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BI-FUNCTIONAL LABELLING OF DNA WITH TRIS(2-AMINOETHYL)AMINE-DERIVED NOVEL FLUORESCENT AGENT

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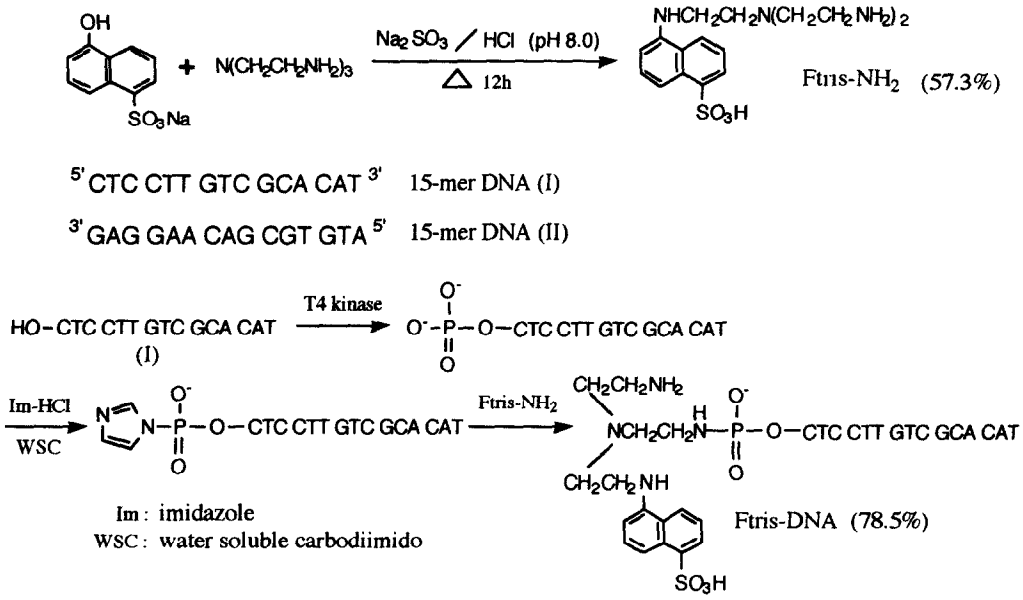
Abstract. A novel bi-functional fluorescent labelling agent derived from 5-hydroxy-1-naphthalene sulfonic acid and tris(2-aminoethyl)amine was synthesized and incorporated into the 5'-terminus of a 15-mer oligoDNA through phosphoroamidate linkage. The labelled oligoDNA bearing a primary and a tertiary amine group along with fluorescent naphthalene moiety exhibits improved hybridization ability with its complementary strand.

Labelling of oligonucleotide with a non-radioactive material, such as a fluorescent dye, has been attracting a great attention for last decade because of its easy handling and relatively inexpensiveness as compared to radiolabelling technique. The labelled oligonucleotide can be used as a probe to detect specific gene or its fragment from a variety of biological samples[1]. Either 3'- or 5'- terminus of the oligonucleotide is often chosen as the site of derivation with non-radioactive material, because the label incorporated to the oligonucleotide will not interfere the duplex formation between the labelled oligomer and its complementary nucleotide strand[2]. Thus, various dye derivatives bearing primary amine group, hydroxyl group or phosphoramidite group have been developed to prepare the oligonucleotide-dye conjugate. In most of the cases, the label is covalently introduced at the terminal position of oligonucleotide through phosphoric ester linkage, phosphoroamidate linkage, or phosphorothioate linkage, etc[2-4]. The dye derivatives hitherto developed are, however, monofunctional and are simply designed to incorporate only one label into one oligomer. On the other hand, if the labelled oligonucleotide can be further modified to enhance the duplex forming ability with its complementary strand then such oligonucleotide would be a more useful prove.

In this report, we describe the synthesis and usage of a novel bi-functional florescent labelling agent derived from naphthalene sulfonic acid and tris(2-aminoethyl)amine. This new reagent is designed to incorporate dye moiety, in this case naphthalene sulfonic acid moiety, and a primary and a tertiary amine group to a oligonucleotide simultaneously through the formation of phosphoroamidate linkage. The amine groups are expected to be charged positively in neutral pH range. Therefore, once the oligonucleotide labelled with the reagent hybridizes to its complement under the physiological condition, the amine groups would interact with negatively charged phosphodiester functions of the complementary strand to enhance the stability of the duplex[5].



Scheme 1

The labelling agent, 5-tris(2-aminoethyl)amino-1-naphthalene sulfonic acid (Ftris-NH_2) was prepared from tris(2-aminoethyl)amine and 5-hydroxy-1-naphthalene sulfonic acid according to the literature[6] and was purified by reverse-phase gel chromatography. 5-Hydroxy-1-naphthalene sulfonic acid was once used for the fluorescent labelling of DNA[7]. The yield of Ftris-NH_2 was 57.3 %[8]. The DNA 15-mer (I) and its complementary strand (II) were prepared by the solid phase phosphoramidite method described previously, using ABI DNA Synthesizer Model 381-B[9]. Phosphorylation of (I) on its 5'-terminal hydroxyl group was accomplished by the action of T4 kinase[10]. Introduction of Ftris-NH_2 to the phosphorylated oligomer (I) was performed according to Orgel's method[11] with slight modification[12]. These are summarized in Scheme 1. After the purification with HPLC(RPC-5 column), the isolated Ftris-DNA (78.5 %) was further analyzed by polyacrylamide gel electrophoresis under the denaturing condition. As shown in Fig. 1, both of the labelled and unlabelled oligomers were easily detected by UV shadowing technique[13,14]. The labelled oligomer, Ftris-DNA, was also easily visualized by its distinct fluorescence (Fig.1-b). Under this condition, Ftris-DNA moved slightly slower than the unlabelled parent oligomer. The slow mobility is due to the existence of positively charged primary and tertiary amine group on the linker moiety of the labelling agent [5].

To see how these amine groups will affect the stability of complementary duplex, we have measured melting curves of duplexes consisted of Ftris-DNA or unlabelled 15-mer (I) and their complement (II) under four different concentrations[15]. The obtained melting curves were all monophasic sigmoidal curves indicating simple two-state transition was going on in each cases (Fig. 2). The melting points were determined by calculating the first derivative $\Delta A/\Delta T$ obtained from these melting curves[16].

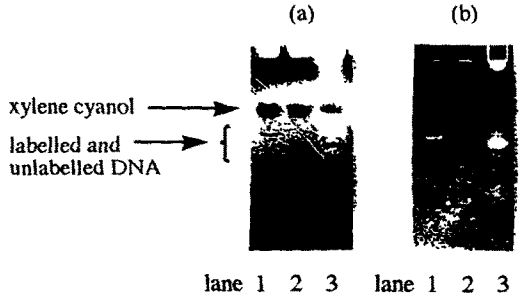


Fig. 1. (a) UV Shadowing of DNAs. The gel was irradiated by UV light at 254 nm. (b) Fluorescent detection of DNAs. The same gel as (a) was irradiated by UV light at 350 nm. lane 1: Ftris-DNA. lane 2: unlabelled DNA (I). lane 3: reaction mixture.

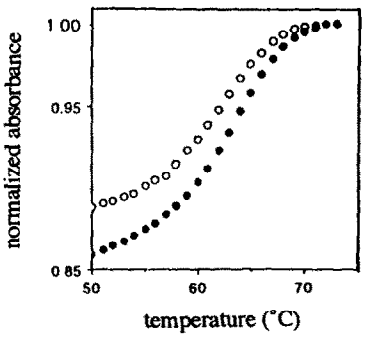


Fig.2. Melting curves of duplexes consisted of (I) : (II) [O] and Ftris-DNA : (II) [●]. In this case, total strand concentration was 10.4 μM for each sample.

The melting point thus obtained increased steadily as the concentration of the sample increased. The duplex consisted of Ftris-DNA with its complement (II) exhibited higher melting points in any case of concentration than the corresponding duplex consisted of the unlabelled oligomer (I) and (II). Thus the labelled oligomer has the improved duplex forming ability as compared to the unlabelled oligomer. This was confirmed by thermodynamic parameters calculated from the above data which are listed in Table 1. The results indicate that the stabilization of the duplex consisted of Ftris-DNA and its complement was mainly brought by an enthalpic factor (ΔH°) even though the entropy factor (ΔS°) was slightly more unfavorable.

Table 1. Thermodynamics for single-stranded to double-helix transition for (I) : (II) and Ftris-DNA : (II). ΔG° represents the free energy change at 25°C.

duplex	ΔH° (kcal mol^{-1})	ΔS° (cal mol^{-1} K^{-1})	ΔG° (kcal mol^{-1})
(I) : (II)	-90.1	-243.8	-17.4
Ftris-DNA : (II)	-104.7	-286.6	-19.2

Since we did not see any measurable interaction of naphthalene sulfonic acid itself with the tested oligoDNAs, we presume that observed stabilization effect of the duplex consisted of Ftris-DNA and the oligomer (II) was due to electrostatic interaction between two positively charged amine groups on the linker moiety of Ftris-DNA and negatively charged phosphodiester backbone of the complementary strand.

The present results will help to design various new bi-functionally derived oligonucleotides in which one can introduce two different functions, such as enhancing the duplex formation and cutting the hybridized

complementary strand, into a oligonucleotide by single reaction. Currently studies to develop such multifunctional oligonucleotides are going on.

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    All samples were formed 1:1 mixture of a strand with its complement in a buffer solution (pH 7.2) containing NaCl (150 mM) and were pre-melted at 80°C and allowed to thermally equilibrate before recording.
    Total strand concentrations examined were 10.4, 20.8, 31.2, and 41.6 μM for each samples.
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