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Modulating fluorescence anisotropy of dye-labeled DNA without involving mass amplification

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ABSTRACT

Fluorescence anisotropy, known as a simple, homogeneous and cost-effective analytical technology, is an invaluable technique for studying the micro-environmental changes of the dye associated with the molecular interactions. An in-depth understanding of the variables affecting the fluorescence anisotropy signal can facilitate better experimental designs to effectively improve the analytical performance. This work is a follow-up effort in evaluating the factors that can significantly influence fluorescence anisotropy. We systematically studied fluorescence anisotropy of dsDNA with the changing length based on dye-DNA interactions, with the fluorophores in the end-labeling, the middle-site-labeling, and multiple number of labeling manners. The fluorescence anisotropy value and the base-pair response dynamic range could be expanded by labeling the fluorophores in the middle of dsDNA and increasing the number of labels on dsDNA. The C overhang configuration in the end-labeling manner could enhance the fluorescence anisotropy signal but not expand the base-pair response range. Results from all the labeling fluorophores reinforced the leveling-off effect, i.e., the fluorescence anisotropy signal does not response to the increased length of the DNA duplex when the length is larger than a critical number of base pairs. These findings provide perspectives about choosing appropriate fluorescent dyes and labeling sites for simple and universal fluorescence anisotropy designs in various applications.

1. Introduction

Fluorescence anisotropy (FA), which measures the emission depolarization when the fluorophore is excited with polarized light, is a simple and sensitive method for a number of important applications in a homogeneous detection manner [1–23]. It allows detection of molecular interactions by monitoring the change in rotational dynamics of the fluorophore [1,24–27]. According to the Perrin equation, the fluorescence anisotropy value is determined by the rotational correlation time and the lifetime of the fluorophore, in addition to the temperature and the viscosity [21,28–31]. As the rotational correlation time is tightly associated with the mass of the rotating object, a large anisotropy signal change of a fluorophore infers a big mass change occurring with the binding event [8,21,24]. Thus, the mass amplification strategy is often adopted for signal amplification [5,6]. However, mass enlargement is not always a convenient approach for simple and fast homogeneous analysis for DNA-based recognitions, especially when the binding of small molecule target with DNA is involved [1,10]. Using the mass enlargement approach, the experimental design and procedure as well as acquired data are often complicated by involving big DNA binding proteins or nanoparticles [4–7,10–13,18,20,32]. On the other hand, the extent of segmental motion of the fluorophore exerts a significant influence on the fluorescence anisotropy signal [1,21,24]. Therefore, confining the segmental motion to increase the fraction of the global motion can also improve the anisotropy signal. By examining the DNA-based recognitions, it can be seen that the nature of the fluorophore, the labeling site and labeling number may imparts an influence on the fluorescence anisotropy signal [21,22,30,33,34]. However, limited studies have been focused on these factors [1], and the resultant experimental designs are neither universal nor simple for most of the small molecule targets [6,7,12]. Using 6-carboxyl-x-rhodamine (ROX) as the fluorescent label, we previously demonstrated that fluorescence anisotropy of dsDNA could be enhanced by using positively charged fluorophore and an overhang on the complementary strand (C overhang), which can be applied to studying the DNA hybridization, and the aptamer-recognition based detection for both proteins and small molecules [1]. To better adapt our previous findings to applications of fluorescence anisotropy based on DNA recognitions, it is imperative to further study the potential factors that can be easily used to enhance the fluorescence anisotropy signal.

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We in this work gave a systematic evaluation of the fluorescence anisotropy performance of double-stranded DNA (dsDNA) labeled with several dyes. Particularly, we interrogated the effect of the labeling site and the number of the covalent labels on the fluorescence anisotropy response of dsDNA. Results reinforced the finding of the leveling-off effect with the increment of dsDNA length, i.e., the anisotropy values do not always increase steadily with the length of dsDNA for all the labeling manners. The middle-site-labeling and the multiple number of labeling effectively increased the overall fluorescence anisotropy and the base-pair response dynamic range. The C overhang configuration in the end-labeling manner enhanced the fluorescence anisotropy signal but did not expand the base-pair response range. This work, together with our recently published work, may provide guidance on choosing appropriate fluorescent dyes and labeling sites in simple and universal fluorescence anisotropy designs for various applications.

2. Experimental

2.1. Materials and instrumentation

All single-stranded DNA (ssDNA) and fluorescently labeled ssDNA were synthesized and HPLC-purified by Sangon Biotech (Shanghai) Co., Ltd. The sequences are provided in the Supplementary Information. Fluorophores, 5-carboxytetramethylrhodamine (TAMRA), indocarbocyanine-5 (Cy5), Texas red (TR), 6-carboxy-x-rhodamine (ROX) were covalently attached with a six-carbon tether to the 5′-end of the ssDNA, 3′-end of the ssDNA or the twentieth base (thymine) of ssDNA in the middle-site-labeling manner. The 10000 × SYBR Green I (SG) was purchased from Sigma, and thiazole orange (TO) was purchased from Shanghai Shifeng Biological Technology. Glycine (AR) and trizma base (AR) were purchased from Beijing Xinjingke Biotechnology Co., Ltd. All other reagents were of analytical grade and were purchased from Beijing Chemical Works. Wahaha® purified water was used throughout the study.

A 50 mM Tris buffer was used throughout the study. The buffer was composed of 50 mM trizma base, 100 mM NaCl, 1 mM $MgCl_2$, and adjusted to pH 7.50 by HCl.

Fluorescence anisotropy was measured using F7000 spectrofluorometer (Hitachi Limited Instruments, Ltd.) with excitation at 585 nm for ROX, 555 nm for TAMRA, 582 nm for TR, 645 nm for Cy5, 495 nm for TO and 495 nm for SG. Circular dichroism spectra were obtained with a J-810 spectropolarimeter (JASCO).

2.2. Fluorescence anisotropy measurements

All fluorescence measurements were carried out with the temperature of the sample compartment maintained by a circulating water bath at 25 ± 0.1 °C unless indicated. Briefly, components of the solution to be tested were added to a 1.5-mL Eppendorf tube to a final volume of 500 μL and mixed by vortex for 10 s. Then DNA strands were annealed by heating the solution to 85 °C and slowly cooling down to room temperature. For noncovalently labeled system, the fluorescent dye was added with the quantity as indicated. The fluorescence anisotropy, r, of the test solution was calculated by $r = (I_{VV}I_{HH}-I_{VH}I_{HV}) / (I_{VV}I_{HH}+2I_{VH}I_{HV})$ where I represents the fluorescence intensity and the subscript defines the orientation (H for horizontal and V for vertical) of the excitation and emission polarizers, respectively.

3. Results and discussion

Fluorescence anisotropy was generally affected by the segmental motion fraction (α) and the rotational correlation time (θ), as described by Eq. 1. For fluorescently labeled, short dsDNA, the rotational motion can be described by the rod model, thus the harmonic mean correlation time (θ_H), an average rotational correlation time of the non-spherical fluorescent entity, is used to replace θ in Eq. 1. The depolarization from the fast segmental motion (θ_F) was much less significant than that from θ_H, thus was neglected in the deduction of Eq. 1 [1,21,30].

$$r = (1 - \alpha)r_0/(1 + \tau/\theta) \quad (1)$$

where r_0 is the fundamental anisotropy of the dye; τ is the lifetime of dyes. According to Eq. 1, the dye-dsDNA interactions and the dye labeling site as well as numbers all may impart the influence on fluorescence anisotropy.

3.1. Effect of dye-DNA interactions on fluorescence anisotropy of dsDNA

The nature of dye-DNA interactions provides the basis for understanding the performance of the fluorescent label in fluorescence anisotropy measurements [25,26,28–30,33–36]. Specifically, the interaction between the dye and dsDNA could affect both the rotational correlation time of dsDNA and the segmental motion fraction of the dye [8,21,23,25,37]. Considering the electronegative nature of dsDNA, the electrostatic interaction, dyes with positively charged center should be effective in confining the segmental motion of dyes [1,25]. In light of this perspective, we chose several representative fluorescent dyes with positively charged centers, TO, SG, Cy5, TAMRA, TR and ROX, as displayed in Fig. 1.

We first examined fluorescence anisotropy of covalently labeled, blunt-ended dsDNA with varied lengths. Well consistent with our previous study, the anisotropy value of all the studied dyes largely reached a plateau with the length of dsDNA larger than a critical value at about 18–25 base pairs (b.p.) rather than increased steadily. Particularly, TR exhibited a larger base-number response slope than Cy5; TAMRA showed the significant fluorescence anisotropy signal fluctuation with dsDNA before reaching the plateau, which might be attributed to the complication by the presence of multiple emitting species with different rotational mobilities caused by rotational coupling of the dye to DNA [22,25,33,38]. There was a slight fluctuation for Cy5 with dsDNA shorter than this leveling-off length, possibly due to the complicated emission mechanism and the occurrence of different binding modes with dsDNA [35,39,40].

Theoretically, the restrained segmental motion of the fluorophore improves fluorescence anisotropy [21,24]. As can be seen from Fig. 2, for TR- and TAMRA-labeled dsDNA, fluorescence anisotropy values fell in the range of 0.10–0.16, showing that fluorophores are very mobile during the lifetime period of the excited state. By contrast, the anisotropy of Cy5 was in the range of 0.21–0.24. The evidently larger fluorescence anisotropy values indicated that the motion of the fluorophore can be well integrated with that of the dsDNA [21]. For Cy5, the planar heterocycles of cyanine dyes on either end of the cyanine dye favor intercalation, while the semi-flexible polymethine bridge can permit the twisting required to follow the curve of the groove [35].

To further understand the influence of the dye-DNA interaction on fluorescence anisotropy of dsDNA, we chose TO and SG, two frequently used fluorescent dyes for noncovalent DNA staining, to explore the fluorescence anisotropy change as a function of the length of dsDNA. A common feature for these two dyes is that they are not fluorescent unless incorporated into the backbone of dsDNA [35,41,42]. It was found that using the intercalative dye TO [42–45], fluorescence anisotropy exhibited a steadily ascending response in the range of 0.18–0.27 with the increment of dsDNA length from 12 b.p. to 39 b.p. (Fig. 3); for the minor groove binding