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ELECTRO-FLUORESCENCE PHENOMENA IN MACROMOLECULAR SYSTEMS

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Abstract - Fluorescence involves the absorption and re-emission of light in which incident polarised radiation is generally depolarised depending upon the directions in space of the corresponding molecular transition moments. Applied electric fields impose order on macromolecules in dilute solutions and the polarised components of fluorescence change. Using a novel apparatus, measurements have been recorded for systems of dye-tagged nucleic acids and polymers which indicate the binding geometry of the dye to the macromolecules.

INTRODUCTION

The principle of the electro-fluorescence method is to record changes in each of the polarised components of fluorescence as dilute macromolecular solutions are subjected to short duration, high voltage electric pulses. In response, the macromolecules orient under the influence of the applied field and the absorption and emission transition moments for chemical groups which are fluorescent (fluorophores) adopt different arrays in space. Hence both the intensity and polarisation state of the emitted light changes(1,2). By measuring these polarised components, information can be obtained on the direction of the absorption and emission moments relative to the backbone of the macromolecules. Many macromolecules are inherently fluorescent for specific incident radiations. Others can be made so by tagging the macromolecules with active dyes. One can then use electro-fluorescence measurements as a means of studying the nature of the interactions.

The use of short pulsed fields has two advantages. Firstly, the experimental procedure becomes very fast. Secondly, the rates of change of the phenomena can be measured. These rates are directly related to the size of the macromolecules.

In this paper illustrative data are given for both polymer and DNA systems. Of special importance in the latter case are studies on the nature of the binding of carcinogens to DNA and the elucidation of any common binding characteristics.

EXPERIMENTAL

A schematic description of the electro-fluorescence apparatus is given in Fig. 1. Light from an argon-ion laser is suitably attenuated and polarised before entering the

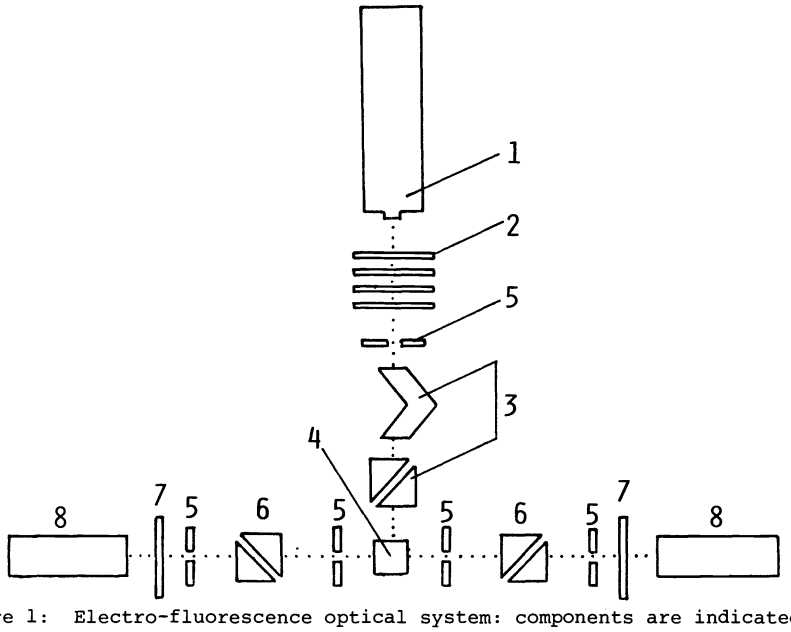


Figure 1: Electro-fluorescence optical system: components are indicated as 1 - Argon laser; 2 - neutral filters; 3 - polarising system; 4 - cell; 5 - apertures; 6 - analysing prisms; 7 - optical filters; 8 - photo-multipliers.

sample cell. The dilute dye-tagged polymer sample is held between a pair of electrodes, which are themselves contained within a glass body. Only a few ml of sample are necessary for these experiments. Fluorescent light from the sample is detected by each of two optical limbs which are arranged to receive light polarised in perpendicular planes. Hence, photomultipliers on each limb record two of the four polarised components of the fluorescence for any given state of linearly polarised incident light. The changes in each of these components are recorded as a pulse of electric field is applied between the electrodes. Typically fields of up to 20 kV cm^{-1} can be applied for durations of up to 400ms. Each photomultiplier records the transient response of the polarised components of fluorescence. The finite times of these transients relate to the rotation rate of the molecules in their viscous environment. Fields must be applied for such a time as to allow molecular orientational equilibrium to be attained as indicated by the build-up of the fluorescence change to a steady value. Decay of the phenomenon after the passing of a pulse leads to measurements of the rotary relaxation time (τ) which is itself a measure of molecular geometry and size(3).

THEORETICAL

Electro-fluorescent responses for each of the polarised components vary with the applied field strength (E). For low field intensities the proportionality is to E^2 but with increasing E orientation saturation is gradually attained. The average absorption transition azimuth angle ϕ and the related emission transition angle ϕ^1, can each be calculated from values of the polarised components of the fluorescence with and without this limiting high field.

Under any condition, the fluorescence intensity is proportional to the total number of excited transitions. For a randomly oriented array of fluorophores, the number excited at any azimuthal angle θ to the electric vector of vertically polarised incident light is proportional, firstly, to the probability of excitation (that is to $\cos^2\theta$) and, secondly, to the number of transitions contained within the angular range θ to $(\theta + d\theta)$. The latter is proportional to $\sin d\theta$. Hence, the total number excited in the absence of an electric field is proportional to

$$\int_{\pi/2}^{0} \cos^2\theta \sin\theta \, d\theta = 1/3 \qquad (1)$$

For a completely vertically aligned collection of macromolecules in which all the active dyes are bound with the same orientation, the total number of transitions excited becomes proportional to

$$\cos^2\phi \int_{\pi/2}^{0} \sin\theta \, d\theta = \cos^2\phi. \qquad (2)$$

The field-free fluorescence intensity is given by the factor $(V_v + 2V_h)$, whilst the field-induced intensity has the form $(V_v + \Delta V_v + 2V_h + 2\Delta V_h)$. Hence, the ratio of the field-free and fully aligned fluorescence intensities is given by

$$\frac{I_{E=0}}{I_E} = \frac{(V_v + 2V_h)}{(V_v + \Delta V_v + 2V_h + 2\Delta V_h)} = \frac{1/3}{\cos^2\phi} \qquad (3)$$

so that ϕ can be evaluated directly from the steady amplitudes of the transient traces of each of the polarised components of the fluorescence. The average emission transition azimuth is simply obtained by comparison of the components of fluorescence polarised in the vertical direction to those in the horizontal plane when a fully orienting field is applied. Hence,

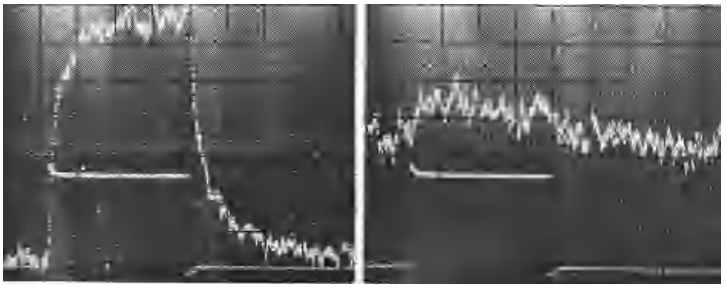
$$\frac{2(V_h + \Delta V_h)}{(V_v + \Delta V_v)} = \tan^2\phi^1 \qquad (4)$$

It can be seen that discrete measurement of the individual components and of the magnitude of their changes in a fully aligning field leads directly to a determination of both ϕ and ϕ^1.

RESULTS

Measurements are reported on three systems. The first is a polymer to which rhodamine B has been attached. The second is for a well-known dye which intercalates DNA. The third represents a study of the interaction of benzo(a)pyrene hydrocarbon, which is known to be an active carcinogen, with DNA. These systems are discussed in turn.

(i) Polystyrene sulphonic acid/rhodamine B; Polystyrene sulphonic acid is a polymer of commercial interest as it is used extensively in water purification and in ion exchange systems. Figure 2 shows the electrically induced fluorescence changes for a dilute aqueous solution of the polymer (of 1.3×10^5 relative molecular mass), in the presence of added



ΔV_v ΔV_h

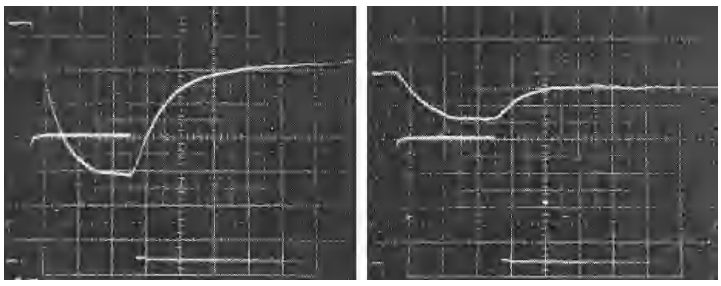
Figure 2: Changes in two polarised components of fluorescence for aqueous polystyrene sulphonic acid with rhodamine B. E = 6kV cm^{-1}. Pulse duration 0.4 ms.

rhodamine B. This dye is itself of current commercial importance in dye-laser technology.

Fluorescence was excited with 488nm radiation and detection was for light exceeding 515nm wavelength. It is known that in aqueous media, the majority of flexible polyelectrolytic molecules extend and align in a field with their major axes along the electric field direction(4-6). The polarised components of fluorescence are designated by a pair of letters V and H, the capitals indicate the polarisation state of the incident light and the subscript those of the detected emitted radiation. From Figure 2 one sees that with vertically polarised incident light, the total fluorescence was enhanced as the molecules were pulled by the electric field into a vertical direction. The absorption transition moment for the active fluorophores was thus predominantly in the vertical plane. It is also seen that of the emitted light, the majority was emitted in the vertical direction when the field was applied. Hence, the emission moment was also predominantly associated with the vertical direction. On the figure, one can see directly therefore that the rhodamine B dye molecules append themselves onto the polymer in such a way that the long axis of their molecular planes associate predominantly parallel to the polymer backbone. When one realises the speed and ease with which one can determine this information the value of the method becomes apparent. Similar reasoning to the above can be obtained from the changes produced in the polarised components of fluorescence with horizontally polarised incident radiation.

Finally, analysis of the rate of decay of the phenomena after the passing of the applied field indicates a molecular relaxation time of the order of 70 μs. By using suitable equations for the rotary relaxation of flexible polymers, one can relate this to the polymer dimensions in solution.

(ii) DNA-proflavine complex. Many dye molecules bind readily to biopolymers, often due to the highly polyelectrolytic nature of the latter. This property is utilised in many biomedical studies as various planar dyes are anti-tumour, anti-viral, anti-malarial or anti-tripanosomal agents in the chromosomes of mammalian cells, (7), presumably through their binding to nucleic acids. An important quest in molecular biochemistry is the elucidation of the geometry of binding of such molecules to nucleic acid helices. The electric field fluorescence appears to be eminently suitable for such studies.



ΔV_v ΔV_h

Figure 3: Field induced changes in polarised fluorescence components for the DNA/proflavine complex in water E = 5kV cm^{-1}. Pulse duration of 0.6 ms.

Figure 3 illustrates transients obtained in the polarised components of fluorescence for a dilute solution (4×10^{-5} g ml^{-1}) of DNA of relatively high molecular mass (5×10^{6}) obtained from calf thymus. Proflavine had been bound to DNA in a concentration corresponding to as little as one proflavine molecule for each 100 base pairs of the DNA. This dye is known to cause mutations in the cells of bacteria and animals(8). Incident light of 458nm wavelength was used to excite the fluorescence and light emitted in excess of 495nm wavelength reached the detectors. Corresponding changes in the components of the fluorescence

are indicated in the figure. DNA is known to align in an electric field with its major axis predominantly parallel to the field direction(9). In our system this direction is vertical. Hence, a comparison with Figure 2 shows that the binding of the proflavine to the DNA is predominantly perpendicular to that previously recorded for rhodamine binding to polystyrene sulphonic acid. With vertically polarised incident light, and a vertical electric field, the fluorescence decreased with field activation. Furthermore, the vertically polarised emergent light was reduced more than the horizontal. Both of these factors indicated that both the absorption and emission transition moments of the proflavine were essentially perpendicular to the aligned and extended DNA helical backbone. Such binding is consistent with the intercalation of proflavine into the DNA helix in the generally accepted manner.

(iii) DNA and benzo(a)pyrene derivatives. This forms perhaps the most dynamic example. Benzo(a)pyrene (BP) is found in tars, smoke and other combustion products. It has long been studied because of its association with carcinogenesis. Although itself relatively inert, BP binds readily to DNA in aqueous solutions, and is fluorescent. Recent biochemical studies have shown, however, that native BP is not itself the carcinogen but is metabolically converted by the host into highly reactive intermediates. Such intermediates are generally found in various stereoisomeric forms which can bind covalently with cellular macromolecules(10-11). Current research has shown that diol-epoxide derivatives are the active metabolites which covalently bind to DNA in vivo and are therefore the precursors to carcinogenesis. More specifically, the form (+)-anti-benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide, hereafter referred to as (±) anti-BPDE is considered to be the ultimate carcinogen (12-14).

An obviously important biochemical quest is to know why this particular derivative is specifically carcinogenic, and to find those characteristics of its binding to DNA which indicate this unique behaviour. It is thus of interest to compare native BP with the various BPDE derivatives in their binding characteristics to DNA.

DNA solutions of 10^{-4}g ml^{-1} concentration were studied after both native BP and specific racemic mixture (±)anti-BPDE had been added to separate samples. In all cases the dye-to-base pair ratio was approximately 1 : 100. Electric fields of up to 25kV cm^{-1} were applied and regular transient changes were obtained in the polarised components of the fluorescence. These are shown in Figure 4 when vertically polarised incident light was used. From the

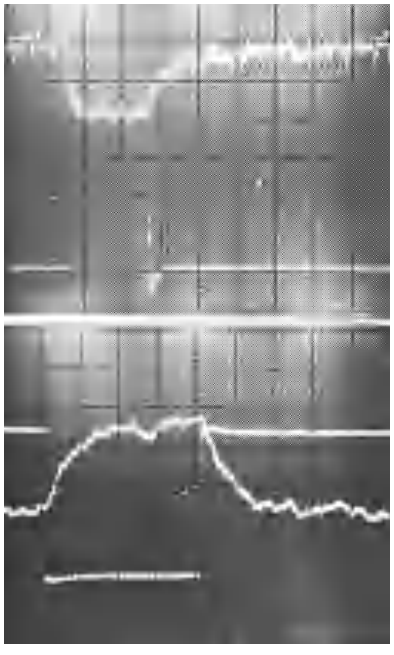


Figure 4: Comparison of changes induced in V_v for DNA-benzo(a)pyrene complexes.

Top: native BP.

Bottom: (±) anti-BPDE

figure one immediately notes the following factors. Firstly, the two materials gave a very different electro-fluorescent response. Secondly, the native BP binds to DNA in a manner very similar to that exhibited by proflavine and previously interpreted as consistent with helix intercalation.

The BPDE/DNA system indicates a binding which, if anything, is more associated with the plane of the dyes being bound parallel with the DNA major axes. In fact, interpreting the experimental data using the equations (3) and (4), one obtains angles of $\phi = 52(\pm 3)°$ and $\phi^1 = 45(\pm 6)°$ for the diol-epoxide complex. These angles are extremely similar to the projection angle of minor grooves of the DNA helix along its major axis. It is known that the N-2 guanine position on the DNA associates with the carbon-10 site of the BPDE(15). If one uses space-filling models and associates these relevant sites along the helix with

the angles ϕ and ϕ^1 indicated above, it is readily possible to fit the BPDE ring into the minor groove of the double helix.

Without knowing the biochemical significance of these observations, it is, however, very apparent that the electro-fluorescent method offers a fast and extremely sensitive means of differentiating between binding geometries of active associating agents with macromolecules. In particular, the behaviour of the (±)anti-BPDE when compared with native BP is very evident and rapidly apparent by this experimental method. Hopefully, the electro-fluorescent method may become a standard means of studying such binding.

CONCLUSIONS

The electro-fluorescence method appears to be a very sensitive indicator of the binding geometry of dye molecules to polymers and biopolymers. Once the apparatus is established and the solutions prepared, the method is very fast and sensitive. In the DNA experiments, the concentrations used are less than those currently employed by any other physical methods and therefore represent situations which more closely relate to those encountered in vivo. Further experiments are envisaged on naturally fluorescent materials where it is hoped that the geometry of fluorescent groups can be determined within the macromolecular structure, with speed and accuracy.

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