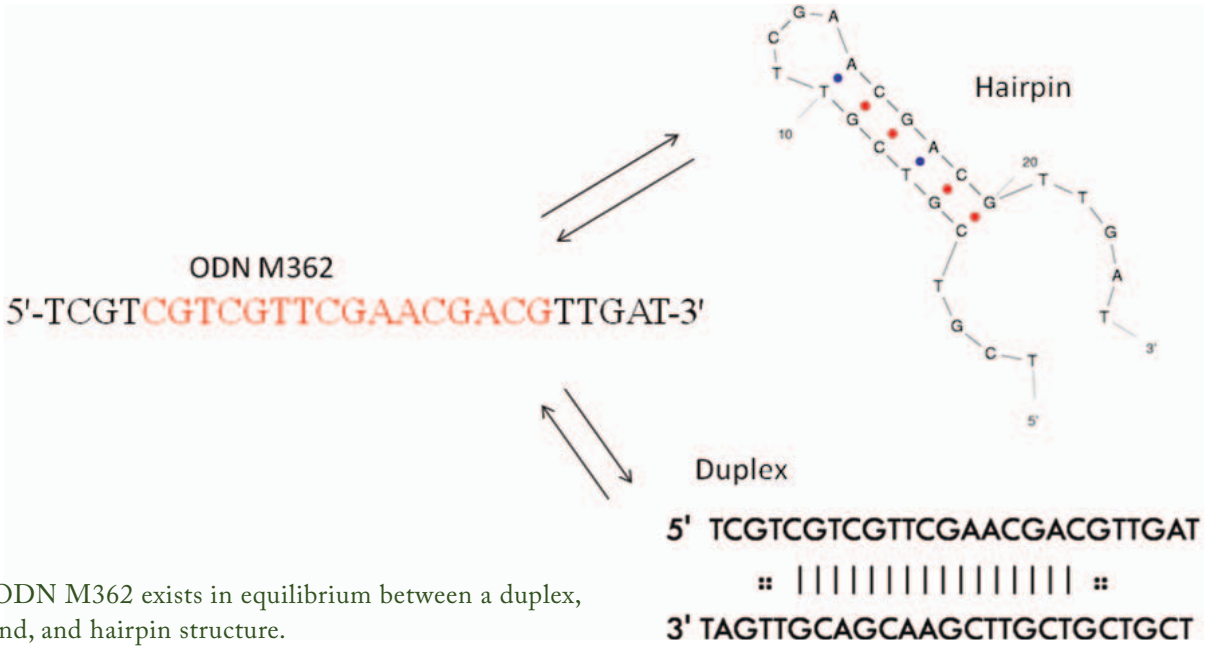

Figure 1. ODN M362 exists in equilibrium between a duplex, single strand, and hairpin structure.

Table 1. Sequence Variations of ODN M362.

Name	Length	Sequence
ODN M362	25	5'-TCGTCGTCGTTCGAACGACGTTGAT-3'
Truncated	16	5'-CGTCGTTCGAACGACG-3'
5' end	20	5'-TCGTCGTCGTTCGAACGACG-3'
3' end	21	5'-CGTCGTTCGAACGACGTTGAT-3'
Trunc + T	17	5'-CGTCGTTCTGAACGACG-3'
Trunc + TT	18	5'-CGTCGTTCTTGAACGACG-3'

Analysis of Structure and Stability of ODN M362:
The molecular modeling program MFOLD was used to predict structural features of ODNs that were shown to exert biological activity, including the induction of TLR9 mediated cellular invasion. This software is used to predict minimal energy structures of DNA and RNA oligonucleotides based on the base sequence, propensity for forming base pairs, base stacking, mismatches, and dangling ends. Using MFOLD, the most stable secondary structures for these oligodeoxynucleotides were determined and probed for biophysical stabilities of base pairing patterns within stems, loop structural and sequence features, and base pair mismatches within the hairpin stems. Results obtained from the secondary structure prediction allowed us to use rational design to incorporate subsequent changes into the base sequence to probe the effects of stem stability, loop sequence and size, and base pair mismatches within the stem on influencing the biological activity of these ODNs in the cell invasion. Accelrys Discovery Studio 2.0 was used to model the hairpin structures of the ODNs and to evaluate their energetic stabilities.

Cellular Invasion Assays:
MDA-MB-231 breast cancer cells were plated onto Matrigel matrices at a cell density of 1 x 10^4 cells per well in 500µL of culture medium. Oligonucleotide treatments containing a phosphorothioate backbone (PS) were added at a concentration of 5 µM. When noted, the oligonucleotides were left unmodified with a phosphodiester backbone (PD). A vehicle treatment of TE buffer was used as the negative control. The cells were allowed to invade for 22 hours after which the inserts were removed and stained with Hema 3 Stain set according to manufacturer recommendations. The number of invaded cells was counted microscopically at five preselected fields using a 40X objective. The results are given as mean ± sd, unless otherwise stated. Student's t test was used to calculate statistically significant differences between the various study groups.

DSC Studies:
DSC experiments were performed with a Microcal VP-DSC from 10°C to 90°C at a heating rate of 0.5°C/min against the appropriate buffer. All samples were prepared to 100µM and were degassed prior to use. At least five scans of buffer in the sample cell were run to acquire an adequate baseline, followed by at least five oligonucleotide melts.

Circular Dichroism Studies:
CD experiments were performed on an Aviv 400 CD spectropolarimeter using a 1 cm pathlength cell. Samples were prepared to 6 µM in 0.01 M sodium phosphate, 0.001 M disodium EDTA, and 100 mM NaCl (BPES) buffer at the designated pH. Data were collected from 215 to 320 nm at every 1 nm with a bandwidth of 1 nm. Time course experiments were monitored at 250 nm over 30 minutes. Spectra were corrected for buffer contributions, and the data were normalized to molar ellipticity (deg□cm^2□decimol^{-2}).

Results and Discussion
In order to discern the structural characteristics of the ODN

that are necessary to induce invasion in breast cancer cells, several sequence variations on the parent ODN M362 were performed. This ODN exists in equilibrium between a duplex with sixteen base pairs and a hairpin structure with six base pairs in the stem and four bases in the loop (**Figure 1**). The sequence modifications are summarized in **Table 1**.

The base sequence of the parent ODN M362 (25mer) was truncated to an entirely self-complementary 16mer ODN. This 16-mer can adopt both a hairpin and a duplex structure, as predicted by MFOLD. The sequence was further modified by introducing additional bases – T and TT – into the loop of the hairpin structure. The addition of the bases in the loop pushes the equilibrium to the hairpin structure, which is more likely to be the dominate species than a duplex with unpaired bases in the center of the sequence. This shift in equilibrium is demonstrated by DSC melting data. The first melt of the truncated 16mer in the absence of salt revealed two species in solution, with melting temperatures (T_m) of 32°C and 53°C (**Figure 2A**). This is indicative of two structures of the self-complementary ODN in solution, which supports the theory of equilibrium between a duplex and hairpin. The less stable structure in the absence of salt is the duplex, and the more stable is the hairpin. After the sample was cooled and melted again, the DSC profile revealed one melting transition at a T_m of approximately 52°C (**Figure 2B**). This corresponds to the annealing of the DNA into its most stable form, the hairpin structure. However, in the presence of salt, the duplex structure is stabilized more than the hairpin is. The DSC melting profile of the truncated 16mer in 100 mM NaCl indicated the presence of two structures, with T_m's of 39°C and 54°C (**Figure 2C**). The presence of salt stabilized the duplex structure by approximately seven degrees, and thereby altered the position of equilibrium between duplex and hairpin. Additional bases were inserted into the loop of the hairpin structure, in order to push the equilibrium to the hairpin. The DSC melting profile of the 17mer, which contains an additional T in the loop of the hairpin structure, revealed that this modification does favor the hairpin structure. The 17mer in 100 mM NaCl melted in a single transition, with a T_m of approximately 58°C (**Figure 2D**). Even in the presence of salt, this ODN does not adopt a stable duplex structure. The hairpin structure is further stabilized for the 18mer, with two additional T's in the loop. This is evidenced by the increase in melting temperatures determined by DSC, as summarized in **Table 2**.

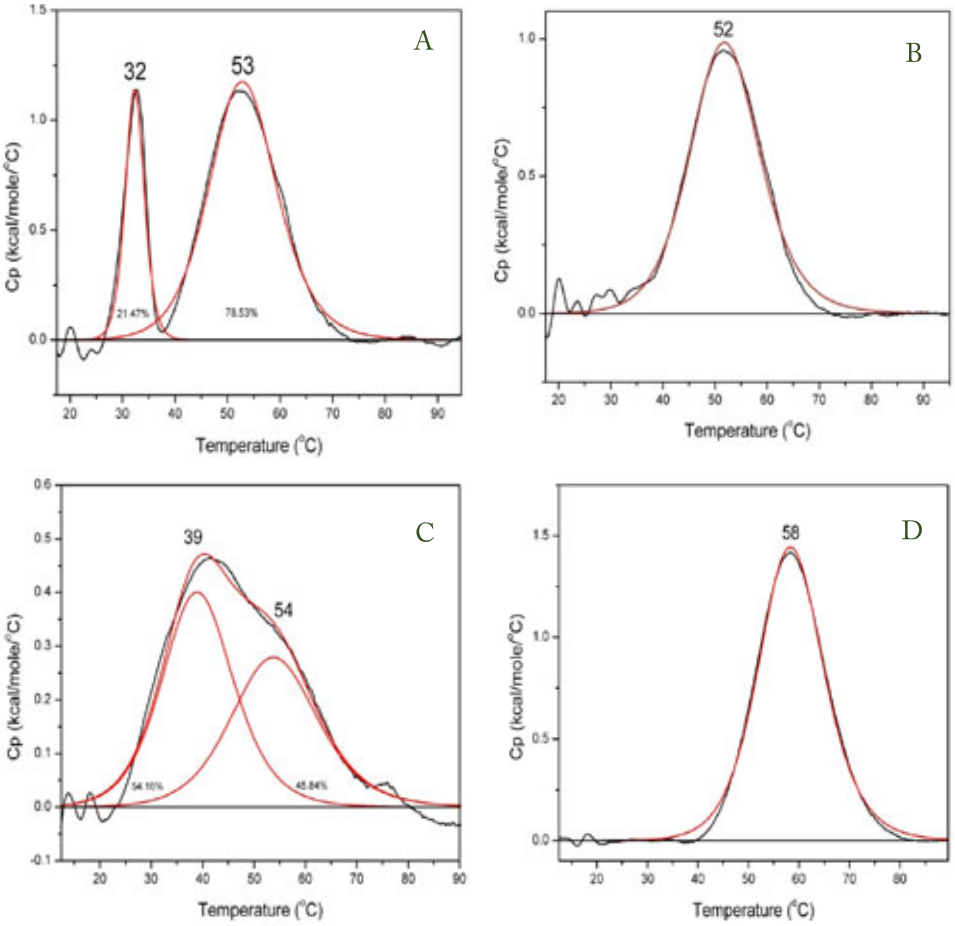

Figure 2. DSC melting profiles of 100 µM truncated 16mer (PS) (A: TE buffer, 1st melt; B: TE buffer, successive melts; C: 100 mM NaCl BPES buffer) and the 17mer (PS) with an additional T in the stem-loop (D: 100 mM NaCl BPES buffer).

Table 2. The melting temperature (T_m) of DNA hairpin structures in 100 mM NaCl BPES as determined by DSC.

Sequence	T_m (°C)
16mer (PS)	54.8 ± 0.53
17mer (PS)	58.15 ± 0.028
18mer (PS)	60.58 ± 0.038

The equilibrium between duplex and hairpin of the truncated 16mer was further evaluated by CD spectrophotometry. The change in the CD spectrum of the 16mer at pH 7 in the presence of 100 mM NaCl was monitored over an increase in temperature. The shift in the spectrum with increasing temperature resulted in two isoelliptical points (**Figure 3**). This is indicative of two species in solution, presumably the hairpin and the duplex. However, because TLR9 is expressed in the endosomal and lysosomal compartments, the interactions between TLR9 and the ODNs may take place in an acidic environment. Therefore, the CD spectrum of the 16mer at pH 5 was monitored over an increase in temperature. At low pH, there was no shift in the spectrum and no isoelliptical points were observed. This demonstrates that at pH 5, the equilib-

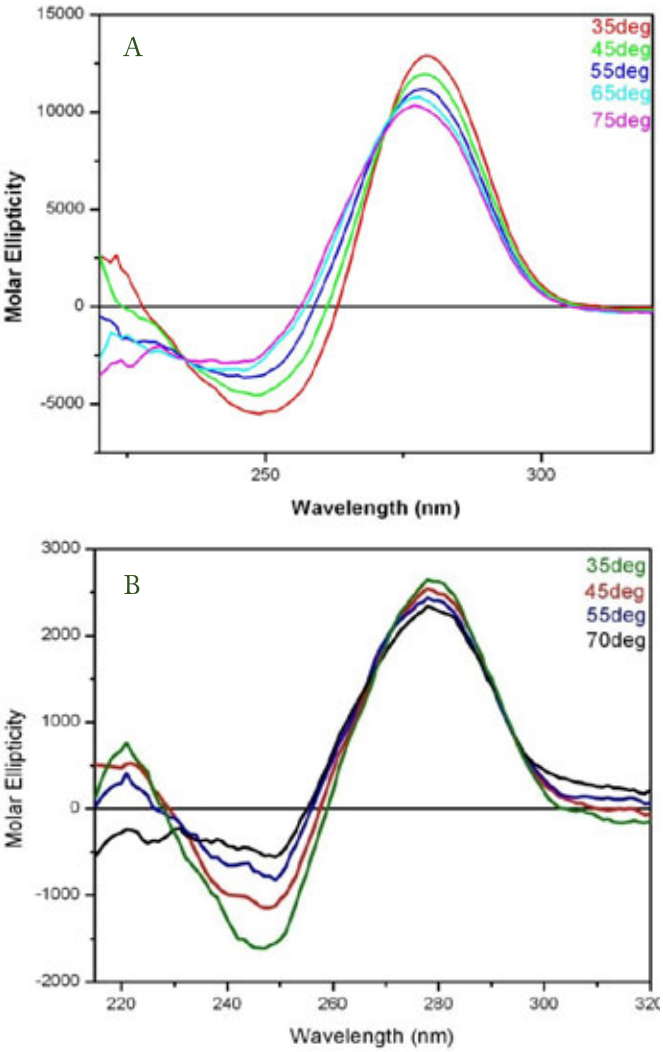

Figure 3. CD spectra of the truncated 16mer (PS) at pH 7 (A) and pH 5 (B) in 100mM NaCl BPES buffer.

rium between the two structures is shifted predominantly to one. Because hairpin structures are favored at lower pH values, it is likely that the hairpin structure dominates at lower pH, even in the presence of salt.

Invasion assays were performed to evaluate the ability of each ODN to induce invasion in MDA-MB-231 breast cancer cells. The resulting fold increase in invasion over a buffer vehicle for each sequence is shown in **Figure 4**. The 16mer induced invasion at a level comparable to that of the parent ODN M362. It has been demonstrated that the 16mer exists in equilibrium between a duplex and hairpin form. However, the 17mer (Truncated + T) and 18mer (Truncated + TT) are more likely to adopt hairpin structures. These sequences also induced invasion comparable to the parent 25mer. Therefore, the 16mer and the 25mer may also adopt hairpin structures in order to produce the invasive response.

In order to perform the invasion assays, the ODNs were modified to a phosphorothioate (PS) backbone, which con-

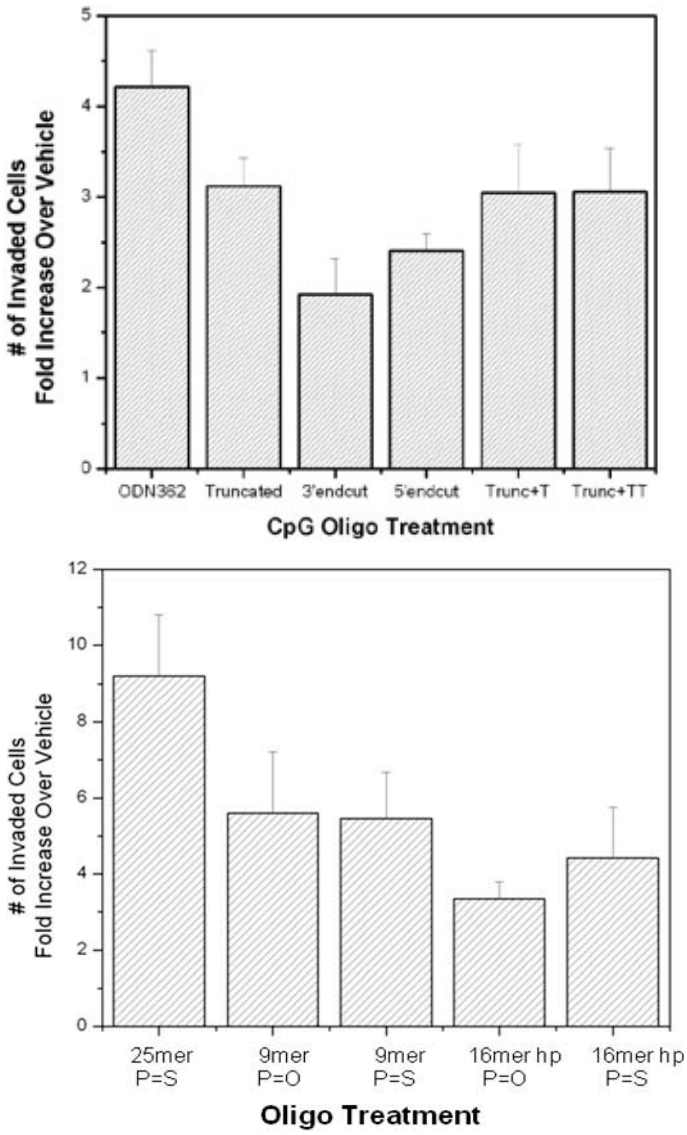

Figure 4. The effects of various oligonucleotides (5μM) on invasion were studied in invasion assays in vitro using MDA-MB-231 cells. The results are expressed as the normalized fold increase in invasion over vehicle. Columns: mean (n = 8) ± SD.

tains phosphate-sulfur double bonds in place of the native phosphate-oxygen double bonds in phosphodiester (PD) backbone. This modification was implemented to make the ODNs resistant to nuclease digestion. However, the apoptotic DNA that is suspected to induce invasion by the same TLR9 mechanism as these ODNs do not contain the modified PS backbone. It has been reported that hairpin structures may offer resistance to nuclease digestion (Yoshizawa, 1994). The effects of a very stable 9mer hairpin with the sequence 5'-d(CGCGAAGCG)-3' on invasion with PD and PS backbone were compared. In addition, the truncated 16mer sequence was modified to contain only purine bases in the loop, in order to provide more favorable stacking interactions to make the hairpin more stable. The effects of this sequence (5'-d(CGTCGTGAAAACGACG)-3', termed "16mer hp") with PD and PS backbone were also studied in invasion as-

says. The results are summarized in **Figure 4**. The increases in invasion due to the PD and PS 9mer hairpin were surprisingly similar. The PD and PS 16mer with purines in the loop also induced invasion at similar levels. This demonstrates that a stable hairpin structure offers resistance to digestion, which allows the ODNs to interact with TLR9 and induce the invasive response.

The hairpin resistance to nuclease digestion was confirmed via CD time course experiments. The CD signal at 250 nm was monitored for a single strand 11mer (PD) that cannot adopt a hairpin structure and for the 9mer (PD) after the addition of S1 nuclease (**Figure 5**). There was a significant change in CD signal for the single strand ODN, while the signal remained constant over thirty minutes for the hairpin ODN.

Conclusions

The work presented here suggests that the biologically active structure is the hairpin. The sequences that contain additional T's in the stem loop of the hairpin structure are more likely to exist as hairpins than in a duplex form. These sequences induce invasion comparable to the truncated sequence. This suggests that the truncated sequence also adopts the hairpin structure. Because the invasive ability of the truncated sequence is not significantly different than that of ODN M362, the biologically relevant species of this sequence may also be the hairpin. These studies also suggest that the predominant and most stable structure is the hairpin.

Furthermore, the hairpin structure provides resistance to nuclease digestion, as evidenced by the ability of hairpin ODNs to induce invasion in breast cancer cells, as well as by the CD data demonstrating resistance to S1 nuclease. This allows for connections to be made from the modified PS ODNs used in invasion assays with PD apoptotic DNA. Plans for future work include the correlation of hairpin ODN stability, as determined by DSC and computational methods, with their ability to induce invasion via TLR9. We intend to undertake a direct investigation of the binding of hairpin ODNs with TLR9 using isothermal titration calorimetry (ITC) studies. Finally, we hope to correlate the ODN sequences that induce invasion with sequences contained in apoptotic DNA.

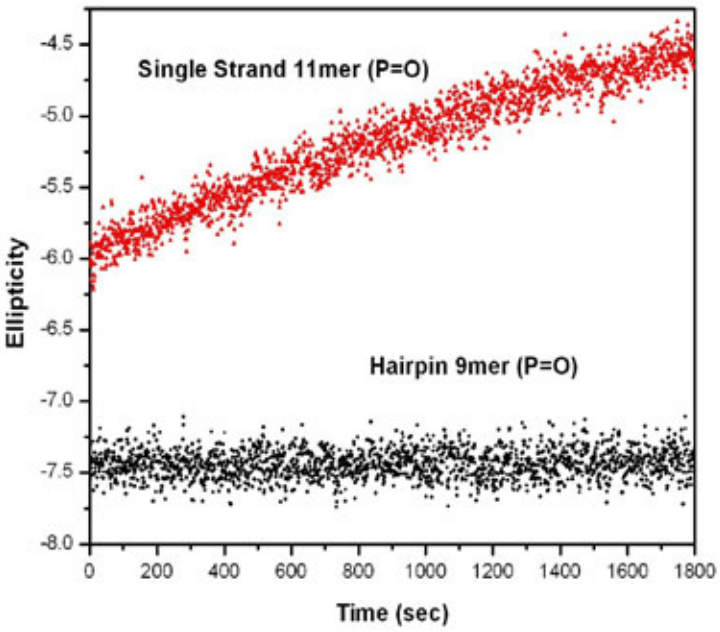

Figure 5. CD signal at 250 nm over 30 minutes after addition of S1 nuclease to a single strand 11mer and the hairpin 9mer.

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