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Direct determination of single-to-double stranded DNA ratio in solution applying time-resolved fluorescence measurements of dye–DNA complexes

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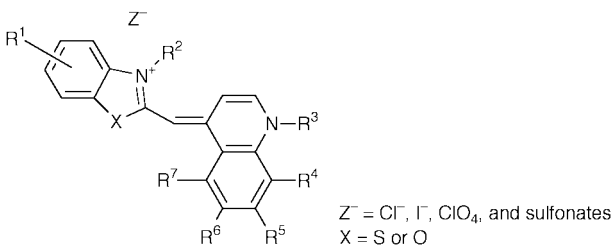
We report the fluorescence lifetimes of the DNA-stain dye PicoGreen and discuss the difference exhibited in the upon binding to single-stranded *vs.* double-stranded DNA; we here developed a direct method for determining single-to-double stranded DNA ratios in solution by measurement of the pre-exponential factors in the fluorescence decay traces of dye–DNA complexes.

The search for new non-radioactive analytical techniques to determine minute amounts of DNA in solution and gels brought about the development of a family of new fluorescent probes.[1,2] Many studies have been concerned with determining the properties of these dyes free in solution,[3] and when complexed to dsDNA.[2,4–6] These recently patented[7,8] fluorescent stains derived from unsymmetric cyanine dyes (Scheme 1) exhibit a high increase (*ca*. 1000 fold) in their fluorescence quantum yields upon binding to double-stranded DNA (dsDNA) as compared to free in solution, making them sensitive probes for DNA detection. These dyes are free to rotate about their central methine bridge while in solution, but this non-radiative deactivation pathway is closed when the dye intercalates between the DNA base-pairs. This explains their high sensitivity as dsDNA sensors.[4,6]

We have explored the possibility that in a complex with single-stranded DNA (ssDNA) a less rotationally restricted dye should be present, as compared to that with dsDNA and that this difference can be reflected in the fluorescence lifetimes. Thus, we have studied the complexes formed with PicoGreen (PG), a representative of the family of cyanine dyes of Scheme 1 which exhibits the greatest sensitivity for selective detection of dsDNA in solution.[7–10] Our results show not only that the dye–ssDNA complexes are more prone to deactivate non-radiatively, but also that the different lifetimes characteristic for each of the complexes (*i.e.* dye–dsDNA and dye–ssDNA) allow a simple approach to quantify their relative amount in solution by determining the pre-exponential factors for the fluorescence decay in a mixture containing both types of DNA. The choice of PG is not accidental, preliminary screening of a range of common dyes showed PG to be that with the best lifetime discrimination between ssDNA and dsDNA.

Our work was performed on calf thymus DNA (type I) (CT DNA) and salmon testes DNA (type III) (ST DNA) suspensions prepared on a TRIS (10 mM) buffer (pH 7.4) solution consisting of distilled and deionized water with Trizma Pre-set crystals, Na_2H_2EDTA (1 mM) and (NaCl) 100 mM. The ssDNA was obtained after boiling a dsDNA solution for 30 min followed by immersion in an ice bath. The fluorescence enhancement of PG upon binding to CT dsDNA, as compared to CT ssDNA was *ca*. 1.89, measured on a PTI 1.2 X luminescence spectrometer. This value is slightly higher than the reported one of 1.56.[9] We also noticed a broader fluorescence band for the dye–ssDNA complex (data not shown), with the maximum red shifted by 3 nm. The unbound dye fluorescence band is also broader than that of the dye–dsDNA complex, though it is hard to quantify this shift since the spectrum in the absence of DNA is very noisy. While the broadening had been previously reported, it was measured at high dye–DNA base pair ratios (*i.e.* 1:1); under this conditions, not only intercalation but groove binding can occur.[5,11] In our work we kept dye/DNA base-pair ratios always lower or around 1/7 to ensure that the predominant binding mode would be that of intercalation.[5] Specifically, the preferred concentrations were 11 mM for PG, and 70 and 150 mM for CT and ST DNA, respectively, both expressed as base pairs.



Scheme 1

Assuming that there is only one kind of complex for each ssDNA and dsDNA, and considering that, for other dyes of the same family, there exists a single lowest energy excited singlet state,[4] we would anticipate that the lifetimes for each of these complexes should be monoexponential. Thus, we measured the lifetimes following picosecond laser excitation using streak camera detection.[12] The results obtained following 355 nm irradiation of the sample with a $\leqslant$50 ps pulse show a virtually pure monoexponential decay with a lifetime of 4.5 ns for dye–dsDNA (for both CT or ST type DNA). The decay obtained for the complex formed with ssDNA was biexponential, with lifetimes of 1.16 ns (51%) and 3.09 ns (49%), where the weight of each exponential is given in parentheses (Fig. 1). The fact that the trace is biexponential may reflect two different phenomena. We are either dealing with two types of binding of the dye (*vide supra*), or there exists some DNA that renatured before data acquisition took place. The dye/DNA ratio employed was low enough as to have only intercalation in the case of dsDNA; however, ssDNA, that may have a lower binding constant,[10,13–15] might show other forms of dye interaction. In relation to the possibility that some DNA may have renatured, we tested our method of DNA denaturation by comparing the absorbance at 260 nm, immediately after the denaturation process, for a treated sample and an untreated one. We determined that within the experimental error, all of the sample had been denatured.[16] In principle, some DNA could renature before data acquisition, however this delay was kept to only 15 min. We cannot rule out either of the two possibilities described, and, conceivably, we may be dealing with a system presenting both some traces of renatured DNA, and some non-intercalated bound dye. This uncertainty does not influence our ability to discriminate between ssDNA and dsDNA.

The fluorescence broadening does not develop over time; rather it is evident immediately after excitation (data not shown). In any event, from the calculated fluorescence lifetime of the free dye in solution, *i.e.* shorter than *ca*. 5 ps, we assume that rotation takes place too fast for our acquisition setup to detect it.

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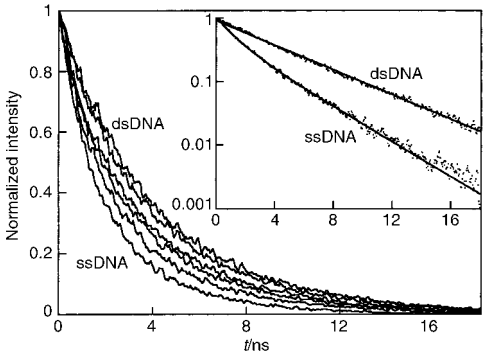

Fig. 1 Normalized fluorescence decay profiles measured on static 2 ml quartz cells in air equilibrated solutions of DNA in TRIS buffer (pH = 7.4) following 355 nm laser excitation; ST DNA base-pair concentration = 150 μM, PG = 11 μM, for 100, 80, 60, 40 and 20% dsDNA and of dsDNA alone. Insert: semi-log plot and fit for dsDNA (single exponential) and ssDNA (double exponential).

In view of the noticeable difference in fluorescence lifetimes between dye–ssDNA complex and dye–dsDNA complex, we attempted and succeeded in developing an analytical technique that would enable us to monitor in solution the ssDNA/dsDNA ratio with a simple method devoid of any operator subjectivity. To that effect, we determined the fluorescence lifetimes in different mixtures containing known amounts of ssDNA, dsDNA (we employed both CT DNA and ST DNA) and PG. From the previous analysis (*vide supra*), we expect a triexponential decay in this system [eqn. (1)]; *i.e.* dye–dsDNA decays with its characteristic rate constant, and ssDNA complexes exhibit their biexponential decay.

$$I = a_{ds}e^{-k_{ds}t} + a_2e^{-k_2t} + a_3e^{-k_3t} \quad (1)$$

where a_{ds} is the preexponential factor for dsDNA, and k_{ds} the reciprocal of its lifetime, while a_2, a_3, k_2 and k_3 are the corresponding parameters for ssDNA, and t is the time.

Though complicated in appearance, we know the three decay rate constants for this system (that of dsDNA, and each of the two for ssDNA); on the other hand, the addition of the preexponential factors for the decays has to be equal to unity for the normalized profile. At this point a six-variable function initially needed to fit this decay (three pre-exponential values, and three decay rate constants), is now reduced to one with two parameters, *i.e.* the two pre-exponential values. Further, since the relative values for the two pre-exponential values for the ssDNA decay are known, and coincidentally they are about equal, *i.e.*

$$a_{ds} + a_2 + a_3 = 1$$
$$a_2 \cong a_3$$

Combined these factors lead to a one-parameter fit according to eqn. (2):

$$I = a_{ds}e^{-0.22t} + [1 - a_{ds}) (e^{-0.86t} + e^{-0.32t})/2] \quad (2)$$

When we plot the recovered pre-exponential value (a_{ds}) corresponding to the dsDNA rate constant *vs.* the fraction of dsDNA in the sample (see inset in Fig. 2) for CT DNA and for ST DNA, a straight line is obtained with a slope of 0.80 and an intercept of 0.048. The theoretical line should go through the origin and have a slope of 1.0. The difference is not surprising; analysis according to eqn. (2) of computer simulated data shows that only perfect data (*i.e.* noise-free) leads to perfect recovery of the pre-exponential factors, particularly for the pure forms, dsDNA and ssDNA; addition of random noise always leads to apparent small weights for other DNA components. Effectively, the percentage of dsDNA present in a sample can be calculated according to eqn. (3), although we note that different instruments may lead to slightly different deviations from the ideal equation; in this sense a recalibration may be desirable.

$$\%(\text{dsDNA}) = 100(a_{ds}^{\text{recovered}} - 0.048)/0.80 \quad (3)$$

It is worthwhile noting that for the fittings, the rate constants used corresponding to ssDNA (0.32 and 0.86 × 10^9 s^{-1}) and dsDNA (0.22 × 10^9 s^{-1}) where those determined in the absence of the other form of DNA, and are the same for CT or ST DNA, and are not adjustable parameters. We believe this new method

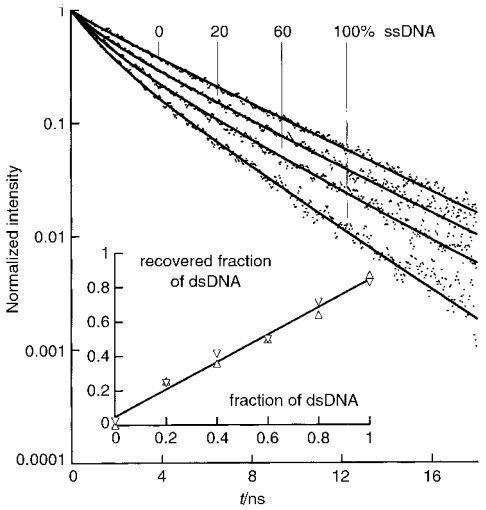

Fig. 2 Normalized fluorescence decay profiles measured on static 2 ml quartz cells in air equilibrated solutions of DNA in TRIS buffer (pH = 7.4) following 355 nm laser excitation; ST DNA base-pair concentration = 150 μM, PG = 11 μM, for various percentages of dsDNA, showing the fit of the data to the one-parameter function of eqn. (2); Insert: linear plot of the recovered dsDNA fraction as a function of the actual fraction in the sample for (△) CT DNA and for (▽) ST DNA.

may find application not only in solution, but also in gels where this information may prove useful for methods such as the comet assay.[17] Finally, we note that while our work involved a sophisticated picosecond fluorescence system, similar measurements could be developed around less expensive light sources, such a short pulse diodes.

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