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Binding of biologically important molecules to DNA, probed using electro-fluorescence polarization spectroscopy

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Abstract

When small fluorescent tag molecules are bound with a single geometry to larger carrier molecules the fluorescence excited using polarized light may itself be partially polarized. If this is the case then alignment of the carrier molecules changes the fluorescence polarization. Electro-fluorescence polarization spectroscopy (EFPS) makes use of the fluorescence polarization changes which occur when the carrier molecules are aligned by an electric field. EFPS allows the determination of the binding geometry of the small tag molecules to the larger carrier molecules. In this study, EFPS has been used to probe the interactions between DNA and a series of chromosomal stains and anti-neoplastic (anti-cancer) agents. Results are presented for calf thymus DNA, treated with the dyes acridine orange, ethidium bromide, proflavine, Hoechst 33258, Hoechst 33342, 4′,6-diamidino-2-phenylindole and the anti-tumour agents, doxorubicin, daunomycin, actinomycin C and actinomycin V.

Keywords: Electro-fluorescence; DNA binding; Polarized fluorescence; Anti-cancer agents; Carcinogens

1. Introduction

There are many compounds which have been found to interact with DNA, and a number of these have medical consequence. Some compounds, such as the anthracyclines [1], are of use as chemotherapeutic agents. Others, such as the diol-epoxide metabolites of polycyclic aromatic hydrocarbons, exhibit varying degrees of carcinogenicity or mutagenicity. The activity of these compounds is thought to be attributable to DNA binding and thus a study of the structure of DNA-molecule adducts and complexes may broaden our understanding of the disease, cancer [2].

There are several techniques that are suitable for studying the structure of these DNA-molecule complexes and electro-fluorescence polarization spectroscopy (EFPS) is particularly useful because it allows the binding geometries of the interacting molecules to be measured in solution.

When an isotropic solution containing absorbing molecules is illuminated with polarized light of suitable wavelength the absorbance is independent of the direction of polarization. If some degree of order can be imposed on the system then a dependence may

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become apparent. This polarization dependent absorption is due to the presence of preferential absorption axes or absorption moments within the molecules. In a dilute solution the order can be imposed by alignment of the molecules using several methods, including electric fields and viscous flow fields, although alignment is experimentally difficult for small molecules. If the small molecules are bound with fixed and uniform geometries to larger carrier molecules, such as DNA, then order within the ensemble of small molecules may be achieved through alignment of their carriers. The ensuing absorption anisotropy is an example of dichroism.

If the bound molecules are also fluorescent then the fluorescence occurs at longer wavelength than the absorption, since some of the absorbed photon energy couples to vibrational motion of the molecules and is unavailable to the emission process. There is again a preferred axis for this emission – the axis of the emission moment of the excited state of the molecule. In general, the emission moment and the absorption moment have different axes since different transitions are involved and different states of the molecule are active. Nevertheless, the fluorescence from an aligned system will also be anisotropic, providing the emission moments of the fluorophores cannot re-orient and randomize within the fluorescence lifetime. Again, this requirement is met when the fluorophores are bound to large carriers but is usually not met for unbound fluorophores within the solution. Anisotropy of fluorescence thus may be used to disclose binding geometries of bound fluorophores to aligned carriers and this fluorescence may be discriminated from the background fluorescence from unbound molecules. EFPS is a technique which allows this anisotropy of fluorescence to be studied further through the changes in fluorescence caused by electric field alignment of the carriers. While standard fluorescence polarization techniques can use the anisotropic fluorescence from isotropic solutions to reveal information about the internal motions and dynamics of bound fluorophores, they cannot match the ability of EFPS to determine the orientations of the absorption and emission moments (and hence infer the binding geometry). Fujimoto and Schurr [3] did analyze geometries of the absorption and emission moments from fluorescence polarization measurements, although their analysis makes numerical assumptions which may not be correct [4]. EFPS however, allows these geometries to be determined without such assumptions.

Electric fields apply aligning torques by coupling with any permanent or induced electric dipole moments in the carrier molecules. If a DC or low frequency AC field is used, coupling can occur to the permanent and induced dipole moments within the carrier as the rotational motion of the carriers is able to follow the field direction. At higher AC frequencies, above the so called Debye frequency, the molecular motion can no longer follow the field direction and only the induced dipole is effective at producing alignment. An AC electric field is also easier to shape and control. For these reasons, EFPS studies usually use an AC field at above the Debye frequency; in the work reported here we have followed this approach and used an AC frequency of 10 kHz. Previous studies have shown that this frequency is well above that required for alignment due to any permanent dipole moment to relax out; the alignment and hence fluorescence polarization changes are independent of frequency above about 5 kHz for DNA samples [1]. Large electric field amplitudes are required to produce full alignment of the carriers and the electrical conductivity of the samples must be made as low as possible, with the electrical fields applied as a short AC pulse. The pulse length is adjusted to reach stationary alignment of the carrier molecules.

EFPS has been used here to study a series of chromosomal stains and anti-neoplastic (anti-cancer) agents, bound to DNA as carrier molecules. These materials absorb in the near UV or blue/green and fluoresce with a shift to yellow/orange wavelengths. Changes in the polarization of the fluorescence when the carrier DNA is aligned by an electric field pulse lead to a determination of the orientation of the absorption and emission moment axes with respect to the DNA helical axis and relative to each other. These orientations then disclose the mode of binding of the fluorophore to DNA. Some of the materials are found to intercalate between the base pairs whilst

[1] Windsor and Tinker, unpublished results.

others bind in the major or the minor grooves of the DNA double helix.

2. Theory

2.1. Dichroism

A sample of absorber is illuminated with monochromatic light, polarized either parallel or perpendicular to the axis of an aligning field. The absorbance of the sample is measured in the absence of alignment (A), then for a system of fully-aligned carriers, with the polarization along the axis of alignment, ($A_{\parallel}$), and then perpendicular to the axis of alignment, ($A_{\perp}$). Eq. 1 shows how these measurements of absorbance can be used to find the angle ψ between the absorption moment and the alignment axis [5,6] of the carrier.

$$\frac{A_{\parallel} - A_{\perp}}{A} = \frac{3}{2}\left(3\cos^2\psi - 1\right) = \frac{3}{2}\left(\frac{A_{\parallel} - A}{A}\right) \qquad (1)$$

In practice, absorbance is measured by observing the transmitted intensity through the sample. Eq. 2 gives the absorbance as:

$$A = \log\frac{I_0}{I} \qquad (2)$$

where I_0 is the intensity of the incident light, and I is the intensity of the transmitted light.

2.2. Electro-fluorescence

Fluorescence, unlike absorption, often exhibits anisotropy in the observed polarization, even from an isotropic sample of absorbers, when illuminated by polarized light. In general, the fluorescence in such cases will be partially polarized, if the lifetime of the excited state is shorter than the time-scale for significant re-orientation of the carrier molecules. Since each individual absorber has both an absorption moment and an emission moment, the polarized fluorescence contains information about the orientations of both these moments within the bound molecules. Experimentally, the polarization of fluorescence is determined by measuring polarization components parallel ${}^{\parallel}F_{\parallel}$ and perpendicular ${}^{\parallel}F_{\perp}$ to the illumination polarization direction. The anisotropy of fluorescence r can then be defined as [3,7]:

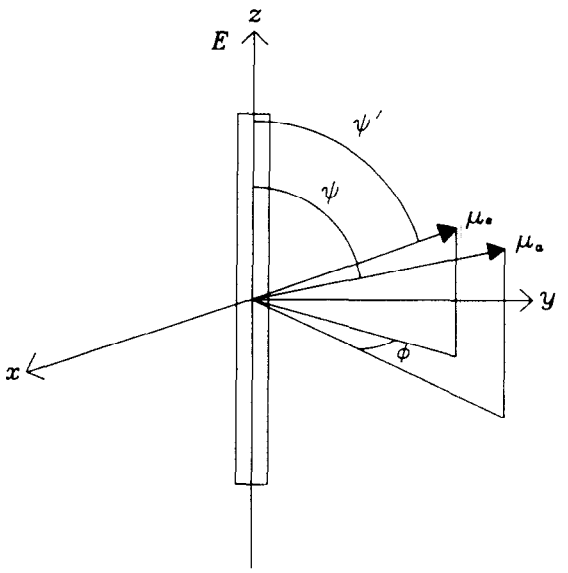

Fig. 1. The angles defining the absorption and emission moment axes of the fluorophore with respect to the alignment axis of the carrier molecule, and each other.

$$r = \frac{{}^{\parallel}F_{\parallel} - {}^{\parallel}F_{\perp}}{{}^{\parallel}F_{\parallel} + 2{}^{\parallel}F_{\perp}} \qquad (3)$$

In Eq. 3 the denominator gives the total intensity of fluorescence whilst the numerator gives the difference in the intensities with polarization along and perpendicular to the incident polarization (which is also the carrier alignment axis). If the molecules re-orientate randomly during the lifetime of the excited state, ${}^{\parallel}F_{\parallel} = {}^{\parallel}F_{\perp}$ and so it can be seen that $r = 0$.

If the carrier molecules are fully aligned parallel to the incident polarization, and remain fully aligned, then the polarization of fluorescence may change. The relative changes in intensity of the polarization components ($\Delta^{\parallel}F_{\parallel}$ and $\Delta^{\parallel}F_{\perp}$) can be used to find the orientation of the absorption and emission moment axes, ψ and ψ' respectively, with respect to the alignment axis of the carrier molecules, and also ϕ, the propeller twist angle between these moments, measured around the alignment axis. Fig. 1 shows the definition of these angles. The relationships be-

tween the polarization components and these angles are given by [8,9] Eqs. 4 to 7.

$$3\cos^2\psi - 1 = \frac{\Delta^{\parallel}F_{\parallel} + 2\Delta^{\parallel}F_{\perp}}{{}^{\parallel}F_{\parallel} + 2{}^{\parallel}F_{\perp}} = \frac{\frac{\Delta^{\parallel}F_{\parallel}}{{}^{\parallel}F_{\parallel}} + 2Q\frac{\Delta^{\parallel}F_{\perp}}{{}^{\parallel}F_{\perp}}}{1+2Q} \quad (4)$$

$$\tan^2\psi' = \frac{2{}^{\parallel}F_{\perp} + 2\Delta^{\parallel}F_{\perp}}{{}^{\parallel}F_{\parallel} + \Delta^{\parallel}F_{\parallel}} = \frac{2Q\left(1 + \frac{\Delta^{\parallel}F_{\perp}}{{}^{\parallel}F_{\perp}}\right)}{\left(1 + \frac{\Delta^{\parallel}F_{\parallel}}{{}^{\parallel}F_{\parallel}}\right)} \quad (5)$$

$$\frac{5r - 3\cos^2\psi\cos^2\psi' + 1}{\sin\psi\sin\psi'\cos\phi + 2\cos\psi\cos\psi'} = 3\sin\psi\,srin\,\psi'\cos\phi \quad (6)$$

where Q is defined by:

$$Q = \frac{{}^{\parallel}F_{\perp}}{{}^{\parallel}F_{\parallel}} \quad (7)$$

3. Apparatus

The EFPS apparatus is a modified version of those previously described [10,11]. A schematic of the experimental arrangement is shown in Fig. 2. It consists of a light source which is either a Spectra Physics series 5000 argon ion laser, capable of operation in the near UV, or a 200 W mercury UV lamp (Oriel Corporation) in combination with a narrow band interference filter or monochromator. The UV lamp, while having a spectral brightness much less than the laser, provides illumination down to 250 nm and allows excitation of a much wider range of compounds, such as the diol-epoxide metabolites of various polycyclic aromatic hydrocarbons (S.A. Windsor, M.H. Tinker, M.R. Osborne and A. Seidel, unpublished results).

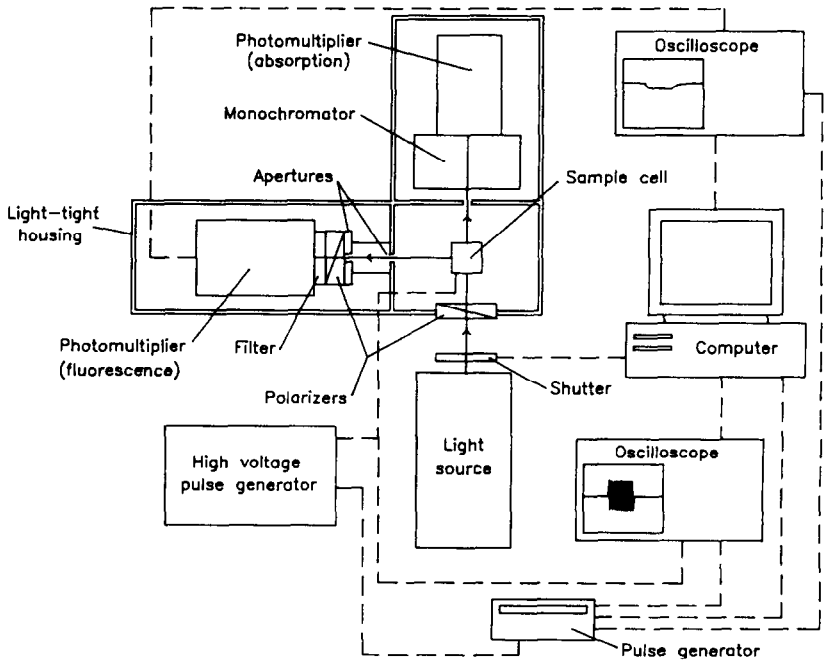

Fig. 2. Schematic experimental arrangement.

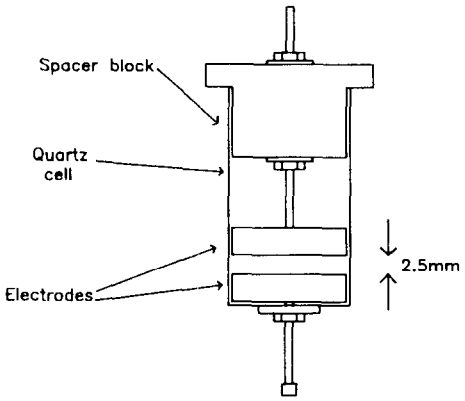

Fig. 3. The sample cell and electrode arrangement.

The incident beam passes first through a shutter, which is opened only during the measurement period, to reduce the exposure of the sample to the potentially damaging UV or laser radiation, then through a polarizer and into the sample cell. The sample cell, shown in Fig. 3, is a modified quartz 10 mm by 10 mm cuvette, with stainless steel electrodes mounted horizontally, one above the other, with a vertical gap of 2.5 mm. This design allows the two electrodes to be maintained parallel and with an electrode gap that is the same for all samples.

Fluorescence is detected orthogonally to the incident beam. The detection arm consists of apertures which ensure a small collection angle, a polarizer, a cut-on filter to eliminate scattered incident radiation and a photo-multiplier to monitor the fluorescence intensity. The photo-multiplier is connected to a digital storage oscilloscope and the data from this may be downloaded to a computer for analysis. The computer also controls the timing sequence for the experiment, triggering the various instruments via a Stanford Research pulse generator. The high voltage aligning AC field pulse is provided by a custom built 0 to 6 kV supply.

The apparatus is enclosed in a light-tight housing and the optics are arranged to maximize the illumination intensity and the fluorescence signals. The apparatus also incorporates a detection arm for the measurement of electric dichroism. Since the dichroic signal under these experimental conditions was negligible, this facility was not used for the measurements reported here.

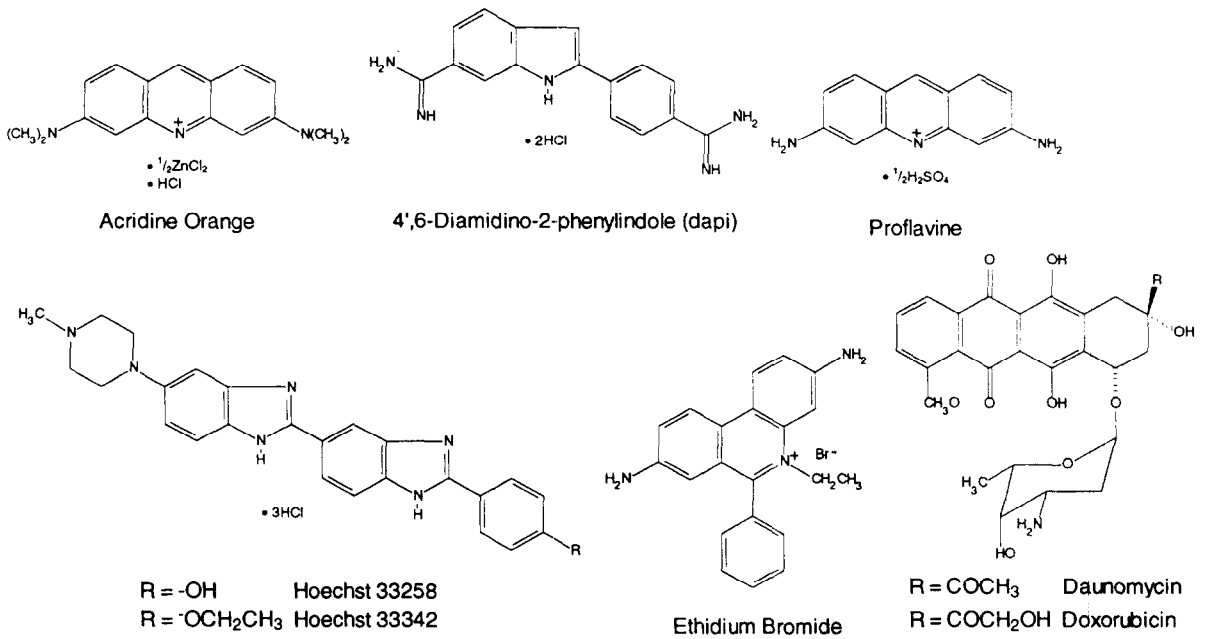

Fig. 4. The molecular structures of the fluorescent dyes and drugs used.

4. Results

In this series of experiments, sodium salt calf thymus DNA was used, provided by Sigma Chemical Company and used without further purification. The DNA was dissolved in ultra pure water (conductivity less than 18 μS cm^{-1}) by very gently stirring for 24 hours [12], to form a stock solution of concentration 150 μg ml^{-1}. Samples of ethidium bromide, proflavine, acridine orange, Hoechst 33258, Hoechst 33342, DAPI, daunomycin, doxorubicin, actinomycin C and actinomycin V were also provided by Sigma and were made into stock solutions, again using ultra pure water. The structures of these compounds are shown in Fig. 4. Each of these dyes or drugs were added to 5 ml aliquots of the DNA stock to give samples for measurement with overall binding levels of approximately 1 dye molecule to 50 DNA base pairs.

Eqs. 4 to 7 require the full alignment of the DNA carriers and the magnitude of the required electric field pulse to produce these conditions must be established initially, before fluorescence data may be interpreted. The magnitude of the required electric field is found by taking a series of measurements of the change in any one polarization component of fluorescence as the electric field is gradually increased. Before the alignment begins to saturate, the field induced changes in the fluorescence are proportional to the square of the electric field [13] and so a plot of these changes against the magnitude of the electric field squared shows the approach to full alignment. At sufficiently high fields the fluorescence changes tend to a limiting value at full alignment (similar saturation effects also have been observed in the dichroism signals with increasing electric field strengths [5,14]). A series of such fluorescence measurements is shown in Fig. 5, for the calf thymus DNA used in these experiments and probed by the fluorescence from bound proflavine. As the graph shows, field magnitudes in excess of about 16 kV cm^{-1} [$E^2 > 250$ (kV cm^{-1})^2] lead to full alignment. Having established this limit, the experiments reported here were then conducted with fields of at least this magnitude.

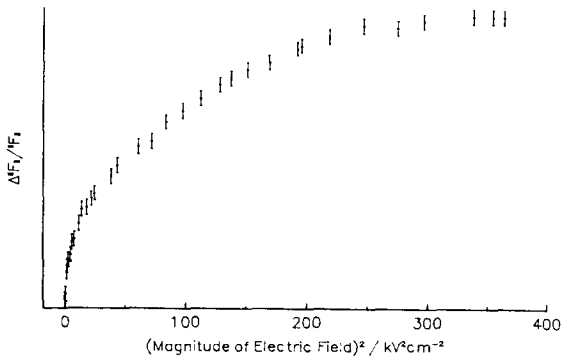

Fig. 5. Saturation of the electro-fluorescence signals with full alignment.

Table 1
Electro-fluorescence results

Molecule	λ_{excite}/nm	$^{\parallel}F_{\perp}/^{\parallel}F_{\parallel}$	$\Delta^{\parallel}F_{\perp}/^{\parallel}F_{\perp}$	$\Delta^{\parallel}F_{\parallel}/^{\parallel}F_{\parallel}$	Ψ (±1°)/°	Ψ' (±1°)/°	ϕ (±3°)/°
Ethidium bromide	515	0.556	−0.522	−0.800	70.1	58.5	35.9
	351/364	0.907	−0.282	−0.743	64.6	66.1	57.0
Acridine orange	488	0.512	−0.376	−0.654	66.3	53.5	31.4
	351/364	0.812	+0.282	−0.634	56.1	67.3	53.3
Proflavine	458	0.492	−0.450	−0.737	68.4	55.1	29.7
	351/364	0.614	−0.151	−0.637	62.7	59.4	43.4
Hoechst 33258	351/364	0.471	+0.134	+0.195	51.4	43.4	36.1
Hoechst 33342	351/364	0.452	+0.164	+0.269	50.4	42.3	33.8
DAPI	351/364	0.789	+0.131	+0.147	52.0	51.3	60.8
Doxorubicin	497	0.404	−0.396	−0.629	66.6	49.0	14.7
Daunomycin	497	0.447	−0.299	−0.570	64.4	50.3	24.6
Actinomycin C	351/364	0.679	−0.021	+0.047	54.6	48.4	53.4
Actinomycin V	351/364	0.669	−0.061	+0.149	54.2	46.3	53.4

Table 1 shows the polarization changes measured and also gives the calculated values of ψ, ψ', and ϕ for each of the complexes. Because the absorption moments for all the measurements made are due to $\pi \rightarrow \pi^*$ transitions, the absorption moments lie in the plane of the chromophore, thus the values of ψ for the long axis transitions (longest wavelength) indicate whether the molecule is an intercalator ($\psi \approx 70°$) or is groove bound ($\psi \approx 50°$).

4.1. Ethidium bromide

Ethidium bromide was originally extensively used as an anti-trypanosomal agent especially in the treatment of trypanosomiasis (or sleeping sickness) which is transmitted by the bite of the tsetse fly [15]. More recently it has found use as a DNA probe [16]. The mode of binding of ethidium bromide to DNA has been well established; the phenanthridinium ring system intercalating between DNA base-pairs [17,18]. The visible absorption peak centred at 525 nm (in methanol), is thought to be due to a $\pi \rightarrow \pi^*$ transition in the plane of the ring system. Measurements in this absorption band using both dichroism [19] and EFPS [20] have revealed that the long axis of ethidium bromide lies at around 70° to the helical axis of the host DNA. Our measurements for this absorption moment are consistent with these observations. Measurements at shorter wavelengths have also been reported for dichroism, although none to the authors knowledge for EFPS. Generally reported angles are less than their visible counterparts, due to the different absorption moments involved in the transition [21]. Our observations give an angle for the absorption moment measured using the UV laser lines from the argon ion laser of around 65°. It is thought that the absorption and emission moment directions are the same, for the lowest energy transition, and so one would expect the measured angles for these to be the same, and also a measured propeller twist angle of 0°. This is not reflected in our results, or those reported elsewhere [3,11,12,15].

4.2. Proflavine

Proflavine, or 3,6-diaminoacridine is a yellow, fluorescent, cationic, acridine dye, which medically has been used as a topical anti-septic [16]. It is known to be an intercalating agent. As with ethidium bromide, there are both visible and UV absorption bands, although the UV band is at shorter wavelength than the corresponding one for ethidium bromide. The visible absorption peak, actually consists of transitions due to several different moments which merge into one. The absorption peak centred at around 450 nm is due to a $\pi \rightarrow \pi^*$ transition, the absorption moment of which lies in the plane of the acridine ring, probably orientated close to the long axis [19]. We have measured EFPS signals at both 458 nm, and 351/364 nm dual laser lines. Our UV measurements are less informative, as they will consist of contributions from both the visible and UV bands, and hence be an average of both moments. Our measurements at 458 nm give angle between the absorption moment (along the long axis) and the alignment axis as 68°. Again the propeller twist angle and the emission moment direction differ from those expected, although the emission moment direction is consistent with previous measurements [11].

4.3. Acridine orange

Acridine orange is a fluorescent, cationic acridine dye, that has a number of applications. The zinc chloride double salt form is used in lithographic applications, for dying leather, in the manufacture of printing inks. Biologically it is extensively used as a stain with many applications [16]. It has also been found to retard cancer-cell growth [22], although it is not in medical use. Recently it was published that acridine orange could not actually intercalate DNA in living cells [23]. Like proflavine, the visible band consists of a number of transitions, orientated along the short and long axes. The absorption maximum at 489 nm (in water) is due to a absorption moment lying along the long axis [19]. We have measured EFPS signals at both 488 nm and using the UV lines of the argon ion laser, although as for proflavine, the UV measurements consist of a mixture of absorption moments, and so the angles reflect this. Our measurements show the long axis absorption moment lies at around 66° to the alignment axis, which is in agreements with measurements by dichroism [19] and EFPS [20]. The emission moment seems not to be co-linear with the absorption moment, which is

different from what would be expected, although consistent with previous measurements [11].

4.4. DAPI

4′,6-Diamidino-2-phenylindole, known as DAPI is a yellow, fluorescent trypanocide which is known to form highly fluorescent complexes with DNA [16]. Although well characterized as a minor groove binding agent, recently it has been shown that DAPI may intercalate at GC sites [24,25]. The absorption spectrum reveals a number of peaks in the UV, with the highest intensity being that centred around 349 nm (in water). We have thus measured EFPS transients using the 351/364 nm laser lines. This transition probably arises from a moment lying in the fluorophore plane close to the long axis. Our measurements for DAPI are the first presented for EFPS. Dichroism results show that with Calf Thymus DNA such as that used in our experiments, DAPI groove binds [24]. Other dichroism studies [26] suggest an orientation angle for the 349 nm absorption moment of around 45°. Our angle of around 52° agrees broadly with this. Measurements using EFPS with Salmon Sperm DNA gives an absorption moment direction of slightly less than this, at 47° [2]. In this case the absorption and emission moments lie at the same orientation to the alignment axis, although the propeller twist angle of 61° indicates that they are not co-linear.

4.5. Hoechst 33258 and Hoechst 33342

Both these compounds are bis-benzimide derivatives, which form highly fluorescent complexes with double stranded DNA. They have a number of biological applications particularly in cytological and histological studies of DNA [16]. The mode of binding of Hoechst 33258 in particular has been extensively investigated; binding in the minor groove with AT preference [24,27,28]. The fluorophore, as with DAPI has been estimated to lye at around 45° to the alignment axis [26]. Our measurements indicate that the highest absorption moment (due to the absorption band centred around 349 nm (in methanol) for both compounds) lies at around 51° for Hoechst 33258 and 50° for Hoechst 33342. These agree favourable with previous EFPS studies [12]. The emission moment orientations are about 10° less than their absorption moment counterparts, again not as expected, but in line with previous results by other workers [12].

4.6. Doxorubicin and daunomycin

Doxorubicin and daunomycin are extremely well documented anti-neoplastic agents. These compounds comprise of an anthracycline fluorophore, which intercalates DNA, attached to a glycan moiety which remains hydrogen bonded in the minor groove [1,29–32]. Although well characterized, no EFPS measurements have been published for these compounds. As the anthracycline is the fluorophore, and it intercalates, then EFPS should give binding geometries for the anthracycline ring system indicative of intercalation. Our results, measured at 497 nm (close to the 496 nm absorption band) give absorption moment orientations of 64° for daunomycin and 67° for doxorubicin, which are similar to those obtained for the classic intercalators proflavine and acridine orange. Again, as with all the other compounds, the emission moment orientations differ from the absorption moments; their orientations given in Table 1.

4.7. Actinomycin C and actinomycin V

Like doxorubicin and daunomycin, the actinomycins are well studied. They consist of a heterocylcic ring which intercalates, along with an oligopeptide chain which lies in the groove [30,31,33–35]. Actinomycin C consists of a combination of different compounds, actinomycin IV, VI and VII, while actinomycin V is a single component [36]. Our EFPS results indicate that the fluorophore (the intercalating component) lies at around 55° to the alignment axis. This would appear to indicate groove binding. The signals from these compounds are also very weak compared to the background fluorescence levels, indicating that there may be a fairly large amount of unbound or loosely externally bound compound present. This would affect the angles observed. This

[2] Windsor and Tinker, unpublished results

could be due in part to the large nature of the complexing agent and the large dye-base pair ratio used in these experiments. EFPS needs to be repeated on these compounds using a better characterized complex, for more of a conclusive determination of binding to be made.

Example transient changes in the fluorescence polarization are shown in Fig. 6 for ethidium bromide (excited at 515 nm), Hoechst 33342, and daunomycin.

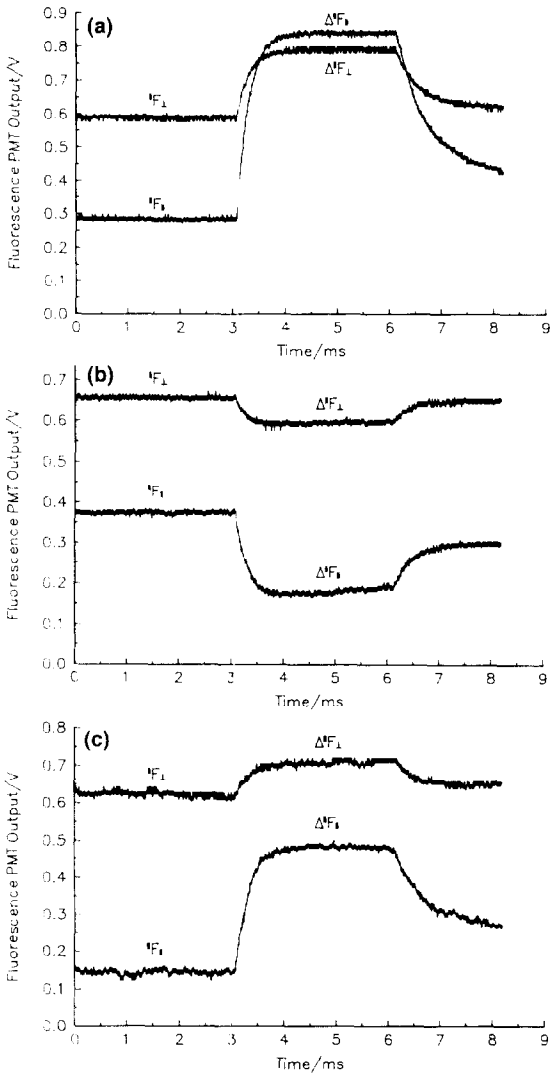

Fig. 6. Changes in the electro-fluorescence signals for several complexes under full alignment of the DNA carriers (a) calf thymus DNA treated with ethidium bromide, 1 dye molecule per 50 base-pairs; (b) calf thymus DNA treated with Hoechst 33342, 1 dye molecule per 50 base pairs and (c) calf thymus DNA treated with daunomycin, 1 drug molecule per 50 base-pairs.

5. Conclusions

Electro-fluorescence polarization spectroscopy is a fast and effective probe of the geometry of binding of fluorescent molecules to DNA. Providing conditions are carefully chosen, the technique gives not only a qualitative indication about the mode of binding but also an accurate quantitative assessment of the geometry of binding. In particular, absorption and emission moment directions can be measured at wavelengths which coincide with absorption due to DNA, where dichroism is difficult to interpret for bound dyes. For the materials studied here, the intercalating dyes have absorptions in the visible and near UV, while those that are groove bound only absorb in the UV. Care must be taken when inferring binding geometries from angles, ensuring that only one moment is excited. For materials which absorb in both the visible and UV regions, the absorption and emission moment directions differ between regions, which is consistent with the existence of different moments.

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