COMMUNICATIONS

Synthesis and Characterization of Circular Structures of i-Motif Tagged with Fluoresceins

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Synthesis of a fluorescein-tagged circular structure of i-motif was accomplished during our investigations. The backbone circularity of this molecular probe and the presence of the fluorescent moiety within its structure were also confirmed through exonuclease hydrolysis and fluorescence spectroscopy.

Held together by hemiprotonated C−C$^+$ base pairing, i-motif is a four-stranded structural entity composed of two parallel-stranded duplexes zipped together in an antiparallel orientation (*1*−*15*). In vitro studies in the past demonstrated that such a tetraplex structural assembly could form by human telomere repeats [d($C_3TAA)_n$] and many other biologically significant cytosine-rich sequences (*8*−*15*) under some physiological-like conditions. Further investigations illustrated that some proteins isolated from *Drosophila* could selectively interact with cytosine-rich repeats of the centromere (*16*). In addition, the cytosine-rich strands of the human telomere were reportedly capable of being bound by some human nuclear proteins such as hnRNP K and ASF/SF2 (*17*). Consequently, it has been assumed that i-motif structures could play some significant roles in certain biological processes (*18*). With the aim of developing molecular probes that could be used to identify new types of i-motif-binding proteins, fluorescein-tagged circular i-motif was constructed in our laboratory recently. We present herein the design and synthesis of these new types of fluorescein-containing circular oligonucleotides as well as characterizations of these circular molecular probes by using exonuclease VII and fluorescence spectroscopy. It is our expectation that such newly designed probes could be useful for identifying a new i-motif-binding protein as well as determining the location and distribution of i-motif-binding proteins in acidic cancer cells upon the introduction of these probes through microinjection.

Our approaches for synthesizing a circular i-motif structure tagged with a fluorescein moiety (oligonucleotide **2**) are illustrated in Figure 1. A linear 32-mer oligonucleotide containing a fluorescein moiety covalently linked to carbon-5 of a thymine (oligonucleotide **1** in Figure 1a) was accordingly designed in our studies. The 5′-phosphate and 3′-hydroxyl ends of oligonucleotide **1** would be proximal to each other once this

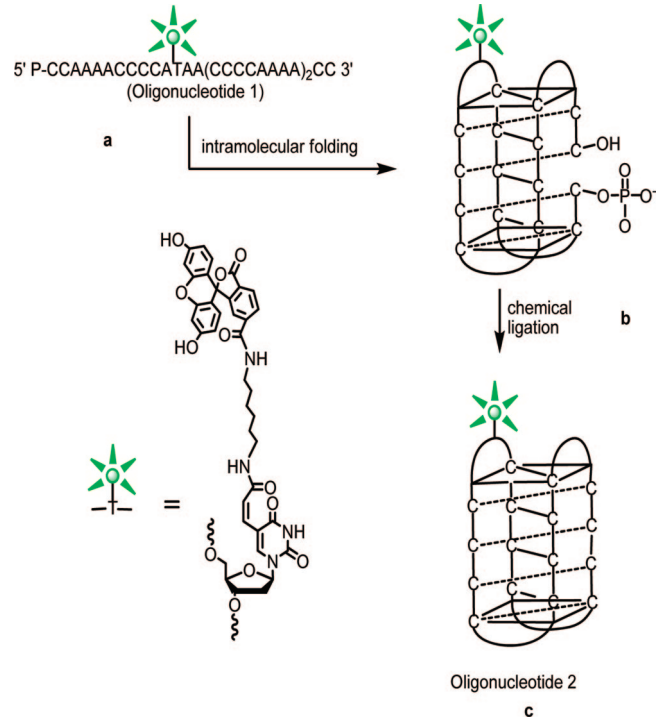

Figure 1. Schematic representation of the synthesis of a fluorescein-tagged circular structure of i-motif.

oligonucleotide self-assembles into intramolecular i-motif conformation (Figure 1b) under slightly acidic conditions (*1, 2*). A new phosphodiester bond was expected to form between 5′-phosphate and 3′-hydroxyl ends upon the subsequent chemical activation. A mixture was accordingly prepared during our investigation that contained 1 μM sequence A, 100 mM MES (pH 5.0), and 50 mM KCl. This solution was heated next to 90 °C and kept at the same temperature for 3 min followed by cooling the mixture to 25 °C over 2 h to allow the formation of the i-motif structure (Figure 1b). The chemical ligation reactions

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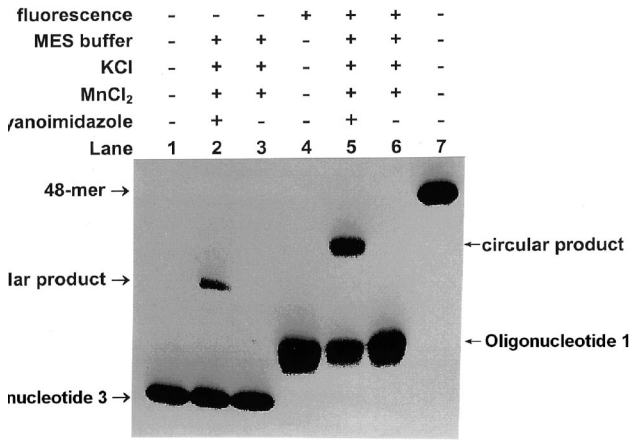

Figure 2. Electrophoretic analysis of the fluorescein-tagged circular structure of i-motif constructed through chemical ligation reactions. A linear precursor (oligonucleotide **1** or oligonucleotide **3**) with free 5′-hydroxyl terminus was labeled with [γ-^{32}P] ATP (GE Healthcare) at its 5′-end catalyzed by T4 polynucleotide kinase (New England Biolabs). A mixture containing 100 mM MES (pH 5.0) and 50 mM KCl and 1 μM linear precursor (oligonucleotide **1** or oligonucleotide **3**) was then kept at 90 °C for 3 min and further allowed to cool down to 25 °C over 120 min. The chemical ligation reactions were next initiated by addition of *N*-cyanoimidazole in the presence of $MnCl_2$, and the resultant solution contains 500 nM annealed linear precursor (oligonucleotide **1** or oligonucleotide **3**), 100 mM MES (pH 5.0), 50 mM KCl, 10 mM $MnCl_2$, and 10 mM *N*-cyanoimidazole. After being further incubated at 4 °C for 24 h, the reaction mixtures were analyzed by polyacrylamide gel electrophoresis (20%) followed by visualization using a PhosphorImager (Typhoon Trio, GE Healthcare). Lane 1: linear prescusor oligonucleotide (oligonucleotide **3**) alone. Lane 2: mixture of ligation reaction containing non-fluorescein-tagged linear oligonucleotide (oligonucleotide **3**). Lane 3: same reaction as in lane 2 except for the absence of **N**-cyanoimidazole. Lane 4: linear precursor oligonucleotide (oligonucleotide **1**) alone. Lane 5: mixture of ligation reaction containing fluorescein-tagged linear oligonucleotide (oligonucleotide **1**). Lane 6: same reaction as the one loaded in lane 5 except for the absence of **N**-cyanoimidazole. Lane 7: 48-mer oligonucleotide as a molecular weight marker.

were then initiated by addition of *N*-cyanoimidazole in the presence of $MnCl_2$ (*19*−*21*). After being further incubated at 4 °C for 24 h, the reaction was terminated by adding loading buffer. The resultant ligation products were analyzed by polyacrylamide gel electrophoresis.

As illustrated in Figure 2, a slow-moving band was observed (upper band of lane 5) when the ligation reaction of oligonucleotide **1** (Figure 1b) was activated by addition of *N*-cyanoimidazole, which corresponds to the desired fluorescein-labeled circular i-motif (*22, 23*) (Figure 1c). For comparison, a linear oligonucleotide (5′-pCCAAAACCCCATAACCCCAAAACCCCAAAACC-3′, oligonucleotide **3**) that contains no fluorescent tag was also designed and circularized (lane 2) during our investigations. As shown in Figure 2, a new band was generated as well in the circularization processes of oligonucleotide **3**. It is worth noting that the circularization product obtained from oligonucleotide **1** (fluorescein-tagged oligonucleotide) (lane 5) has a slower rate of mobility than that of oligonucleotide **3** (non-fluorescein-tagged oligonucleotide) (lane 2). The possession of an extra fluorescein moiety within oligonucleotide **1** could be the cause of the mobility shift difference between these two circular products (Figure 1c).

With the aim of confirming the absence of open ends in oligonucleotide **2**, the newly formed ligation product (upper band in lane 5 of Figure 2) was purified and further hydrolyzed with exonuclease VII (an exodeoxyribonuclease that degradates linear DNA from both 3′ and 5′ termini). As seen in Figure 3, there was an absence of degradation product observed when the purified ligation product was incubated with exonuclease VII

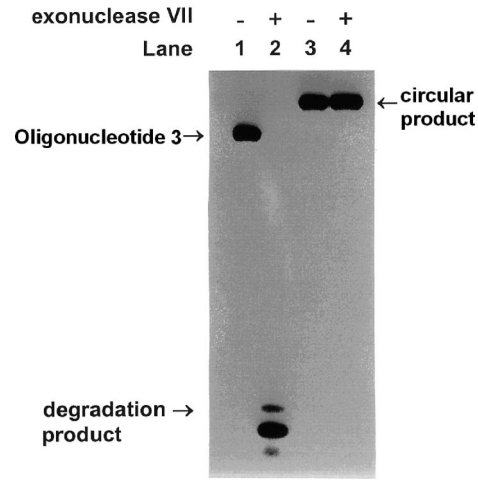

Figure 3. Hydrolysis of purified fluorescein-tagged circular i-motif (oligonucleotide **2**) by exonuclease VII. The ^{32}P-labeled reaction products in the upper band in lane 5 in Figure 2 were cut out and further purified. Solutions containing 100 mM Tris-acetate, 100 mM potassium phosphate, 16.6 mM ethylenediamine tetraacetic acid, and 20 mM 2-sulfanylethanol, pH 7.9, 10 units of exonuclease VII, and the newly purified ^{32}P-labeled oligonucleotides were then incubated at 37 °C for 2 h. The hydrolysis products were further analyzed using polyacrylamide gel electrophoresis (20%). Lane 1: 32-mer linear precursor oligonucleotide (oligonucleotide **3**) alone. Lane 2: the reaction mixture of 32-mer linear precursor oligonucleotide (oligonucleotide **3**) and exonuclease VII. Lane 3: fluorescein-tagged circular products (oligonucleotide **2**) alone. Lane 4: the reaction mixture of purified fluorescein-tagged circular products (oligonucleotide **2**) and exonuclease VII.

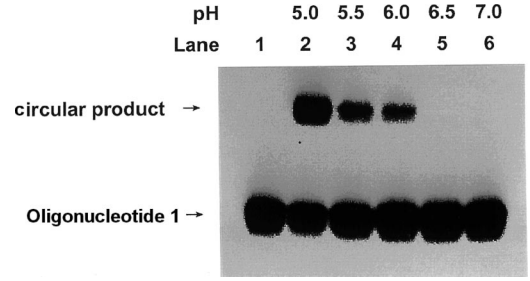

Figure 4. pH dependency of the circularization reaction of fluorescein-tagged linear oligonucleotide (oligonucleotide **1**). Ligation reactions were carried out in the same way as the one loaded in lane 5 in Figure 2 except that pH values of the buffer solutions were 5.0 (lane 2), 5.5 (lane 3), 6.0 (lane 4), 6.5 (lane 5), and 7.0 (lane 6), respectively. Lane 1: linear precursor oligonucleotide (oligonucleotide **1**) alone.

(lane 4 in Figure 3). For comparison, oligonucleotide **3** (a linear precursor) was also degraded by exonuclease VII under the same reaction conditions, which led to the complete disappearance of this linear precursor and generation of fast-moving degradation products (lane 2 in Figure 3). The complete resistance of the purified ligation product (oligonucleotide **2**) to the hydrolysis by this exonuclease is the indication that there is absence of open terminus within its backbone (*24*).

In addition, the variation of pH value on our circularization reactions was examined during our investigations. As shown in Figure 4, the efficiency of these ligation reactions decreased with the increase of pH values of the corresponding buffer solutions. The circularization reaction of oligonucleotide **1** proceeded in 45%, 11%, and 6% yields at pH 5 (lane 2), pH 5.5 (lane 3), and pH 6.0 (lane 4), respectively, while there were no circularization products from the reaction observable at pH 6.5 (lane 5) and pH 7.0 (lane 6). The above observations suggest

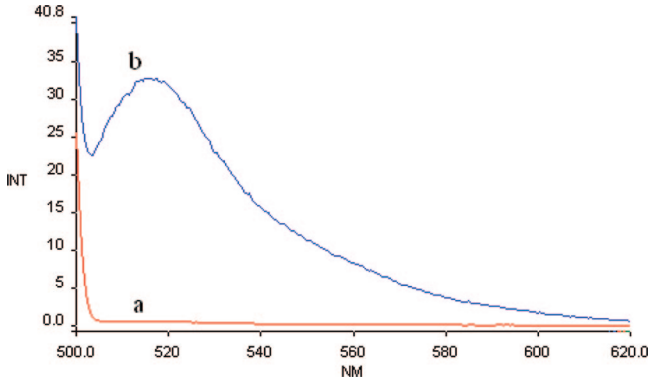

Figure 5. Fluorescence emission spectra of fluorescein-labeled circular i-motif (oligonucleotide **2**) (a) and non-fluorescein-labeled circular oligonucleotide (b). Samples containing 1 μM oligonucleotides and 10 mM Tris buffer (pH 7.0) were examined at 20 °C with excitation set at 495 nm using a fluorescence spectrometer (Perkin-Elmer LS 55).

that the formation of the i-motif structure is a prerequisite for the i-motif-based ligation reactions (*1*−*15*).

With the aim of confirming that the molecular moiety of fluorescein was indeed present in oligonucleotide **2**, fluorescence spectroscopic examinations were carried out in our studies. As seen in Figure 5, oligonucleotide **2** displayed an emission maximum at 520 nm when excited at 495 nm wavelength. For comparison, the circular product of oligonucleotide **3** (a non-fluorescence-tagged oligonucleotide) was also examined during our studies, which lacks emission from 500 to 620 nm. In addition, MALTI-TOF mass spectroscopic examination on oligonucleotide **2** and oligonucleotide **1** was carried out during our investigations. It turned out that the obtained molecular weights of the ligation product (oligonucleotide **2** in Figure 1) and its linear precursor (oligonucleotide **1** in Figure 1) were 10 145.35 Da (calculated molecular weight: 10 141.8 Da) and 10 163.09 Da (calculated molecular weight: 10 159.8 Da), respectively. The observed molecular weight difference between oligonucleotide **2**and oligonucleotide **1** was ∼18, which is consistent with the suggestion that a condensation reaction took place between the two termini of oligonucleotide **1**.

In conclusion, circular i-motif structure tagged with fluorescein was synthesized in our laboratory through chemical ligation reactions. Our subsequent studies confirmed that this newly obtained oligonucleotide resists the hydrolysis by exonucleases and the fluorescent moiety is indeed present in the ligation product. Since many cancer cells are acidic (*25*−*27*), it is our expectation that these newly designed fluorescein-labeled circular i-motifs could serve as useful probes for identifying new types of i-motif-interacting enzymes and other functional proteins in these fast-dividing cells.

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Supporting Information Available: Additional experimental details about oligonucleotide **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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