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Facile preparation of non-self-quenching fluorescent DNA strands with the degree of labeling up to the theoretic limit†

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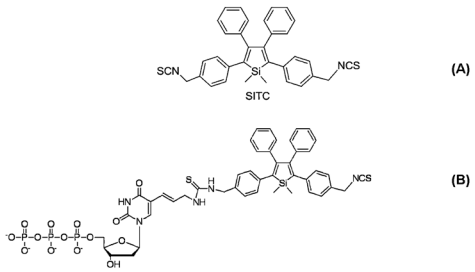
A new aggregation-induced emission (AIE) compound 1,1-dimethyl-2,5-bis[4-(isothiocyanatemethyl)phenyl]-3,4-diphenylsilole (SITC) was synthesized and used to conjugate with aminoallyl-dUTP. The SITC–dUTP can be incorporated enzymatically into DNA strands with the degree of labeling (DOL) up to the theoretic limit.

Fluorescent DNA segments have attracted great interest because of their applications as fluorescent probes for gene detection through fluorescent *in situ* hybridization (FISH) or hybridization in solutions with the target nucleic acids.[1,2] Enzymatic incorporation and solid-phase oligonucleotide synthesis are two main approaches to construct fluorescent DNA.

Solid-phase oligonucleotide synthesis is a classic method for preparing functional nucleic acids by using functionalized nucleoside phosphoramidites.[3–5] The fluorescent products can be synthesized by using dye labeled nucleoside phosphoramidites or through poststaining of synthetic oligonucleotides. Wonderful results were achieved through postsynthetic modifications of synthetic oligonucleotides.[6] The matter of solid-phase synthesis is the necessity of using protecting groups in most cases.[7]

Enzymatic incorporation constructs functional nucleic acids by polymerase incorporation of base-functionalized dNTPs through nick translation, random priming and polymerase chain reaction (PCR).[8] Fluorescent DNA can be obtained by labeling the base-functionalized dNTPs before or after enzymatic incorporation. For the former, it is reported that incorporation of traditional dye labeled dNTPs usually has a poor labeling efficiency, which is quantified by the degree of labeling (DOL) and commonly expressed as the number of dyes per 100 bases.[9] For the latter, the two-step labeling process requires selective and mild reactions. Furthermore, the DOL of the fluorescent DNA prepared with traditional fluorescent dyes must be controlled at a relatively low level to avoid quenching of fluorescence, and thus the sensitivity of the probes is significantly weakened.[10]



Scheme 1 Chemical structures of (A) SITC and (B) SITC conjugated aa-dUTP (SITC–dUTP).

Great effort has been made on the development of luminescent materials with aggregation-induced emission (AIE) property.[11–13] In this work, an amine modified dUTP, 5-(3-aminoallyl)-2′-deoxyuridine-5′triphosphate (aminoallyl-dUTP or aa-dUTP), was labeled with an AIE-active silole derivative 1,1-dimethyl-2,5-bis[4-(isothiocyanatemethyl)phenyl]-3,4-diphenylsilole (aka silole isothiocyanate, SITC, Scheme 1A) prior to the enzymatic incorporation and the fluorescent product was subsequently incorporated into DNA through nick translation, random priming and PCR methods. Fluorescent DNA products were obtained with high labeling efficiency by all three enzymatic incorporation methods. The DOL values of the SITC labeled products were comparable to that of the fluorescent DNA labeled with traditional dyes for nick translation and random priming but were 10 times higher with the PCR method. The effects of DNA sequences and base pair numbers on the preparation of SITC labeled DNA using the PCR method were tested. The control of DOL was realized by adjusting the ratio of SITC conjugated aa-dUTP to dTTP through the PCR method. The AIE property of SITC removed the limitation on the DOL and made it possible to prepare highly emissive fluorescent DNA without quenching but enhancing effect.

The silole derivative SITC was prepared according to the synthetic route shown in Scheme S1 (ESI†) and its AIE property is demonstrated in Fig. 1. The wavelength at maximum absorption of SITC was at 363 nm and the emission maximum was at 490 nm (Fig. 1A). Similar to all other AIE molecules,

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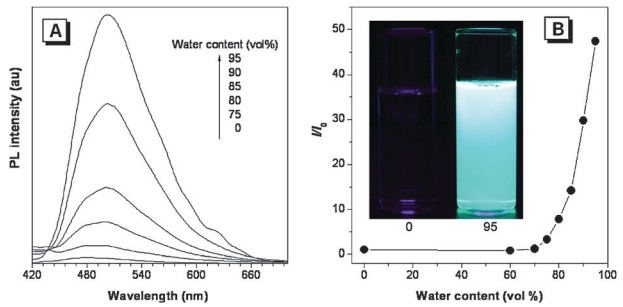

Fig. 1 (A) Photoluminescence (PL) spectra of SITC in THF–water mixtures with different water contents; concentration of SITC: 10 μM; excitation wavelength: 365 nm. (B) Plot of PL peak intensity *versus* water content of the THF–water mixture. I_0 is the PL intensity in pure THF solution. Inset of (B): photos of THF–water mixtures of SITC with 0 and 95 water fractions taken under 363 nm UV irradiation.

SITC was weakly emissive in good solvents such as THF, while its PL intensity in the water–THF solution with a water fraction of 95 vol% was approximately 50-fold higher than that in the mixture with 70% water (Fig. 1B). The quantum efficiency of SITC was 45.8% in the solid state.

Compared with the highly reactive isocyanate compounds, isothiocyanate products are more stable in water and most organic solvents, but have a comparable reactivity with the primary amine groups. Therefore an amino group containing dUTP, 5-(3-aminoallyl)-2′-deoxyuridine-5′triphosphate was selected and labeled with SITC in aqueous media. The modification site of aa-dUTP is the C5 position of the pyrimidine ring, which does not participate in the base-pair hydrogen bonding (Scheme 1B). To avoid the reaction of two aa-dUTPs with one SITC which contains two isothiocyanate groups, a solution of SITC in DMSO was added into aa-dUTP solution with the amount of SITC equal to that of aa-dUTP. Because of the limited amount of aa-dUTP we got, purification of the labeling mixture was not carried out before the preparation of fluorescent DNA. It should be noted that trace amount of DMSO has little effect on enzymatic incorporation.[14]

A PCR product of plasmid DNA containing 1061 bp was used as a template (Table S1, ESI†) for both nick translation and random priming. Nick translation of this template by using DNase I and DNA polymerase I was employed to prepare a pool of labeled DNA fragments (Fig. 2A, lane 2). On the other hand, degenerate oligonucleotides (*e.g.*, random hexamer primer) were used together with DNA polymerase in random priming to synthesize uniformly labeled DNA strands (Fig. 2A, lane 3).

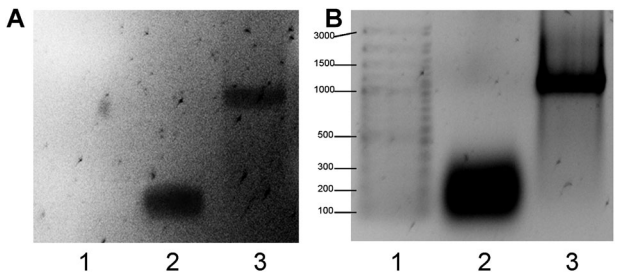

Fig. 2 (A) Fluorescent image of agarose gel of fluorescent DNA prepared by nick translation and random priming with SITC–dUTP. (B) Fluorescent image of the same gel restained with GelRed™. Lane 1: DNA ladder, lane 2: nick translation, lane 3: random priming.

Table 1 DOL values of fluorescent DNA prepared by nick translation and random priming

dTTP/ μM	SITC–dUTP/ μM	DOL of nick translation	DOL of random priming
50	50	2.57 ± 0.61	2.64 ± 0.81
30	70	4.01 ± 1.13	3.47 ± 0.70
0	100	5.00 ± 1.19	3.52 ± 1.65

The 1061 bp template DNA was a PCR product of plasmid DNA. dTTP was replaced with increasing amount of SITC–dUTP.

The labeled double-stranded DNA (dsDNA) products were confirmed by GelRed poststaining (Fig. 2B). According to the results shown in Fig. 2A and B, SITC labeled DNA products prepared by nick translation had a broad band with the bp number in the range of ~100 to ~300, while the random priming fluorescent products appeared as a uniform band of about 1 kbp.

The DOL of labeled DNA, number of dyes per 100 bases, was calculated using the Beer–Lambert law. The details about the calculation are described in the experimental section. The calculated molar extinction coefficient (ε) of SITC is 10 277 cm^{-1} M^{-1} and its correction factor (CF) is 2.08. Table 1 shows the DOL of the fluorescent DNA prepared by nick translation and random priming using an increasing amount of SITC–dUTP. The DOL values of all the SITC labeled products are comparable to the results of DNA products labeled with traditional fluorescent dyes.[9]

Polymerase chain reaction (PCR) has many advantages over other enzymatic incorporation methods in preparing fluorescent DNA. Through the PCR method, simultaneous amplification and labeling of a specific DNA or a specific region of a DNA can be achieved. Thermophilic Deep $Vent_R$ exo-DNA polymerase (New England Biolabs) with high efficiency in incorporation of modified dNTPs to DNA was utilized to prepare SITC labeled DNA. Plasmid DNA with bp numbers of 94, 204, 304 (Table S2, a–c, ESI†) were selected as templates to prepare SITC labeled DNA products. The results confirmed the successful amplifications of these three templates through normal PCR (Fig. S1A, ESI†). On the other hand, preparation of SITC labeled DNA products was conducted by using Deep $Vent_R$ exo-DNA polymerase and SITC–dUTP. Only the template with 94 bp could be replicated during this process (Fig. S1B, ESI†). As mentioned above, modified nucleoside triphosphates are incorporated less efficiently than the natural ones. It is suggested that the PCR method could work efficiently only with the templates of ~100 bp or even shorter ones.

Three SITC labeled DNA products with similar bp numbers of ~100 but different sequences (Table S2, a, d, e, ESI†) were obtained through PCR with corresponding templates (Fig. S2A, ESI†). The products were also confirmed to be dsDNA by GelRed poststaining (Fig. S2B, ESI†). This result implied that the incorporation of SITC–dUTP through PCR with Deep $Vent_R$ exo-DNA polymerase can be achieved successfully with various DNA sequences.

A 103 bp DNA template replicated from a region of a plasmid DNA (Table S2, f, ESI†) was chosen for testing the effect of the relative amount of SITC–dUTP on the PCR products' fluorescence.

Table 2 Characterization of fluorescent DNA prepared by PCR

dTTP/ μM	SITC–dUTP/ μM	Fraction of SITC–dUTP[a]	Theoretic DOL	Experimental DOL
150	50	0.25	5.34	5.31 ± 1.95
134	66	0.33	7.04	6.40 ± 1.61
100	100	0.50	10.68	7.41 ± 3.56
66	134	0.66	14.10	9.49 ± 2.48
50	150	0.75	16.02	17.96 ± 0.69
20	180	0.90	19.22	18.95 ± 7.07
0	200	1	21.36	21.56 ± 8.07

[a] Fraction of SITC–dUTP is defined as SITC–dUTP/(SITC–dUTP + dTTP).

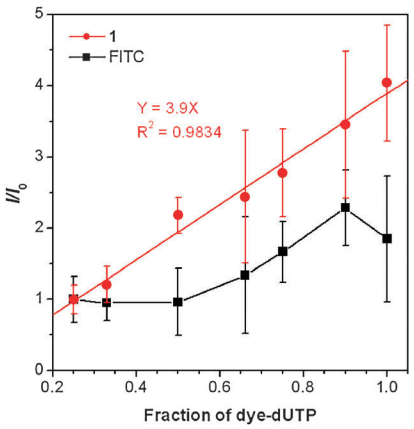

Fig. 3 Plot of normalized fluorescence intensity of the fluorescent DNA *versus* fraction of dye-dUTP. Fraction of dye-dUTP is defined as dye-dUTP/(dye-dUTP + dTTP). I_0 is the fluorescence intensity of the DNA prepared with fraction of dye-dUTP being 0.25.

This template had the highest yield of amplification through PCR with Deep $Vent_R$ exo-DNA polymerase and SITC–dUTP in our study. dTTP and SITC–dUTP with a total concentration of 200 μM were added into the 100 μL PCR system. The UV absorption and PL spectrum of the fluorescent products was obtained and the DOL values of the products prepared under various SITC–dUTP/dTTP ratios were calculated (Table 2).

The highest DOL value we obtained was nearly 10 times than that was ever reported. There were 47 dTTP on the dsDNA template and 3 dTTP on the primers, so the number of dTTP which could be replaced by SITC–dUTP was 44. The theoretical DOL could be calculated accordingly (Table 2). The experimental data matched reasonably well with the theoretical ones.

To demonstrate the advantage of SITC labeled DNA over traditional dye labeled DNA on fluorescence quenching effect, FITC, a widely used traditional fluorescent dye, was selected to prepare the fluorescent DNA with the same PCR protocol as that used by SITC. The PL intensities of both FITC labeled DNA products and SITC labeled DNA products were measured at the same time. The PL peak intensities of the products prepared with the lowest fraction of dye-dUTP were used as the reference and plots of the normalized fluorescence *versus* the fraction of dye-dUTP were obtained (Fig. 3). The fluorescence intensity of SITC labeled DNA increased linearly with an increase in the fraction of dye-dUTP and reached the highest value when dTTP was completely replaced with SITC–dUTP. On the other hand, fluorescence quenching effect was observed for FITC labeled DNA when the fraction of dye-dUTP was over 0.9. The quenching effect would be even more obvious when T-rich templates are used. This inherent fluorescence quenching effect of the traditional fluorescent dye inhibits the complete substitution of normal dNTPs with dye modified ones and therefore the fluorescent DNA with high DOL could not be obtained.

In conclusion, AIE-active silole derivative SITC was synthesized and used to label amine modified dUTP. The modified nucleotide SITC–dUTP was enzymatically incorporated to DNA by nick translation, random priming and polymerase chain reaction (PCR). The fluorescent products obtained by nick translation and random priming had acceptable DOL which could be controlled by varying the concentration of SITC–dUTP used in the enzymatic incorporation. The PCR products showed extremely high DOL without any fluorescence quenching when a large amount of SITC–dUTP molecules was used to replace dTTP. The fluorescence intensity of SITC labeled DNA increased linearly with the increase of the fraction of dye-dUTP, unlike the FITC labeled DNA which met with the fluorescence quenching effect when the fraction of dye-dUTP was over 0.9. Based on its AIE property, SITC is quite suitable for the preparation of fluorescent DNA which could be used in various bio-applications.

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