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Direct determination of single-to-double stranded DNA ratio in solution using steady-state fluorescence measurements

Lori Beach, Claude Schweitzer and J. C. Scaiano *
Department of Chemistry, University of Ottawa, 10 Marie Curie, Ottawa, Ontario, Canada K1N 6N5. E-mail: tito@photo.chem.uottawa.ca

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We report a simple and rapid method for quantitation of single-to-double stranded (ss : ds) DNA ratios in solution, using steady-state measurements of fluorescence from two simultaneously excited intercalated dyes; the ratio of fluorescence intensities from PicoGreen (525 nm) and ethidium bromide (610 nm) is directly proportional to the ss : ds DNA ratio.

The development of analytical methods for detection and quantitation of radiation-induced DNA damage has become a rapidly growing field of research during recent years, especially due to increasing demands for control of agricultural samples. Irradiation causes strand breaks in the DNA, which, after treatment with alkaline unwinding buffer, can be quantified by measuring the so-obtained ratio of single-to-double stranded (ss : ds) DNA. Under carefully chosen unwinding conditions, the ss : ds DNA ratio is directly proportional to the irradiation dose applied to the sample.

Very promising methods for such determinations have been proposed on the basis of measurements of luminescence from the recently patented cyanine dye PicoGreen (PG), which exhibits a particularly strong and structure-dependent (*i.e.*, ds *vs.* ss) fluorescence enhancement upon intercalation in DNA.[1] For example, Rogers *et al.*[2] have successfully used PG fluorescence intensities to quantify irradiation doses applied to calf thymus (CT) and plasmid DNA in solution, relative to non-irradiated control samples. Their method is very simple and sensitive, but obviously limited to situations where non-irradiated samples of the same DNA are available, which may not be the case in many applications.

More recently, Cosa *et al.*[3] have used time-resolved measurements of PG fluorescence to determine ss : ds DNA ratios in solution, and to quantify irradiation doses applied to cells.[4] This method (based on fluorescence lifetime determination) does not require non-irradiated control samples, but it is more complicated and more expensive, as it requires a significant investment in equipment and expertise in order to carry out picosecond fluorescence measurements.

In the present report, we propose a novel method for quantitative determination of absolute ss : ds DNA ratios by steady-state fluorescence spectroscopy, thus combining the advantages of both previous methods. Our approach relies upon the simultaneous detection of fluorescence from two intercalating dyes, one of which discriminates significantly between ss and ds DNA (*i.e.*, PG), while the second one exhibits nearly identical signals in the presence of both ds and ss DNA (this has been most prominently observed in the case of ethidium bromide (EB)).[5] Use of these two particular dyes is especially interesting, because both absorb in the same wavelength region, but fluoresce in different ones (see Fig. 1). Thus, the fluorescence spectrum of a mixture of PG and EB in the presence of DNA should contain information on its relative ss : ds content, and especially the ratio of fluorescence intensities from both dyes should be directly proportional to the ss : ds DNA ratio.

PG and EB have similar emission quantum yields in the presence of DNA, but their extinction coefficients differ significantly. Significant absorption of light by both dyes can be obtained at a EB–PG ratio of 9 : 1. In this case, the absorbance of both compounds is identical at 503 nm. Excitation of the mixture at this wavelength in the presence of DNA yields fluorescence spectra as shown in Fig. 2, where the 525 nm centered PG band is strongly dependent on the ss : ds DNA ratio, and the EB band at 580 nm is almost constant.

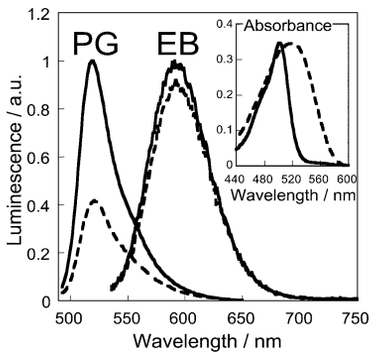

Fig. 1 Normalized fluorescence spectra of PG and EB in the presence of ds (solid line) and ss (dashed line) DNA. Inset: absorption spectra of PG (solid line) and EB (dashed line) in the presence of DNA (50% ds, 50% ss).

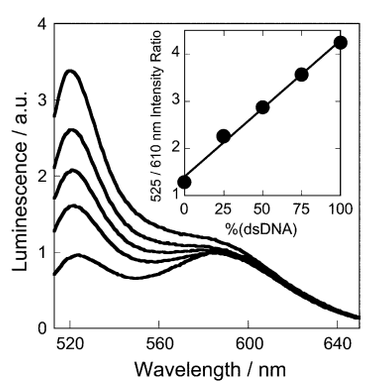

Fig. 2 Fluorescence spectra obtained by 503 nm excitation of a solution containing 0.2 μM PG, 1.8 μM EB, and 50 μM CT DNA (total ds + ss DNA concentration in base pairs), for 0, 25, 50, 75, and 100% ds DNA. Inset: dependence of the ratio of fluorescence intensities at 525 and 610 nm on the percentage of ds DNA.

All experiments shown in Fig. 2 were carried out on fresh solutions of 0.2 μM PG, 1.8 μM EB, and 50 μM CT DNA (total ds + ss DNA concentration, in base pairs), in distilled and deionized water containing 0.01M tris buffer (pH 7.4), 1 mM EDTA, and 0.1 M NaCl. ss DNA was obtained after boiling a ds DNA solution for 30 minutes followed by immersion in an ice bath, as previously described.[3–5] No changes are observed in the fluorescence spectrum of PG in the presence of ss CT DNA, when the sample is kept for 6 hours at room temperature, thus indicating that no significant renaturation occurs during that

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time. The spectra shown in Fig. 2 were obtained upon 503 nm excitation of a mixture obtained by adding 1.5 mL of a buffer solution containing 3.6 μM EB and 0.4 μM PG to 1.5 mL of different solutions of 100 μM CT DNA, obtained by mixing variable volumes of heat-denatured and untreated 100 μM CT DNA solutions. EB and PG solutions were prepared from EB powder as received from Aldrich, and by diluting the 320 μM PG solutions obtained from Molecular Probes. In the case of EB it was necessary to work with fresh solutions, since aging introduced some fluorescence fluctuations, when EB was kept at low concentration in aqueous buffer solution. DNA concentrations were determined by absorbance spectroscopy, using the extinction coefficient of 6600 M^{-1} cm^{-1} at 260 nm.[6]

It has been shown that, at least in ds DNA and at high DNA : dye ratio, both dyes preferentially intercalate between two base pairs, but at low DNA : dye ratio, both compounds also associate with the exterior of DNA.[5,7] Thus, a high ratio of DNA to total dye concentration, such as the 25 : 1 ratio used here, is essential to avoid additional non-intercalating modes of association, as well as competition of both dyes for the same binding site.

The inset of Fig. 2 shows the dependence of the ratio of relative fluorescence intensities at 525 and 610 nm (from Fig. 2), on the percentage of ds DNA. The former wavelength corresponds to the emission maximum of PG in ds and ss DNA, while the EB fluorescence intensity was monitored at a longer wavelength, in order to minimize contributions from PG fluorescence. The solid line is given by Equation (1),

$$\%(\text{ds DNA}) = 34.6\,F - 49.13 \qquad (1)$$

where F is the ratio of fluorescence intensities measured at 525 and 610 nm, respectively, and %(ds DNA) is the percentage of double-stranded DNA. Thus, the method allows the determination of the ss : ds DNA ratio with high accuracy (correlation coefficient $R^2 = 0.992$), and should lend itself to determinations of a broad range of irradiation doses applied to biological samples. For example, it has been shown that, under variable unwinding conditions, measurement of the ss : ds ratio on DNA extracted from irradiated white blood cells allows quantitative determinations of ionizing radiation doses between 0 and 100 Gy, with a sensitivity of a few Gy.[4] At higher doses reliable qualitative determinations are possible. The present method allows quantitative determinations of the ss : ds DNA ratio on a quantity as small as 150 nmol of DNA. The %(ds DNA) value is directly obtained from the fluorescence spectra of Fig. 2, and no recalibration is required, as opposed to the previously proposed method relying on time-resolved measurements.[3]

Similarly good correlations are also obtained at different DNA : dye ratios, however, both the slopes and intercepts display some variation, due to an increase of PG fluorescence intensity with increasing DNA concentration, which is not observed in the case of EB. For example, at PG and EB concentrations of 0.2 and 1.8 μM, respectively, and a total DNA concentration of 100 μM, we obtained %(ds DNA) = $26.0F - 56.60$, with $R^2 = 0.986$, and at 200 μM, we found %(ds DNA) = $17.1F - 59.51$, with $R^2 = 0.994$. Experiments have been performed for several other concentrations of dye and DNA, and similar results were obtained even at a DNA concentration as low as 10 μM, and a total dye concentration of 1 μM. Thus, the method has a high sensitivity at a broad range of DNA : dye ratios, however, knowledge of the total DNA concentration is required. Extrapolation of the present data indicates that uncertainties on total DNA concentration should be no larger than 5%, in order to achieve an error limit smaller than 10% on the ss : ds DNA ratio.

In conclusion, we note that measurements of steady-state fluorescence from PG and EB provide one of the most simple methods for quantitation of ss : ds DNA ratios developed thus far, and the method definitely constitutes a promising new approach for detection of radiation-induced DNA damage. Quantitative determinations are possible on very small amounts of DNA, and require only the measurement of relative fluorescence intensities at two wavelengths, *i.e.*, a simple fluorimeter is the only piece of equipment required; its only significant limitation is the need to know the total DNA concentration well. The need for non-irradiated control samples is eliminated, and the experimental procedure is simple and rapid enough to envision automated screening procedures on the basis of this method.

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Notes and references

1 V. L. Singer, L. J. Jones, S. T. Yue and R. P. Haugland, *Anal. Biochem.*, 1997, **249**, 228–238.
2 K. R. Rogers, A. Apostol, S. J. Madsen and C. W. Spencer, *Anal. Chem.*, 1999, **71**, 4423–4426.
3 G. Cosa, K.-S. Focsaneanu, J. R. N. McLean and J. C. Scaiano, *Chem. Commun.*, 2000, 689–690.
4 G. Cosa, A. L. Vinette, J. R. N. McLean and J. C. Scaiano, *Anal. Chem.*, 2002, in press.
5 G. Cosa, K.-S. Focsaneanu, J. R. N. McLean, J. P. McNamee and J. C. Scaiano, *Photochem. Photobiol.*, 2001, **73**, 585–599.
6 S. R. Gallagher, Quantitation of DNA and RNA with Absorption and Fluorescence Spectroscopy, in *Current protocols in molecular biology*, ed. F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, Wiley Interscience, New York, 1994.
7 E. Nordmeier, *J. Phys. Chem.*, 1992, **96**, 6045–6055.